

PERSISTENT INFECTION OF A BOVINE KIDNEY CELL LINE WITH
NEWCASTLE DISEASE VIRUS

by

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I certify that the work presented in
this thesis is my own.

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SUMMARY

SUMMARY

Continuous passage of undiluted supernatant fluids from cultures of a bovine kidney cell line MDBK infected with the Herts 33 strain of NDV, led to the establishment of a carrier state [MDBK_{cs}].

The general properties of this virus-cell system were compared with those of a regulated-type of NDV persistent infection of MDBK cells [MDBK_{pi}] which was accidentally induced in this laboratory in 1962 [Edwards, 1972].

The intracellular polypeptide composition of the cell associated virus in MDBK_{pi} was different from that in MDBK_{cs} cells. In MDBK_{cs} virus, the usual pattern of composition and distribution of NDV polypeptides was observed whereas the MDBK_{pi} virus lacked the M protein which is necessary for viral assembly.

Experiments using both RNA and protein synthesis inhibitors [actinomycin D and cycloheximide, respectively] failed to induce an increased release of infectious virus from MDBK_{pi} cell cultures. This indicates that the blockage is not dependent on cellular RNA or protein synthesis.

The presence of a cellular control mechanism of NDV replication was detected in the persistently infected MDBK_{cs} cultures as well as in MDBK cells primarily infected with NDV and there is evidence that a similar mechanism occurs in MDBK_{pi} cells. It is emphasised, however, that the cellular block does not prevent the release of fully infectious virus from primary or carrier state cultures.

Although the virus released from MDBK_{pi} does not undergo a productive replication cycle in permissive cell culture systems or in embryonated eggs, it is able to attach, replicate, haemadsorb and induce cell fusion at the first passage level. The available

evidence suggests that a defect in the synthesis of M protein might account for failure to assemble complete viral particles at the cell surface.

Besides promoting the release of non-infectious particles, the absence of the viral M polypeptide may also be responsible for the intracellular accumulation of viral nucleocapsids. These structures were found to be of two different types, namely granular and smooth, according to the presence or absence of a sheath covering the RNP component. The accumulation observed in the nucleus of some cells in aged cultures was always restricted to smooth nucleocapsids. Furthermore, only the granular nucleocapsids were shown conclusively to be NDV-specific by immunoperoxidase techniques.

Biochemical experiments on Herts NDV and MDBK_{CS} virus, propagated in embryonated eggs, showed that both types consist of a mixture of two distinct kinds of virus particles. The first of these sedimented at a density of 1.12 in sucrose or tartrate gradients and revealed an unusual polypeptide composition including a significantly reduced NP/F peak, low haemagglutinin and neuraminidase activities, and low infectivity titres; whereas the virus sedimenting at a density of 1.18 was of the standard type.

A viral inhibitory factor [VIF] was detected in the supernatant fluids of MDBK_{CS} cell cultures. This viral-induced component was NDV-specific, could not be sedimented by ultracentrifugation, and was able to protect indicator cell monolayers against infection by homologous virus but not by related or unrelated viruses.

On the other hand, absence of the viral inhibitory factor in MDBK_{pi} cell culture fluids suggested that a different mechanism is involved in the maintenance of the regulated type of infection.

Unsuccessful attempts were made to transfect the putative integrated viral information in DNA from MDEK cells persistently infected with NDV. However, the results of several experiments involving DNA analogues and the fact that a transient "cure" of MDEK_{pi} monolayers was obtained after prolonged propagation seem to suggest that viral integration is a possible hypothesis, at least so far as regulated infections are concerned.

ABBREVIATIONS

ABBREVIATIONS

The following conventional abbreviations have been used throughout this thesis:

BHK-21	-	clone 21 of baby hamster kidney established cell line
BMG	-	stable line of African green monkey kidney cells
BMV	-	bovine mammillitis virus
BSC-1	-	a monkey cell line
CEF	-	chick embryo fibroblast culture
c. p. e.	-	cytopathic effect
DI	-	defective interfering particles
DNA	-	deoxyribonucleic acid
EDTA	-	ethylenediaminetetracetic acid
HA	-	haemagglutinin activity
HAIU	-	haemagglutinating inhibition units
HAU	-	haemagglutinating units
HEF	-	hamster embryo fibroblast culture
HEF/MV	-	persistent infection of MV in HEF cells
HEPES	-	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IdU	-	5 iodo-2'-deoxyuridine
Lu106	-	human embryonic lung cells
M6	-	a rhabdovirus similar to VSV
MDBK	-	Madin and Darby bovine kidney cell line
MDBK _{cs}	-	<u>Idem</u> , carrier state of NDV
MDBK _{pi}	-	regulated persistent infection of NDV in MDBK cells
MEM	-	minimal essential medium (Eagle's)
MOI	-	multiplicity of infection
MV	-	measles virus
NDV	-	Newcastle disease virus
PAGE	-	polyacrylamide gel electrophoresis
PLA	-	peroxide-labelled antibody

PBS	-	phosphate buffer saline
RNA	-	ribonucleic acid
cRNA	-	complementary RNA
mRNA	-	messenger RNA
RNP	-	ribonucleic protein
SDS	-	sodium dodecyl sulphate
TCD ₅₀	-	50% tissue culture infectious doses
TCTF	-	trichlorotrifluorethane
tris	-	2-amino-2-hydroxymethylpropane-1, 3-diol.
t. s.	-	temperature-sensitive
U-V	-	ultraviolet light
VIF	-	viral inhibitory factor

INTRODUCTION

INTRODUCTION

A - History of Newcastle disease

Although the first descriptions of Newcastle disease came from Java (Kranefeld, 1926) and Great Britain (Doyle, 1927), there are a number of reports which suggest that an illness of poultry resembling Newcastle disease may have occurred in other parts of the world late in the nineteenth century (Camara and Valadao, 1950; MacPherson, 1956).

These early observations, together with the fact that Newcastle disease frequently occurred as a subclinical disease with low mortality and little disability (Burnet, 1960), support the suggestion that the disease could well have existed as an established infection before it was first described as a distinct clinical entity.

In the original outbreaks, Newcastle disease was characterized both by its fulminating nature and high mortality. The infection spread rapidly from the initial focus to many other areas, retaining its pathogenic properties. However, it quickly became apparent after a number of outbreaks, that milder forms of the disease existed and that many could remain in an endemic state which persisted in the population for considerable periods of time (Allan, 1971).

On the other hand, in North America, the initial outbreaks of Newcastle disease showed characteristics of a relatively mild respiratory infection which was called "avian pneumoencephalitis". Only later was this infection shown by serologic procedures to be due to Newcastle disease virus (NDV) (Beach, 1944).

More recently, because of the intensification of poultry production and the build up of air and sea communications, together with the introduction of widespread vaccination, isolation and slaughter control measures, the virus of Newcastle disease has readily become adapted to new environments and to large populations of susceptible hosts. Indeed,

there is clear evidence that a number of recent outbreaks of the disease were caused by mutants of the virus with higher affinity for the respiratory tract and, consequently, an increased facility to spread by droplet infection, thereby infecting a greater number of birds (Allan, 1971; Chu and Rizk, 1972).

In early outbreaks, the epidemiology of Newcastle disease was closely associated with local wild birds, or bird migrations, due to the high innate susceptibility shown by many of these species towards the virus (Lancaster, 1966). Similarly, world-wide movements of psittacine birds from endemic to healthy areas may also have played an important part in the spread of NDV (Lancaster and Alexander, 1975).

It is generally agreed that warm-blooded animals are not very susceptible to natural or laboratory infections with NDV and that the virus does not spread spontaneously to other mammals under field conditions (Hanson et al., 1951). However, since Brandly (1959) has shown evidence of a discrete but wide-spread mammalian susceptibility, this indicates a greater potential range of infectivity of the virus. In this connection it is of interest that a number of cases of clinical Newcastle disease have been confirmed in man, and that human patients may spread the infection to poultry (Blood, 1950; Trott and Pilsworth, 1965).

B - The Virus

1 - Morphology, structure and chemistry

a) The virion

Newcastle disease virus generally appears as a pleomorphic, approximate spherical particle measuring about 200nm in diameter.

The mature virion consists of an inner helical nucleocapsid enclosed by an outer coat or envelope covered with a fringe of short, radially-arranged projections or spikes (peplomers). It is not a

robust virus, and the particles frequently appear deformed or disrupted in electron microscopic preparations.

Chemical analysis show that NDV contains approximately 67% protein, 1% ribonucleic acid (RNA), 24% lipids, and 7% carbohydrates (Cunha et al.; Robinson and Duesberg, 1968; Blough and Lawson, 1968). A number of polypeptides (varying from four to ten) have been described and two of these have been identified as glycoproteins (Evans and Kingsbury, 1969; Mountcastle et al., 1971; Alexander and Reeve, 1972; Moore and Burke, 1974; Nagai et al., 1976 a,b). Haemagglutinin, neuraminidase and transcriptase activities are associated with those components, as are a number of other enzymes including ADPase, ATPase and phosphodiesterases which are considered to be products of cellular origin. (Neurath, 1965; Huang et al., 1971; Pristasova and Rosenbergova, 1974; Charlton and Sabina, 1975).

b) The substructures

I - Nucleocapsid

Morphologically, the inner component is a long and flexible structure measuring approximately 1000nm in length by about 18nm in width (Compans and Choppin, 1967; Kingsbury and Darlington, 1968; Finch and Gibbs, 1970). It has a hollow core, approximately 5nm in diameter and a striated surface with a periodicity of 4 to 6nm, surrounding a filament of single-stranded RNA (Hosaka, 1968). The viral nucleic acid has a molecular weight of 7.5×10^6 and is intimately associated with a series of identical protein subunits arranged in a single left-handed helix which seems to protect the RNA molecule from the environment (Duesberg and Robinson, 1965).

It is generally considered that the intact nucleocapsid contains a single NDV genome, which not only carries all the genetic and chemical information necessary to code for the structural proteins of the

particle but also for the enzymes necessary for its own replication and for the synthesis of viral proteins. However, it is interesting to note that other workers have indicated the existence, in NDV populations, of virions containing more than one genome (Granoff, 1959; Dahlberg and Simon, 1969; Kingsbury and Granoff, 1970).

According to Mountcastle et al., (1970) the nucleocapsid proteins may exist in at least two different forms. One, with larger protein subunits can be obtained from mature virions or from infected cells dispersed by ethylenediamine tetracetic acid (EDTA), while a second type consisting of smaller protein subunits is obtained after the cells have been dispersed by trypsin. Electrophoretic studies have shown that these viral proteins migrate as a single component (NP) having a molecular weight of 52000 to 62000 (Bikel and Duesberg, 1969; Evans and Kingsbury, 1969; Moore and Burke, 1974).

Chemically, the NDV nucleocapsid contains approximately 4 to 5% RNA and 95 to 96% protein (Kingsbury and Darlington, 1968).

Purification of the virus after detergent disruption and separation of its components on the basis of sedimentation velocity and buoyant density, indicate that the sedimentation coefficient (S_w) in sucrose gradients is about 200S for the nucleocapsid of NDV and about 50 to 57S for the intact viral RNA (Duesberg and Robinson, 1965; Kingsbury and Darlington, 1968). Most of the virus-specific RNA that is synthesized in NDV-infected cells has been shown by specific annealing techniques to be complementary in base sequence to 57S RNA from virus, although it sediments slower at 18, 22, or 35S (Bratt and Robinson, 1967; Kingsbury, 1967).

II - The envelope

The inner component is protected by a fragile but well defined outer membrane invested with a fringe of radially arranged surface projections about 12.5nm in length. The membrane is believed to be

a lipid bilayer which has a non-glycosylated protein (M) associated with its inner surface, and measuring approximately 6nm in width, giving the viral envelope an overall thickness of some 18.5nm (Mountcastle et al., 1971; Cheville et al., 1972; Landsberger et al., 1973).

Several biological activities of NDV are associated with its coat. Some, such as haemagglutinin, neuraminidase and hemolytic activity are believed to be viral proteins whereas others including adenosine diphosphatase (ADPase) and adenosine triphosphatase (ATPase) are considered to be proteins of host cell origin (Neurath and Sokol, 1963).

Disintegration of the virion by treatment with ether or with a nonionic detergent such as polyoxyethylene sorbitan monooleate (Tween 80) was used by earlier workers mainly to separate the virus specific components of the envelope and to release the nucleocapsid (Rott and Schäfer, 1961). Although intensive efforts were made to separate the polypeptides responsible for haemagglutinin and neuraminidase activities by using detergents such as sodium dodecyl sulphate (SDS) and sodium deoxycholate (DOC), the final activity of the isolates were reduced appreciably (Laver, 1969). However, by using Tween 20 at high pH, Webster and Darlington (1969) were able to achieve separation of the surface components in biologically active form.

More recently, following similar studies on a number of related viruses using a labelled carbohydrate precursor, a nonionic detergent or a protease, it has been possible to identify two glycoproteins of different molecular size (Mountcastle et al., 1971; Chen et al., 1971; Scheid et al., 1972).

The larger and more abundant glycoprotein has a mol. wt. of about 65000 - 74000, whereas that of the smaller component is approximately 52000 - 56000, and both are associated with the surface projections

(peplomers) of the viral envelope. It has also been shown that the major glycoprotein possesses both haemagglutinin and neuraminidase activities (Scheid and Choppin, 1973; Tozawa et al., 1973). Later work involving extraction of viral protein by nonionic detergents followed by affinity chromatography in fetuin-Sepharose columns, enabled each of the two glycoproteins to be isolated and identified. It was also possible to show not only that both biological activities are associated with the same viral protein and are situated on the same spike, but also that the smaller glycoprotein is involved in the phenomenon of virus-induced haemolysis and cell fusion (Scheid and Choppin, 1974). Similar studies by Klenk and Choppin (1969; 1970 a,b) on other members of the same virus group indicate that the viral coat also contains lipid which is probably derived from the plasma membrane of the infected host cell.

Associated with the inner surface of the viral envelope is a nonglycosylated protein which is thought to have a structural role in the viral coat and to be the recognition site for nucleocapsid during the process of virus assembly at the cell membrane (Mountcastle et al., 1971; McSharry et al., 1971; 1975; Choppin et al., 1972). This protein has been designated "membrane" or "matrix" (M) protein based on its location within the virion (Kilbourne et al., 1972; Laver, 1973).

2 - Classification of NDV

In the most recent classification of viruses proposed by the International Committee for the Classification of Viruses, (Fenner, 1976), members of the family Paramyxoviridae have been defined as enveloped single-stranded RNA viruses, with helical symmetry, and some possessing haemagglutinating properties.

In this new scheme, the family Paramyxoviridae has been subdivided in three genera, namely: Paramyxovirus, in which NDV is the prototype

and also includes several other members e.g. mumps, Yucaipa, and parainfluenza 1, 2, 3 and 4 viruses; Morbillivirus, comprising the measles-rinderpest-distemper (MRD) triad of viruses; and Pneumovirus represented by respiratory syncytial viruses.

The present classification of the paramyxoviruses is based on the diameter of the helical ribonucleoprotein component, as well as the biological and replication characteristics of the different members. By this method, pneumoviruses and morbilliviruses have an RNP component 12-15nm in diameter, compared with more than 15nm for true paramyxoviruses.

3 - Viral replication

The processes by which paramyxoviruses replicate are, in many respects, closely related to the fact that their genetic material consists of a single piece of single-stranded RNA which is complementary to that of the messenger-RNA.

In the typical productive type of growth cycle a number of distinct and sequential steps have been described, namely:

a) Adsorption

Attachment of virus particles to the cell surface is, basically, a phenomenon involving a series of electrostatic interactions in which the frequency of collision between virus and cell does not necessarily lead to attachment. Usually, this only occurs when there is affinity between the surfaces of the virion and those of the specialised areas of the cell membrane called virus receptors (Fenner et al., 1974).

It is generally agreed that adsorption of NDV to susceptible host cells is mediated by attachment of viral haemagglutinin to specific receptor sites on the surface of the cells and, while it was believed that neuraminidase plays little part in either adsorption or entry of the virus (Fazekas de St Groth, 1948), more recent evidence has shown that both haemagglutinin and neuraminidase activities are required for virus

uptake (Zhdanov and Bukrinskaya, 1962; Portner et al., 1975).

b) Penetration and uncoating

Fazekas de St Groth (1948) was the first to propose that host cells play an important role in engulfing the intact virion, and called the phenomenon "viropexis". Later work, including electron microscopic studies on NDV pioneered by Silverstein and Marcus (1964) supported the concept that enveloped viruses are phagocytosed as intact particles. Furthermore, Mussgay and Weibel (1962) showed that complete virions could be seen in the cytoplasm half an hour after infection.

An alternative proposal for viral penetration and subsequent uncoating is based on observations on fusion of viral envelopes with cell surface, and was put forward originally by several authors including Hoyle (1962), Dourmashkin and Tyrrell (1970), Apostolov and Almeida (1972). The latter workers have also shown that the nucleocapsid of the infecting virus becomes extruded and is released into the cytoplasm of the host cell. More recently, however, it has been suggested that penetration of paramyxoviruses into host cells can be achieved either by viropexis or, concomitantly, by viropexis and fusion (Dales, 1973; Haywood, 1975).

It has not yet been established whether disruption of the viral envelope begins at the cell membrane so that only the nucleocapsid penetrates into the cytoplasm, or if the uncoating process is a strictly intracellular event whereby disaggregation of the engulfed virions occurs within the cytoplasmic vacuoles due to cellular enzyme activity. According to Durand et al., (1970), envelope digestion is a consequence of phagocytic activity of the cell lysosomal enzymes. On the other hand, Dourmashkin and Tyrrell (1974) have produced evidence in support of the theory that uncoating of the virus particle

takes place while the virions are in direct contact with the host cell cytoplasm, but without the intervention of lysosomal enzymes.

c) Eclipse phase

Following its release within the cytoplasm, the RNA-containing ribonucleoprotein component "migrates" to the site where replication begins (Bukrinskaya et al., 1969). In the case of paramyxoviruses, this is believed to be confined to the cytoplasm (Wheelock, 1963; Bratt and Robinson, 1967), although a number of other workers including Zhdanov et al. (1965), consider that the nucleolus may also play an important role in the replication of NDV.

The synthesis of novel viral RNA begins between 2 to 4 hours post-infection and reaches a maximum approximately 3 hours later. During this early phase, a virus-associated enzyme named RNA-dependent RNA polymerase, which is probably closely linked to RNP, appears to play the major role in viral RNA transcription (Robinson, 1971 and Huang et al., 1971). This is characterized by the production of large amounts of RNA molecules which are smaller than viral strands but are complementary in their base sequences to those of viral genomes. The presence of "de novo" viral structures can only be detected 3-4 hours post-infection, towards the end of the so-called eclipse phase, by which time appreciable amounts of viral antigen have been synthesized. In most instances, the RNP antigen accumulates in specialised areas of the cytoplasm in the immediate vicinity of the nuclear membrane.

i - Synthesis of early RNAs

The presence of a RNA-dependent RNA polymerase associated with the nucleocapsid of paramyxoviruses was first described independently by Huang et al. (1971) and Robinson (1971a), and it is interesting to note that the latter worker also found that viral transcriptase

begins to transcribe the genomic RNA into complementary RNA in the nucleocapsid immediately after the viral RNP enters the cytoplasm. The RNA species synthesized in this manner differ from genomic 50-57S RNA but resemble more closely those which accumulate in the cells during a late stage in the infection cycle.

Studies using drugs such as cycloheximide and actinomycin D also revealed that viral RNA transcription is not affected by inhibitors of protein or cellular RNA synthesis (Robinson, 1971b).

ii - Production of different types of NDV-specified RNA

The insensitivity of NDV replication in infected cells treated with actinomycin D was first reported by Scholtissek (1969), and has proved of value in further studies of viral RNA synthesis.

Four types of virus-specific RNA were isolated by Bratt and Robinson (1967) and were found to have sedimentation coefficients of 50-57S, 35S, 22S and 18S but only the first, which occurred in the lowest amount, corresponds in size to viral RNA.

Another striking feature of RNA replication of paramyxoviruses is that up to 90% of the virus-specific RNA produced by infected cells is complementary RNA, although some parental RNA is invariably produced also (Kingsbury, 1966; Bratt and Robinson, 1967; Blair and Robinson, 1968). The detection of those heterogeneous complementary RNA (cRNA) strands in cells infected with NDV and other paramyxoviruses, and the fact that they appear earlier in the infection cycle than viral RNA, led to the conclusion that they have a biological function other than being degradation products of the 50-57S viral RNA or its complementary strands (Bratt and Robinson, 1967; Barry and Bukrinskaya, 1968; Blair and Robinson, 1968).

Kingsbury (1966) has suggested that the biological functions of the intracellular 35S to 18S cRNAs are related to the mRNA responsible

for early protein synthesis. In fact, observations made by Blair and Robinson (1968) suggest that the viral RNA strands are associated with the polysomes. However, the reason why much of the cRNA in the cytoplasm is not associated with the ribosomes remains obscure (Kingsbury, 1974).

iii - Translation and replication

The cRNA transcripts, which are smaller than the 50-57S RNA, are translated on polyribosomes to give virus-specific replicases, haemagglutinin, neuraminidase, transcriptase, nucleocapsid structural units and a number of other viral proteins. However, there is some evidence that translation begins before transcription is complete and gives rise to a complex consisting of a transcriptive intermediate and ribosomes (Bukrinskaya et al., 1969; Kingsbury, 1972).

Virus-specific replicase directs the synthesis of a complementary strand, equal in size to that of the viral genome, which in turn serves as a template for genome replication. Although these newly synthesized 50-57S RNA components are now sensitive to inhibitors of protein synthesis the effect can only be demonstrated during the first few hours after infection (Wilson and LoGrefo, 1964; Scholtissek and Rott, 1965). The nucleocapsid structural units and transcriptase are synthesized at the ribosomal level, and are believed to associate with the nascent progeny genomes to form complete viral nucleocapsids. These may have three possible fates: some may become involved in transcription and are indistinguishable from the original input infectious units, others may take part in replication, while the remainder tend to migrate towards the cell membrane (Kingsbury, 1972).

iv - Control of RNA and protein synthesis

The existence of a mechanism which regulates the relative proportions of small complementary RNAs and 50-57S RNA produced in cells

infected with paramyxoviruses has been suggested by several authors including Kingsbury (1972) and Fenner et al. (1974). The evidence available indicates that the production of viral polypeptides is regulated at the transcriptional level. On the assumption that RNA replication and transcription are competing processes occurring through an encapsidated single-stranded RNA template, Kingsbury (1974) has suggested a control role for the capsid proteins in RNA replication.

d) Virus assembly and release

Early electron microscopic studies by Bang (1953) revealed that NDV is assembled in epithelial cells at the plasmalemma. This has been confirmed by Morgan et al. (1956) who showed that few if any mature particles are present within the cytoplasm and that practically all the virus is formed at the cell membrane. Complete virions are generally found outside the cell.

In 1962, Marcus observed that incorporation of viral haemagglutinin into the cell membrane, approximately 3-4 hours post-infection, initiates the haemadsorption of red blood cells. But recent work by Duc-Nguyen (1968) suggests that a simple change of electrostatic charge of the cell membrane can also produce haemadsorption without the presence of viral material at the cell surface.

It has been suggested that neuraminidase may play an important role in the release of newly formed virions from infected cells, concomitant with the development of cytopathic changes (Seto and Chang, 1969). Supporting this view, Palese et al. (1974a) reported that the addition of neuraminidase-specific inhibitors resulted in a significant reduction in the size of plaques produced by NDV in chick embryo fibroblasts.

Incorporation of viral envelope proteins into cell membranes accounts for one of the earliest changes that occurs in the cellular surface. The glycoproteins start to assume their spike-like appearance and an electron-dense layer develops on the inside of the plasma membrane, adjacent to these spikes. It seems that the nucleocapsid structures have the ability to recognise and align themselves under the altered membrane because the matrix protein, which seems to constitute the dense layer, is the target site to which the RNP is attracted (Compans and Choppin, 1971; Choppin et al., 1971). Later, the modified membrane protrudes locally, and a constriction develops at the base of the protrusion until the budding process is complete.

4 - Virulence and strain differences in NDV

Virulence has been defined as the ability to cause damage or death in the host cell (Wilson and Miles, 1964).

Isolates from outbreaks of ND show remarkable variation in the capacity to cause disease and deaths in chickens. To assess the virulence of NDV, three methods have been adopted namely: the mean death time of embryonated eggs (MDT), the intracerebral pathogenicity in chicks, and the intravenous pathogenicity index (Anon., 1971).

On the basis of the MDT, strains of NDV have been divided into velogenic, mesogenic and lentogenic forms (Hanson and Brandly, 1955). Such classification is arbitrary, mainly because the strains already isolated show a continuous spectrum of virulence, varying from acute systemic disease to asymptomatic infections (Waterson et al., 1967). The differences in virulence seem to be related to variations in the affinity of viral haemagglutinin for cell membranes, and the capacity of viruses to destroy the lymphoid system and suppress immunological function (Cheville, 1975).

Almost all of the lentogenic and most of the mesogenic strains have been used to produce live vaccines. The milder forms are generally employed in the immunisation of young birds, while the mesogenic strains provide a long-lasting immunity for adult stock. Moreover, adaptation of NDV to mammalian tissue culture systems, such as pig and bovine kidney cells, has also resulted in the production of attenuated vaccines (Markovits and Toth, 1962).

Contrary to what happens with influenza virus, NDV does not show any clear evidence of variation in antigenic structure (Pennington, 1967). Similarly, no morphological or structural differences have been observed among NDV strains (Andrews and Pereira, 1972; Moore et al., 1974).

5 - Biological activities

Newcastle disease virus is not a robust virus and treatment with ether or detergents, or manipulations during viral purification breaks the viral envelope and gives rise to small fragments bearing surface projections, or 'rosettes' formed from clusters of detached projections (Rott et al., 1962). There is evidence that these clusters are due to the rearrangement of the viral projections, according to their hydrophobic and hydrophilic terminals. The released spikes retain both haemagglutinin and neuraminidase activities. In contrast to the orthomyxoviruses, it has been shown recently that only one virion glycoprotein is responsible for the haemagglutinin and neuraminidase activities of paramyxoviruses and, furthermore, that it plays a leading role in virus-induced cytopathology (Scheid et al., 1972; Scheid and Choppin, 1974; Portner et al., 1975). The other biological properties related to the viral envelope are believed to be associated with the subjacent membrane layer (Waterson, 1964).

a) Haemagglutinin

The phenomenon of haemagglutination demonstrated by NDV is due to its ability to bind to specific receptors on the surface of the red blood cells of various animal species. The capacity to haemagglutinate amphibian, reptilian, avian and mammalian erythrocytes is common to all members of the genus Paramyxovirus (Lancaster, 1966), and it is through this mechanism that NDV first becomes attached to the cell.

It is now known that the haemagglutinin of NDV and other paramyxoviruses is associated with the virus spikes but contrary to what happens in the orthomyxoviruses, the same spike contains both haemagglutinin and neuraminidase activities. Indeed, these two activities are part of the same viral glycoprotein (HN) which migrates, with a molecular weight of about 70000, on SDS-acrylamide gels (Scheid and Choppin, 1973). Under laboratory conditions, non-infectious haemagglutinins are sometimes released into the cell culture medium or are produced in the fluids of infected embryonated eggs, together with fully infectious particles (Granoff, 1955; Rott and Schäfer, 1964). The main feature of the non-infectious form is that both its haemagglutinating and neuraminidase activities are similar to those of normal virus except that they tend to be deficient in viral RNA (Rott et al., 1962).

b) Neuraminidase activity

The biological role of neuraminidase has been the subject of considerable discussion since its discovery, and the controversy still continues. According to some authors the function of the enzyme is probably related to the release mechanism of the virus particle (Seto and Chang, 1969), and an early report by Ackermann (1964) suggested that the phenomenon of elution of virus from the receptor areas of agglutinated erythrocytes is due to the activity of viral

neuraminidase. More recently, however, Becht et al., (1971) showed that the release of influenza virus is unaffected by the presence of anti-neuraminidase antibodies in the medium, whereas Morein and Bergman (1972) in an in vivo study with parainfluenza-3 virus, suggested that the nasal secretions probably act as a natural substrate for paramyxovirus neuraminidase and, further, that the enzyme is probably involved in penetration of the mucus barrier by the virus.

Investigations concerning a large number of NDV strains of different virulence showed no significant disparity in the neuraminidase content of the strains examined (Alexander et al., 1970). However, in vitro studies have indicated a role for NDV neuraminidase in the development of cytopathic changes in cell cultures (Reeve et al., 1970; Palese et al., 1974a,b). And in a comparative study, McNulty et al. (1975) reported that velogenic strains possess more than three times the neuraminidase activity per virus particle as do lentogenic strains. Such findings support the hypothesis that the enzyme is closely associated with the development of cytopathic changes in the infected cells.

c) Haemolytic and cell fusion activities

The haemolytic activity of NDV and other paramyxoviruses is distinct from their haemagglutinin and neuraminidase properties. This was first reported by Sokol et al. (1961) who discovered that isolated surface projections of the virus lacked the ability to haemolyse red blood cells. Later, Scheid and Choppin (1974), working with Sendai virus, found that activation of haemolytic and cell fusion activities occurred together following cleavage of the precursor glycoprotein and, furthermore, that the two properties were derived from similar biochemical mechanisms. A precursor glycoprotein (F_0) in NDV has also been described by several authors (Samson and Fox, 1975; Kaplan

and Bratt, 1973), and the available information suggests that proteolytic cleavage of Fo into F polypeptide is necessary for the activation of haemolytic and cell fusion activities, and for acquisition of viral infectivity (Nagai et al., 1976b).

In a series of electron microscopic studies of paramyxoviruses, Apostolov and Almeida (1972) observed the rapid formation of holes on erythrocyte membranes due to a mechanism of integration of the viral innermost layer with the cellular membrane. They also proposed that haemolysis and cell fusion are closely related and that these are, perhaps, different manifestations of the same phenomenon occurring in diverse types of cells.

C - The Host Cell

1 - Permissibility and incompetence

The cell's susceptibility to infection is usually controlled by the cellular genes. These determine the presence of cell membrane receptors which, in turn, interact with specific viral proteins. Neither the physiological state nor the degree or type of cell differentiation seem to be important considerations (Pontén, 1971). Nevertheless there are circumstances in which the cell's membrane barrier to infection can be bypassed by means of cell fusion.

The capacity of a cell to initiate and sustain synthesis of infectious virus varies with the virus, the strain of virus, or with the cell itself. Indeed, it has been clearly shown both in the case of infection of different host cells by the same NDV strain (Alexander et al., 1973; Huppert et al., 1974) and when same host cell is infected with different NDV strains (Reeve and Waterson, 1970; Reeve et al.; 1970), that the host cell is just as important as the

infecting virus in determining the degree and type of cytopathic effects produced. Thus, comparative studies on highly permissive and incompetent cells do not reveal an all-or-none effect, but suggest rather that there is a wide range in the degrees of cellular susceptibility (Huppert et al., 1974).

2 - Viable cell systems

a) In vivo

It is well known that NDV has a wide range of susceptible avian hosts and the role played by migratory and non-migratory birds has been extensively studied, mainly in relation to the potential risks of spreading the disease (Lancaster, 1966; Lancaster and Alexander, 1975).

Apart from poultry, which are the most susceptible natural hosts, other domestic birds have been shown to be susceptible to NDV. They range from pigeons, which are highly susceptible, to water-fowl which are said to be almost wholly refractory. In chickens, several factors including age, climatic and environmental influences as well as methods of management and husbandry, markedly influence the clinical and pathological manifestations of the disease. There are also a number of reports of both naturally occurring and laboratory infections of mammalian species by NDV, but the results appear to indicate that the ability of the virus to replicate in these hosts is not so great as that in birds (Hanson et al. 1951). On the other hand, Malik and Dhawedkar (1970) were able to isolate a lentogenic strain of NDV from a pig which gave satisfactory results when used as a live vaccine. It is also known that NDV is capable of producing natural infections in humans, particularly those working in close contact with clinically affected birds. This has been confirmed by serological studies on human populations and it is possible, therefore, that agriculture workers, vaccinators, and others connected with the poultry industry may play a role in spreading the infection from flock to flock (Trott and Pilsworth, 1965; Pastor and Galiano, 1969).

b) In vitro

Newcastle disease virus was first propagated in vitro by Topacio (1934), using explants of chick embryo tissues maintained in Tyrode's solution. Since then, the virus has been cultivated successfully on a wide range of avian and mammalian cell cultures. Although cells derived from chicken embryos are probably the most susceptible to all strains of NDV, a number of authors including Mason and Kaufman (1955) and Huppert et al. (1974) have observed differences in susceptibility among certain types of avian cells.

A variety of primary, secondary and continuous cell cultures of mammalian origin have been used to propagate the virus and some, such as epithelial cells derived from a carcinoma of the human cervix (HeLa) and a line of baby hamster kidney cells (BHK-21), are particularly susceptible to NDV and are widely used for identification and titration studies (Bankowski, 1964; Hanson, 1972). Others, such as mouse L cells, produce only a very small yield of infectious virus and manifest a discrete cytopathic effect (Wilcox, 1959; Rodriguez and Henle, 1965).

D - Virus-Host Cell Relationship

1 - Cytopathogenicity

The manner in which viruses damage cells and cause cytopathic effects (CPE) provides one of the most important aids in diagnostic virology.

Viruses grown in cell cultures can cause either destruction or transformation of infected cells, or multiply with no obvious cytopathic effect. Paramyxoviruses and other enveloped viruses can induce characteristic cytopathic changes which are frequently useful

as markers in multiplication and titration studies.

a) Haemadsorption

The ability of infected cells to adsorb mammalian and avian erythrocytes was originally described by Shelokov et al., (1958), and is a useful means of detecting virus activity in cell cultures infected with NDV or other membrane-forming viruses. The mechanism of haemadsorption is not fully understood but it seems that the incorporation of newly synthesized viral envelope material into the cell membrane leads to modification of the cellular surfaces which permits haemadsorption of healthy erythrocytes (Marcus, 1962). Other workers have shown significant differences in the time required for virulent and avirulent strains to produce haemadsorption in chick embryo cells (Bankowski, 1964).

Reeve and his colleagues (1970) observed that virulent strains of NDV which have a greater ability to induce syncytial formation, usually produce larger amounts of cellular haemagglutinin than do avirulent strains. They also found that inhibition of viral protein synthesis reduces or abolishes haemadsorption by infected cells (Reeve et al., 1971).

b) Cell fusion

Fusion of adjacent cell membranes by virus action involves different phenomena associated with the formation of two types of polykaryocytes, and have been named respectively, "fusion from within" (FFWI) and "fusion from without" (FFWO) (Bratt and Gallaher, 1969). "Fusion from without" occurs when cells are treated with very high multiplicities of virus (over 1000 particles per cell) of either infectious or ultraviolet inactivated virus. The phenomenon was first described by Okada (1962) who obtained fusion of Ehrlich tumour cells previously treated with Sendai virus.

With NDV, artificially induced polykaryons usually contain between 3-150 nuclei per cell and the process begins within the first hour and reaches its maximum 2-4 hours post-infection. The extent of cell fusion varies only with the input multiplicity and does not require host-specific or virus-specific macromolecular synthesis (Kohn, 1965; Bratt and Gallaher, 1969).

The ability of different strains of NDV to induce FFWO is not associated with virulence, although higher rates of cell fusion are more likely to be produced by virulent strains of virus (Kohn and Fuchs, 1969; Poste et al., 1972).

Cell cultures infected with low or moderate doses of infective NDV show syncytial formation at about 8 hours post-infection. This "fusion from within" is believed to be related to intracellular viral synthesis (Reeve and Poste, 1971; Reeve et al., 1971), and it has been suggested that modification of the cell surface by virus products appears to be the ultimate cause (Ejercito et al., 1968; Alexander et al., 1973).

A theory that differences in the composition of the cell membrane are responsible for variance in cell fusing ability was put forward by Klenk and Choppin (1969; 1970a) who found that cells with higher cholesterol/phospholipid ratios are more difficult to fuse. However, these findings were not confirmed by Poste et al. (1972).

The ability to produce FFWI and the size of the syncytia formed, are directly related to the virulence of the infecting strain of NDV, and can be used as a method of differentiating avirulent from virulent field isolates (Bankowski, 1964; Poste et al., 1972).

Although the speed with which cell fusion occurs depends on the strain of virus and the multiplicity of infection employed it seems that the phenomenon is related to the presence of specific viral

proteins, and is not dependent on virus multiplication (Rifkin and Quigley, 1974).

c) Plaquing ability

As a result of viral infection, areas of cell destruction on monolayer cultures can lead, in certain circumstances, to the formation of macroscopic foci. Such evidence was first recognized by Dulbecco and Vogt (1954) who used it as a parameter for the isolation of pure lines of polioviruses. Since then, the method has been modified and adapted in several viral cloning techniques, or used as a marker to differentiate virus strains by their capacity to produce macroscopic lesions (plaques) of various sizes in monolayer cultures. These plaques are normally examined after staining with neutral red since the lysosomes of viable cells adsorb the dye so that normal cells appear red in colour and are readily seen against dead or dying cells which do not take up the stain.

Plaque formation is related to the virulence of NDV strains for chickens (Daniel and Hanson, 1968). Large plaques are produced by the more virulent strains whereas most avirulent strains do not form plaques (Schloer and Hanson, 1968; Reeve and Poste, 1971).

2 - Alterations of cell metabolism

a) Inhibition of protein synthesis

Many viruses which induce cytopathic effects also inhibit the mechanisms of cellular synthesis. Eablanian et al. (1965), working with polio viruses, has shown that there is no direct relationship between CPE and the inhibition of cellular macromolecular synthesis. Although Thacore and Youngner (1970) reported that the shut-off mechanism of host RNA and protein synthesis in CEF cells infected with NDV was slower and less efficient than the "shut-off" seen in L cells, viral production was greater in chicken cells and caused more severe cell damage than was obtained in L cells.

Wheelock and Tamm (1961b) observed that inhibition of host protein synthesis in NDV-infected cells occurs before any cytopathic effect appears, and that cell protein synthesis seems to be affected directly by viral replication. These results have been confirmed by other workers who also showed by using actinomycin D and cycloheximide as inhibitors of DNA transcription and protein synthesis respectively that synthesis of early NDV proteins was responsible for the inhibition of cellular protein synthesis, and also that the synthesis of these viral proteins requires the prior synthesis of RNA. The degree of inhibition of cell protein synthesis by different strains of NDV appears to be directly related to the virulence of the infecting strain for chickens and chick embryos (Reeve et al., 1971). It should be noted, however, that Moore et al. (1972) found similar levels of inhibition with either virulent or avirulent strains.

b) Effect on cellular RNA and DNA synthesis

Inhibition of cellular RNA synthesis by NDV infection was demonstrated by Wheelock and Tamm (1961) and later confirmed by Wilson (1968) who also showed that the inhibitory effect follows the early synthesis of viral proteins. Wilson also emphasised that the use of cycloheximide and azauridine, as inhibitors of protein synthesis, enhanced the part played by novel viral proteins as a cause of cellular RNA inhibition. Similarly, it was revealed that the extent of inhibition of cellular RNA synthesis varies with the virulence of the infecting NDV strain.

Moore et al. (1972) studied 13 strains of NDV and showed that cellular RNA synthesis was blocked by both virulent and avirulent strains of virus. Recently, it was reported that there is a direct relationship between the virulence of the NDV strain and the cessation of host RNA production (Alexander et al., 1973). Several authors

have also proposed that one of the consequences of NDV infection is inhibition of DNA synthesis (Wheelock and Tamm, 1961; Ensminger and Tamm, 1970a).

Further experiments have supported these earlier findings and have shed some light on the shut-down mechanisms of cellular DNA synthesis, by revealing that NDV inhibits cellular DNA synthesis only after the cessation of protein production. Also that viral blockage of cellular protein synthesis affects the process required for the initiation of DNA production (Ensminger and Tamm, 1970b).

3 - Interferon production

Isaacs and Lindenmann (1957) reported that a soluble substance produced by virus infected cells plays a major role in the phenomenon of viral interference. They named the substance "interferon" and showed that it could be induced in a wide range of cells infected with either live or inactivated viruses.

The virulence of NDV is related to its sensitivity to interferon, as has been shown in some other viral systems where less virulent strains are more sensitive to interferon than are more virulent strains (Wagner, et al., 1963). However, Baron (1964) has shown that in nine strains of NDV which differed in virulence, all seemed to be relatively insensitive to interferon.

It has also been suggested that the lack of interferon production by some infectious virus might be due to the more rapid inhibition of cell macromolecular synthesis compared with that of less aggressive virus (Fenner, 1968; Gandhi and Burke, 1970). However, studies conducted with NDV in L cells and chick embryo fibroblasts showed that interferon production continues in L cells even though the shut-down of cellular synthesis is faster and more efficient than in CEF cells (Thacore and Youngner, 1970). Furthermore, the absence of interferon

induction in CEF cells was not related to the general stoppage of the mechanisms of protein synthesis in host cells but rather to the failure of some other more specific event.

Lomniczi (1973) confirmed the insensitivity of NDV to interferon and also discovered that mesogenic and velogenic strains are better inducers of interferon in embryonated eggs than are lentogenic strains.

E - Phenomenon of Viral Persistence

1 - Definition of persistent viral infection

The commonest trend shown by pathogenic viruses in living cells is that of a productive growth cycle which normally leads to the release of newly assembled virions and destruction of the host cell. However, there are many factors which may modify this productive cycle, mainly because the cellular pathways are the unique alternatives employed by viruses for their replication. Thus, any deficiency or inability at the cell level to adsorb, uncoat, synthesize or assemble the virions may result in failure or other abnormality of the infectious viral cycle (Johnson, 1974).

Such occurrences yield mainly defective viral particles which are unable to destroy the host cell and, even with highly cytotoxic viruses, this may lead to the establishment in vivo or in vitro of viral infections that persist for variable lengths of time in the host cell or its progeny.

The relationship between persistent infections and defective viruses shows a number of unusual features. For example Huang and Baltimore (1970) consider that the shorter nucleic acid molecules of defective virus particles compete more effectively for replicase and are produced in greater numbers than those of complete virions. It

is generally agreed that the production of defective virus is a characteristic of many types of persistent infections (Fucillo *et al.*, 1974), and recent work with NDV has shown that spontaneous selection of temperature-sensitive (t.s.) mutants play a decisive role in the establishment and maintenance of persistent infections in mammalian cell lines (Preble and Youngner, 1972; 1973b; Youngner and Quagliana 1975). In the same way, studies of chronic viral infections generally show that t.s. mutants differ in several ways e.g. virulence, plaque size and antigenicity from the original virus population used to establish the persistent infection (Preble and Youngner, 1975).

More recent evidence suggests that the persistence of virus in living cells provides a mechanism for the exchange of genetic information in the biosphere (Reaney, 1974; Zhdanov and Tikchonenko, 1974). This hypothesis is based on the well established fact that the number of defective particles in virus populations greatly exceeds the number of complete virions. Indeed, cytopathic viruses may be the exception to the general rule in that many potentially infectious agents remain in the latent state in their natural hosts.

Furthermore, defective particles are known to be capable of genetic interaction, and of interfering specifically with the intracellular replication of non-defective homologous virus. In this way they may act as reservoirs for the persistence of pre-adapted variants (Markushin, 1975).

Finally, the association between the information contained in the genome of RNA viruses and the chromosomes of infected cells, which is considered to be an exclusive feature of oncogenic viruses, has recently been detected in a number of other groups of RNA virus (Simpson and Iinuma, 1975; Haase *et al.*, 1976). These observations besides providing an hypothetical tool in evolution, may also fulfil

a decisive role in the maintenance of a number of persistent viral infections.

2 - Classification of persistent infections

a) In vitro

The first attempt to classify persistent infections was made by Ginsberg (1958) who suggested that there were three main types, namely: a) cell cultures in which the majority of cells were primarily resistant to infection; b) resistant cells which were selected by viral pressure from a population of which the majority of cells had been killed by the viral infection; and c) cell cultures which were highly susceptible to infection.

The mechanisms responsible for these different categories of persistent infections include i) natural or selected resistance, ii) the presence of non-infectious material and iii) the presence of inhibitory substances in cell culture fluids. An alternative theory was proposed by Walker (1964), who also based his classification on the mechanisms by which cells escaped destruction.

However, a number of features concerning the general characteristics of persistent infections should be considered before an attempt is made at their classification. These include the necessity for specific antibody to be incorporated in the cultural medium; cloning of the infected cells in the culture; resistance to superinfection; the percentage of infected cells in the culture; and the presence of interfering factors apart from antibody in the surrounding medium.

In Walker's report, the following four categories were proposed in an attempt to classify the majority of persistent infections: I) infections in cultures of genetically resistant cells; II) infections in cultures of genetically susceptible cells protected by antiviral factors in the medium; III) infections in cultures of genetically

susceptible cells protected by interference or interferon; and
IV) regulated infections of cells in culture.

The types of persistent infection listed above still prevail today although some modifications have been introduced more recently mainly with regard to the introduction of a more generic approach to the simplified nomenclature used.

Fraser (1967), on the basis of virus production studies in cell cultures infected with myxoviruses, suggested that there are two types of imperfect growth cycles, namely, a) an abortive infection in which viral replication may have begun but no progeny virus is obtained, and b) defective infections in which the amount of infectious virus released is minimal compared to the quantity of viral material produced. Two additional categories of defective infections have been proposed and are named "self-limiting" and "perpetuated" depending on whether the infection is terminated or the virus enters into a type of persistent infection.

On the assumption that oncogenesis is a form of persistent infection, Fenner et al. (1974) suggested that there are three main categories a) tumour viruses in which the viral genome is integrated with that of the cell; b) steady-state infections in which non-cytocidal viruses are continuously produced by all cells; c) carrier cultures in which a cytotoxic virus is inhibited by any factor present in the medium or when a minority of susceptible and a majority of genetically resistant cells are present in the culture.

A similar classification, comprising only two groups, was postulated by Davis et al. (1973) for persistent infections both in vitro and in vivo namely: a) endosymbiotic infections where there is an association between the viral nucleic acid and the cellular chromosome; and b) the carrier state, when infection of a few cells in the culture is kept low by a number of factors such as partial resistance of the cells

to viral infection, the presence of antiviral agents in the medium, and cell-to-cell transmission of infection.

b) In vivo

The behaviour of a virus in cell cultures may bear no relationship to the disease pattern caused by the agent in its normal animal host. Nevertheless, it has been suggested that several examples of virus persistence in vitro, due to cyclical transient infection, bear a close similarity to naturally occurring infections in animals (Hotchin, 1974).

Moreover, it should be stressed that host defence mechanisms may also play a decisive role in the establishment and evolution of persistent infections in vivo. In a recent review, Mims (1974) drew attention to various components of the host antiviral defence, with special emphasis on the role of the lymphoid system as a potential shelter for viruses awaiting to reappear as direct or opportunistic pathogens.

The effects of viral activity in vitro and in vivo are not dissimilar. In the animal host, the usual pattern of a pathogenic virus is the production of an acute infection characterized by viral multiplication either locally or following haematogenous dissemination, or both. The symptoms of disease are often associated with tissue damage and, unless the disease proves fatal, the host's antiviral defences eliminate the infecting agent.

In contrast to acute infections are those in which the virus persists for months or years in the animal host. Fenner et al. (1974) classified persistent infections in vivo into three categories. These are a) latent or recurrent infections characterized by the fact that the virus is not demonstrable after primary infection, and disease does not occur until provocative factors initiate active viral multiplication, cell damage and a localized clinical infection;

b) chronic infections in which absence of disease is the common feature although infectious virus is always demonstrable, and the infecting agent is often shed or integrated with the cellular genome of the host cells; and c) slow infections when, after a long incubation period, a slowly progressive disease develops.

3 - Induction "in vitro" of persistent infections

a) Carrier state

Animal cell cultures often become persistently infected after they are deliberately inoculated with any one of a wide range of viruses. This gives rise to persistent replication of the virus while the culture survives and continues to grow.

The majority of known persistent infections have been categorized as carrier state cultures, the name being derived from a similar phenomenon that occurs between certain bacteria cells and bacteriophages (Lwoff, 1953). This carrier state is characterized by the fact that only a small proportion of the cells in the culture seem to be infected. In these, a regular viral replication cycle takes place usually leading to cell death, whereas the released infectious virus is capable of infecting only a small number of the cells remaining in the monolayers.

Several conditions are necessary before a cell culture becomes a carrier. For example, the culture may be genetically resistant or becomes resistant by the selective action of the virus. On the other hand, it is possible that some susceptible cell variants may become infected by extracellular virus in the medium allowing virus replication and thereby maintaining the infection (Puck and Cieciura, 1958; Takemoto and Habel, 1959). In this type of carrier culture

only the innate resistance of the cells protects them from destruction by the virus, although some antiviral factors are frequently needed in the medium to maintain equilibrium. The antiviral factors may be of two types: extracellular, as in the case of antibody that must be supplied in the culture medium to protect the cells and limit the transfer of the virus from cell to cell (Wheeler, 1960); or intracellular with the production of substances such as interferon or other inhibitory factors which renders most of the cells temporarily resistant (Henle, 1963). In some cases, the cell-virus relationship shows a consistent pattern of cyclical rising and falling in the levels of viral infection, this so-called "crisis" of the persistent infections seems to be related either to the genetic characteristics of the cells or to the presence of viral interference factors, such as interferon (Wiktor and Clark, 1972; Ehrnst et al., 1974).

Apart from the features mentioned above which relate to the proportion of infected cells in carrier cultures, another general characteristic of the carrier state is the case that cultures can be cured of infection either by cloning the cells in the presence of antiviral antibody or simply by maintaining the cultures in a medium containing antiviral serum. In this situation infected cells may divide for a limited number of generations but only viable clones of uninfected cells are ultimately obtained (Fernandes et al., 1964)

Because frequent changes of media may remove inhibitors of viral replication (e.g. interferon or interfering particles) from the medium, this may alter the state of equilibrium in the carrier culture and lead to increased virus production and cell damage.

b) Endosymbiotic infections

The term endosymbiotic infection was introduced by Fernandes et al. (1964) in view of the fact that a close and non-pathogenic relationship develops between the virus and the cells. Regulated

infections (Walker, 1964) and steady-state infections are synonyms used to describe identical virus-cell systems.

Paramyxoviruses are among those RNA viruses which are said to be capable of inducing this type of infection. In these persistent infections the majority of the cell population is infected, and the affected cells divide and grow into infected clones. However, since the infection cannot be cured by the addition of antiserum to the medium, this suggests that an intracellular transmission mechanism is present. Furthermore, the virus does not seem to go through the usual replication cycle and appears to be under some sort of intracellular regulation or control. With the exception of those examples that are caused by cytotoxic DNA viruses (Hinze and Walker, 1971), the majority of persistent infections are induced by enveloped RNA viruses which bud from the plasma membrane of infected cells sometimes without killing them (Walker and Hinze, 1962b).

Several theories have been proposed in an attempt to explain the mechanisms responsible for the establishment and perpetuation of these forms of persistent infection. It has been suggested, for example, that the infection may persist because the rate of replication of the virus is low enough to allow the cells to survive and multiply; or that there is a sufficient number of viral nucleic acid molecules or viral particles in the cell to ensure infection of most daughter cells by random segregation at mitosis (Davis et al., 1973). On the other hand, a number of workers consider that there is an association between the viral nucleic acid and the cellular chromosomes and this latter phenomenon, which was previously considered to be related only to oncogenic RNA viruses, has recently been shown by Simpson and Iinuma (1975) to occur with other viral infections, also.

4 - Virus-host cell relationship

There is abundant evidence to show that the virus in persistently infected cultures is evolved in response to various selective pressures within the carrier system. Similarly, it is believed that the presence of the virus affects the host cell in some way and leads to a wide range and degree of metabolic or functional alterations; and may even induce cellular transformation.

a) Properties of defective virus

i - Physical properties and virulence

The paucity of information available concerning the physical properties of the virus released from persistent infections is mainly due to the fact that mature virions are released in very low levels, if at all, and because the methods used to isolate and purify such agents in practical quantities are inadequate.

However, results obtained from studies on carrier cultures of human embryonic lung (Lu106) persistently infected with measles virus show that the agent produced bands at lower density in caesium chloride gradients than were obtained with the wild-type Edmonston strain used to induce the infection (Norrby, 1967).

Similarly, comparative studies of the properties of the RNP of Sendai virus in three different host systems suggest that the viral polypeptide isolated from the cytoplasm of infected non-permissive Ehrlich tumour cells has a slower migration in CsCl gradients and is sensitive to ribonuclease digestion, in contrast to egg-grown or mouse fibroblast passaged virus (Vorkuna et al., 1974). Studies on chronic infections in cattle with foot-and-mouth disease lead to the recovery of the virus many months after the onset of the disease. The agent was shown to be less virulent for experimental animals and had a higher stability at 56°C and at low pH than field strains of the virus (Fellowes and Suttmoller, 1970; Straver and van Bekkum, 1972).

Furthermore, Thacore and Youngner (1971) have reported that both the haemagglutinin and neuraminidase of an NDV mutant isolated from persistently infected L cells are more resistant to thermal inactivation than those of the original wild-type virus.

The detection and study of infectious virus released from persistently infected cultures are probably the most intensively studied parameters in carrier state infections. In some virus-cell systems a low but continuous release of infectious virus has been reported which often shows a decreased infectivity and/or virulence, as measured by the size of plaques produced in appropriate indicator cell systems, or by the survival of experimentally inoculated animals or embryonated eggs (Takemoto and Habel, 1959; Walker and Hinze, 1962b; Fernandes et al., 1964; Thacore and Youngner, 1969; Schwobel and Ahl, 1972; Gavrilov et al., 1972). In a recent report of persistently infected mammalian kidney cells with NDV, Fraser et al. (1976) associate the failure to detect infectious virus in two of these cultures (ox and pig) as well as the inability of haemagglutinin to elute from agglutinated erythrocytes, with the virtual absence of neuraminidase activity in the persisting virus.

Many persistent infections are characterized by the fact that the equilibrium between cell and virus is broken and, consequently, infectious virus is released into the cultural medium in greater amounts than normally, and cytopathic effects appear in the host cells. Such is the case in Lul06 cells persistently infected with measles virus in which the titre of the extracellular agent shows temporary variation (Ehrnst et al., 1974). Also, Perekrest et al. (1975) studying a persistent infection of pig embryo kidney cells with an influenza virus variant (PEK_{WSN}) showed that infectious haemagglutinating virus was demonstrable only at certain passage

levels. By contrast, a number of persistent infections have been described in which there appears to be no release of infectious virus into the culture fluids; nor can the release of extracellular virus be induced by cell fusion or co-cultivation techniques. It is emphasised, however, that the inability to detect cell-free virus in these circumstances might be due to lack of sensitivity in the methods used (Rustigian, 1966; Fraser et al., 1976; Flanagan and Menna, 1976).

ii - Defective viral synthesis

Studies on the mechanisms involved in initiating and maintaining persistent viral infections in animal cells have mostly been directed towards an understanding of viral synthesis and maturation of the virus at the cell surface.

The question of defective virus assembly has been approached from two main angles, namely: a) possible blockage by a viral or cellular event of virus completion and b) a qualitative or quantitative defect in viral polypeptide synthesis, prior to assembly of the component parts of the intact virion. In the first instance, several authors have shown that the viral proteins required for virus assembly are synthesized and accumulated at the cell membrane, while the control of viral maturation by cellular repressors has been put forward as an explanation of the continued suppression of the release of measles virus in persistently infected HeLa cells (Rustigian, 1966).

In 1969, North^rop investigated a line of human conjunctival^c cells persistently infected with mumps virus (C-M) and showed that when puromycin was added to the cultures to inhibit protein synthesis, a dramatic increase occurred in the percentage of haemadsorbing cells and in the proportion of infectious virus recovered from the cultures. These observations suggested that failure in viral assembly could be due to the presence of viral or cell products capable of blocking

any event in viral maturation. Furthermore, it was confirmed by experiments using actinomycin D, that the control of such repressor activity was DNA-dependent.

More recently, it has been demonstrated in African green monkey kidney cells persistently infected with measles virus (BGM/MV) that induction of haemagglutinin activity requires protein synthesis, and that a host-cell protein is responsible for suppressing the production of viral protein (Flanagan and Menna, 1976).

Similarly, Knight et al. (1972) induced the early release of large quantities of infectious virus, which are insensitive to inhibition of protein synthesis, from hamster embryo fibroblasts (HEF) persistently infected with measles virus. The method used was that of co-cultivation of the persistently infected cells with a susceptible cell line of monkey cells (BSC-1).

In turn, studies on an abortive system consisting of mouse L cells infected with NDV showed that synthesis of virus-specific polypeptides is not inhibited, and that the major structural viral proteins become associated with the cell membrane. Even so, only a tiny fraction of the progeny virus is released into the culture medium and most of the intact particles are non-infectious. These results indicate that the blockage in the production of infectious NDV in L cells is more likely to be involved with the composition of the virion itself than with the process of maturation. Hecht and Summers (1974) proposed that the cause of the defect might be associated either with a lack of virion-associated transcriptase or with a viral protein that is absent or produced in insufficient quantity.

A comparison of virus-specific RNA synthesis in primary and persistent infections of HeLa cells with measles virus showed no difference in the type of virus-specific RNA produced, although the primary infection yields three times more low-molecular weight RNA

species (18S) (Winston et al., 1973) than does the other type of infection. This result is probably associated with differences in the method of production of viral-specific proteins in persistent infections.

iii - Temperature sensitivity

A number of reports in the past few years have described temperature-sensitive mutants (t.s.) that are spontaneously selected from persistent infections in vitro. They have also stressed that natural selection of t.s. mutants may be involved in outbreaks of the disease in vivo.

Preble and Youngner (1972, 1973a,b) have conducted a number of extensive studies on the role of virus t.s. mutants isolated from mouse L cells persistently infected with a Herts strain of NDV. They found that NDV from persistent infections does not replicate in primary CEF cells or in bovine or canine kidney cell lines at 42-43°C, whereas the original virus produces approximately normal yields at that temperature. They also showed that the virus variants produce smaller plaques than those of the original virus seed.

Nagata et al. (1972) also described the production of considerable amounts of haemagglutinin at 31°C, in a cell line of baby hamster kidney (BHK-21) persistently infected with Sendai virus, but only restricted quantities of the viral progeny were released at 37°C. Increased virus yields were also obtained following incubation of the persistently infected HEF cells at 33°C rather than at 37°C, but only for a certain number of passages of the carrier cultures (Haspel et al., 1973).

Selection of t.s. mutants also take place in vivo during chronic infections. Isolates of FMD virus from the pharynx of carrier cattle

showed inhibition of plaque production at 40-41°C in contrast to the isolates from vesicular fluids in cases of acute infections (Fellowes and Suttmoller, 1970; Straver and van Eekum, 1972). More recently, monolayer cultures of human brain cells persistently infected with a strain of parainfluenza type 1 (6/94) isolated from a patient with a slow viral disease, produced much higher yields of virus at 33°C than at 37°C, while yields of the putative wild-type virus were the same at both temperatures (Wroblewska et al., 1976).

Populations of wild-type paramyxoviruses are known to contain a small percentage of spontaneous t.s. mutants (Preble and Youngner, 1973a; Portner et al., 1974). The emergence of t.s. mutants in persistent infections does not appear to occur at random and it seems likely that these variants play a role in the establishment or maintenance of the carrier state. It has been suggested, therefore, that these virus clones may be selected during early stages of the persistent infection and that the structural alterations which occurs late in the growth cycle are induced by altered thermal sensitivity (Preble and Youngner, 1973b).

Co-selection of temperature-sensitivity with other viral parameters is not uncommon. However, studies using revertants for t.s.-markers showed that plaque size, virulence or thermal stability do not revert simultaneously, and this suggests that mutation for t.s. and other markers probably occurs independently (Simizu and Takayama, 1971; Preble and Youngner, 1973b; Haspel et al., 1973).

Biochemical characterization of the t.s. mutants isolated from L_{NDV} cell cultures has shown that no virus-specific RNA synthesis takes place at non-permissive temperatures, and that all RNA clones appear to have some defect associated with RNA polymerase activity (Preble and Youngner, 1973a). Later evidence suggests that the t.s. mutants naturally selected in two mammalian cell lines persistently infected

with NDV were also defective in RNA synthesis at non-permissive temperatures (Youngner and Quagliana, 1975).

b) Cell modifications

i - Selective and repressive factors

Little is known about the mechanisms involved and the consequences of viral permanence in host cells from the point of view of cell tolerance, but the available literature suggests that certain properties of both host cell and virus must be altered to allow perpetuation of the infection.

Some authors maintain, or tacitly agree, that the resistance shown by the cell population in persistent infections is achieved through selection of part of the initial colony. Either resistance can develop from a minority of resistant variants in the original cell population under the selective pressure of the virus, or a few susceptible cells may be segregated to start producing interfering factors which protect the majority of the population (Henle et al., 1958; Takemoto and Habel, 1959; Knight et al., 1972; Haspel et al., 1973).

On the other hand, some reports suggest that cell-dependent factors may be responsible for viral control. Huppert et al. (1975) investigated the problem of variability of NDV production according to the cell type used and postulated that permissiveness of a cell for the replication of the virus is not an all-or-none phenomenon but covers different degrees of sensitivity. They also pointed out that the amount of cellular components is critical for a transcriptional step of NDV RNA synthesis to occur. Similarly, the presence of cellular factors which affect the efficiency of continued viral production or assembly of the synthesized viral components in persistent

infections has been proposed (Rustigian, 1966b; Menna et al., 1975a).

A number of biochemical studies have been made in an attempt to explain the molecular events of the cellular mechanisms responsible for maintaining viral repression. Sometimes a cell protein regulator seems to control a phase of viral RNA synthesis and may be decisive as a restrictive device of viral replication (Northrop, 1969). In turn, viral maturation followed by release of fully infectious particles appears to be sustained by cellular blockage at the cell membrane. (Flanagan and Menna, 1976).

At the cytological level, it is apparent that nearly all cells are affected in some way by the presence of the virus. This is evident when comparisons are made between the carrier cultures and the original uninfected cell lines; and some authors have noted lower growth rates and final yields of cells together with increased aerobic glycolysis and marked resistance to other viruses (Henle et al., 1958; Norrby, 1967; Zhdanov et al., 1973).

However, in the majority of reports on persistent infections no significant morphological or functional cellular alterations have been reported, other than those biological activities associated with the presence of the virus at the cell membrane, e.g. haemadsorption and cell fusion.

Regulated persistent infections seem, nevertheless, to bear some of the general characteristics shown by transformed cell cultures infected with tumour viruses. In both systems a very stable cell-virus relationship is developed, and the probable integration of a pro-viral intermediate into the cellular chromosomes of the carrier cell may explain the transformation that occurs in persistently infected cells (Walker et al., 1966; Zhdanov and Parfanovitch, 1974; Simpson and Iinuma, 1975).

ii - Accumulation of viral material

A common feature of persistent viral infections is the very low yield of virus released from infected cells. In general, viral RNA and polypeptide synthesis gives rise to a large amount of viral aggregates that can be seen by immunofluorescence staining and in electron microscopic preparations, lying within the cytoplasm and/or nucleus, or in close association with the cell membrane.

The rapid release of assembled virions and the fact that no intracellular accumulation of infectious virus can be detected, except at the cell surface, lead to the assumption that the intracellular aggregation is mainly due to defective viral antigens (Walker and Hinze, 1962a). Likewise, there are a number of reports from studies on persistent infections demonstrating the presence of large masses of viral antigen, sometimes filling most of the intracellular area, without any apparent alteration in cell morphology or function (Norrby, 1972; Menna et al., 1975a). Such material appears to be mainly composed of viral ribonucleoprotein in which viral RNA is associated with viral and cellular proteins, and has been shown to be viral specific by immunological techniques (Norrby, 1972; Zhdanov et al., 1973; Dubois-Dalcq and Barbosa, 1973). These RNP filaments may be arranged at random or in an orderly disposition, and thus reflects a change from early to late stages of cellular infection (Tawara, 1965).

The mechanisms by which viral material is prevented from reaching the cell membrane but accumulates in certain areas of the cell interior are still not known. The sites at which those viral inclusions develop depend upon the characteristics of the virus replicative cycle, although some viruses, including certain paramyxoviruses, seem to contradict the normal pattern. In fact, the measles-rinderpest-distemper (MRD) triad which are now included in the family Paramyxoviridae frequently shows both types of viral inclusions, within the cytoplasm and nucleus of the infected cells.

Other members of same viral family, e.g. NDV, which are thought to have only a cytoplasmic replication phase are capable of inducing both types of inclusions either in primary or persistently infected cell cultures (Omar, 1965; Fraser et al., 1976).

c) Role of interfering agents

The interfering agents present in the fluid phase of some persistently infected cell cultures play a decisive role in perpetuating the infection. There can be no doubt from the data available that two different mechanisms of viral interference take place in carrier cultures. One is related to the production of defective viral particles, while the other is involved in the production of interferon or interferon-like substances, by the induced cells.

The question of protective mechanisms being generated in persistent infected cultures was raised in a number of early reports and it was suggested that the key factor in the protective process is due to the temporary refractory status of the cell arising from interference by non-infectious virus in the monolayers (Chambers, 1957; Henle et al., 1958). Additional evidence from subsequent reports suggested that the effect was probably due to induction of interferon production by defective particles in the viral population (Henle, 1963; Rodriguez and Henle, 1965). Furthermore, Rodriguez et al. (1967), working with NDV obtained from embryonated hens' eggs and persistently infected mouse L cells, clearly showed that avirulent virus contained one or more components which interfered with the replication of any infectious virus particles present in the inoculum, and they suggested that this component was the most likely inducer of first-cycle interferon synthesis.

However, observations made by Youngner and Quagliana (1975) on BHK cells persistently infected with NDV seemed to indicate that

although a significant amount of non-defective haemagglutinating particles was present, insufficient interferon was available for the establishment or maintenance of the persistent infection.

The data which have been accumulated concerning biologically active defective particles in animal virus systems, led Huang and Baltimore (1970) to suggest that since such particles are capable of specific interference with homologous, original virus, they may play a role in the evolution of a number of viral diseases, including self-limiting and persistent infections.

Recently, a defective-interfering particle (T particle) generated during the induction of carrier cultures has been implicated in the establishment and maintenance of persistent non-cytocidal infections, even when it is present in infected cells along with virulent wild-type virus (Holland and Villarreal, 1974).

However, the need for interferon as a means of establishing a persistent infection in cell cultures is widely accepted. The fact that interferon or interferon-like substances are released into the fluid phase of cell cultures chronically infected with virus was originally reported by Ho and Enders (1959) and Henle *et al.* (1959) who showed that frequent changes of the medium upset the equilibrium of the carrier system and led to increased viral production and cell damage. They also found that there was a positive correlation between the levels of cell resistance and interferon production. Evidence that the addition of exogenous interferon could establish, maintain and even cure persistent infections was reported by Glasgow and Habel (1962) and confirmed later by Hallum *et al.*, (1972).

It is well known that the induction of interferon synthesis by viruses requires the production of both cellular RNA and protein. On the other hand, observations made on L cells persistently infected with NDV, led to the conclusion that replication of viral RNA is not related

to the induction of interferon synthesis. However, only a small residual fraction of the host-synthesizing activity is necessary for the production of significant amounts of interferon (Thacore and Youngner, 1970).

5 - Virus-cell association

Most species of cells are known to have one or more types of virus associated with them (Reaney, 1974). Three types of relationship may be distinguished so far as the consequences of viral infection on the viability of the affected host cell are concerned. These are named lytic, asymptomatic, and symbiotic. In the latter two groups, the progeny virus causes practically no damage to the cell and may even prove beneficial. In addition viruses may be divided into two main categories according to the relationship established between the viral and cellular genomes, namely: infectious viruses in which the viral genome remains autonomous, and integrated viruses where the viral genome may interact with the cell chromosome. It should be noted, however, that the same virus may behave either as an infectious or an integrated virus, according to the species and physiological state of the cell infected (Zhdanov and Tikchonenko, 1974).

a) Viral integration on host-cell DNA

The discovery of an enzyme called RNA-directed DNA polymerase (RDDP) or reverse transcriptase, that induces the synthesis of double-stranded DNA that is capable of being integrated into the cell chromosome from the template of the virion RNA, was made during studies on RNA tumour viruses (Temin and Mizutani, 1970; Baltimore, 1970). However, there is increasing evidence that the distribution of RDDP is not only concerned with tumour viruses but is also present in several non-oncogenic RNA viruses. So far, the majority of these

viruses are related to slow virus diseases or to persistent infections in vitro (Lin and Thormar, 1970; Parks and Todaro, 1972; Furman and Hallum, 1973).

Only later did it become known that the formation of proviral DNA intermediates by RNA viruses could be achieved through the mechanism of RNA-dependent DNA polymerase. The transcription of the RNA template into single-stranded DNA seems to be the first step in the reaction. The second phase comprises synthesis of double-stranded DNA, which is actinomycin D sensitive (Faras et al., 1971).

However, controversy still exists as to whether reverse transcriptase is present as a virion enzyme or if it is associated with an endogenous virus already residing in the cells. In this situation, transcription of the RNA virus into a DNA intermediate could be mediated by the enzyme of the latent virus (Zhdanov and Parfanovitch, 1974; Simpson and Iinuma, 1975).

Three theories have been formulated recently to explain the origin and mechanisms of integration of the genetic information of RNA tumour viruses into the host cell genome. Originally, a theory was put forward by Temin (1964) who considered that RNA tumour viruses have a DNA synthetic step in the course of their replication cycle which facilitates integration of genetic information into the genome of the transformed cell. The discovery that a reverse transcriptase enzyme is carried by members of this viral group seems to confirm this provirus hypothesis. Based on extensive studies concerning the inducement of C-type viral particles from apparently virus-free cell lines, Huebner and Todaro (1969) proposed that the cells of most or all vertebrate species contain genomes of RNA tumour viruses and that the virus-specific information is transmitted vertically. A control mechanism exists to regulate the expression of the virogene, and activation is achieved when the regulation system breaks down (Huebner et al., 1970).

Support for the oncogene theory has consistently been obtained from studies on the induction of C-type viruses in a number of healthy vertebrate cells (Aaronson et al., 1969; Klement et al., 1971; Lieber et al., 1973; Todaro et al., 1974; Panem et al., 1975).

More recently, Temin (1971) proposed a so-called provirus theory which includes a possible mechanism of genetic amplification and cellular differentiation. According to this hypothesis, viral integration is believed to be created by successive RNA to DNA to RNA information transfers, and could lead to modification of the original information.

b) Rescue of integrated provirus

The induction of viral oncogenes or provirus can be obtained by several biological, physical or chemical methods. For example, Aaronson et al., (1969) described the release of mouse leukemia virus from uninfected BALB/c mouse embryo cells after prolonged propagation. Another method often chosen to induce repressed viral information in living tissues is co-cultivation. By this method explants or cell suspensions of animal tissues are co-cultivated with indicator cells for variable periods of time, when an increased amount of the repressed virus is released into the culture fluid (Watkins and Dulbecco, 1967). The mechanisms involved are not clearly understood but it is interesting to note that even greater yields of virus are obtained if UV-inactivated Sendai virus is added to the mixed cell suspensions. It has been established that Sendai virus promotes cell fusion and it seems likely that the rescued virus is produced by heterokaryons obtained by fusion of carrier cells with those virus-susceptible cells (Gerber et al., 1968).

Although ionizing radiation techniques and the isolation of infectious proviral DNA have also been used successfully for the rescue of integrated genomes (Hill and Hillova, 1972b; Lieber et al., 1973),

chemical induction is still the commonest procedure for the recovery of proviral DNA.

Most reports refer to the induction of C-type particles and other RNA oncogenic viruses from a wide range of cell species, either in primary cultures or in established cell lines. In so far as the methods employed to help expression of integrated genomic information are concerned, one of the most useful procedures involves the utilization of chemical compounds like the halogenated pyrimidine derivatives, for example, 5-iodo-2-deoxyuridine (IdU) and 5-bromo-2-deoxyuridine (BrdU).

Activation of integrated viral DNA requires incorporation of pyrimidine analogues into the cellular DNA, but little is known about the mechanisms involved in the induction of the virus (Rowe et al., 1972). The analogues, although mutagenic, are known to have a specific effect on the cellular metabolism. This is in contrast to other mutagenic drugs which can induce a much lower degree of viral expression simply as a result of non-specific cell toxicity (Aaronson, 1971).

c) Isolation and infectivity of cellular DNA

Hill and Hillova (1971) pioneered the rescue of integrated RNA viral genomic material by the isolation of cellular DNA of infected cells, followed by inoculation of the isolates into suitable indicator cell systems. The circumstances in which exogenous DNA changes the phenotype of recipient cells has been called a transfection event, and the DNA is known as the infectious proviral DNA.

In the uptake of foreign DNA the cellular membrane apparently does not function as a barrier for DNA molecules that are present in the extracellular environment, and it is claimed that exogenous DNA is readily taken up by the cells and penetrates into the cell nucleus (Hill and Hillova, 1974). It is believed that both phenomena may

occur in vitro or in vivo (Bhargava and Shanmugan, 1971).

The uptake of infectious DNA is greatly increased by prior or simultaneous treatment of the recipient cells with DEAE-dextran (McCutchan and Pagano, 1968), or by the formation of co-precipitates of DNA with calcium phosphate (Graham and van der Eb, 1973).

Foreign DNA can integrate into the host cell genome by a recombination process which requires cellular DNA synthesis. Two kinds of recombination events have been described in studies of chicken cell cultures transfected with mouse DNA. One relates to nascent pieces of cellular DNA which are inserted into free mouse cell DNA, while the other consists of single-stranded units of donor DNA recombined with nascent strands of chicken cell DNA and integrated into the cellular chromosome (Hill and Hillova, 1971).

More recent evidence suggests that genetic recombination at the DNA level with either host cell genomes or other viruses, is not an exclusive property of RNA tumour viruses, but may also occur in a number of other RNA enveloped viruses. If these findings are confirmed they may have important implications on the mechanisms of information transfer in eukaryotic cells and on the origin of novel viruses in nature (Zhdanov and Parfanovich, 1974; Zhdanov et al., 1974; Simpson and Iinuma, 1975).

F - Persistent Infections of Cell Cultures by NDV

1 - Survey of literature

Persistent viral infections are relatively common in natural and artificial biological systems, and accounts of enveloped RNA viral persistent infections with the characteristics of regulated infections have frequently been reported in the scientific literature. The main features shared by this large group of viruses, which includes Paramyxovirus, Togavirus, Rhabdovirus and Retravirus are related to the

fact that they do not shut down cellular metabolism and are released from the cell membrane by a process of budding.

Although NDV is the type species of paramyxoviruses and has an ubiquitous capacity for infection of host species, most of the in vitro studies of persistent infections with NDV have been confined to a limited number of cell types. In their original report, Puck and Cieciura (1958), succeeded in inducing a persistent infection of HeLa cells following several challenges with NDV and were able to maintain the infection for over two years.

In 1958, Henle and collaborators gave a detailed description of the establishment and characteristics of persistent infections of Earle's mouse strain of L cells and Lung-To cells (human embryo lung cells) with NDV (Henle et al., 1958; Bergs et al., 1958; Deinhardt et al., 1958). Their later studies were made using the L_{NDV} cell system with emphasis on interference and the role of interferon in persistent infection (Henle et al., 1959; Henle, 1963).

Meanwhile, Wilcox (1959) described a transient persistent infection of L cells with NDV in which the cultures eventually regained their susceptibility to the original inducer virus. Mason and Kaufman (1961) observed differences in the ability to maintain persistent infections of L and human uterus (U/12) cells probably due to factors inherent in each cell type. Attempts by Rodriguez et al. (1965; 1967) to identify the factors responsible for establishment of NDV persistent infections of L cells marked a turning point in this field of study. Indeed, L cells persistently infected with NDV became the most widely used model for this type of investigation, and the works of Thacore and Youngner (1969; 1970; 1971) who isolated and characterized a considerable number of viral mutants, showed clearly that these differed from the original virus. Using this model as a basis, Preble and Youngner (1972; 1973a,b) showed that several viral mutants had

temperature-sensitive properties, and their findings led them to propose a theory to explain the aetiology of persistent infections in vivo and in vitro (Preble and Youngner, 1975). The fact that certain mutants in persistent NDV infections of BHK-21 and canine kidney (MDCK) cells were temperature sensitive seem to confirm this hypothesis (Youngner and Quagliana, 1975).

The only information available about the existence of persistent NDV infections in cells other than those of mammalian origin is contained in a recent report of prolonged viral infection in embryonic chicken tracheal explants (Cummiskey et al., 1973). Finally, Fraser et al., (1976) reported an accidental persistent infection of NDV in three lines of mammalian cells (pig, ox and sheep) having all the characteristics of regulated infections.

2 - Objectives

This current study is based upon experimental work relating to two types of persistent NDV infections in an established line of bovine kidney cells (MDBK).

The original model, MDBK_{pi}, has been previously identified and characterized and refers to an accidental regulated infection which had been maintained in this laboratory for more than ten years (Edwards, 1972; Fraser et al., 1976; Ruben, unpublished observations). The second system concerns an experimentally induced persistent infection with NDV also in MDBK cells (MDBK_{CS}) with features that have some bearing upon the carrier state model.

One of the aims of the present study was to obtain knowledge of the mechanism involved in the establishment of a carrier cell culture according to the conditions employed for the inducement of the infection.

The presence of an inhibitory factor in the culture media of MDBK_{CS} cells, other than interferon, was detected, and this observation

raised the question as to whether other mechanisms at the cellular level could be responsible for initiating and maintaining the carrier state in cell culture systems.

Following the achievement of a stable carrier culture, studies were made in relation to the mechanisms of viral synthesis in both models, in an attempt to correlate the production of defective virus with possible errors or blockage of viral replication at the cellular level.

Similarly, the unexpected discovery of an accumulation of viral material either within the cytoplasm or nucleus of MDBK_{pi} cells prompted further investigations into the viral specificity and morphological characteristics of these unusual structures.

Attempts were also made to transfect any integrated information of the two persistent infections in order to shed some light on the mechanisms involved in viral perpetuation. This was done, following recent reports of reverse transcriptase activity in NDV mutants found in persistent infections, and of an increased production of virus in cells containing integrated viral material in cellular chromosomes, after treatment with DNA analogues.



MATERIALS AND METHODS

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A - REAGENTS

I - Radioactive material

The following radio chemicals were obtained from the Radiochemical Centre, Amersham, Buckinghamshire:

[5,6-³H] - uridine with a specific activity of 43Ci/mmol

L - [U-¹⁴C] - leucine with a specific activity of 324Ci/mmol

L - [4,5-³H] - leucine with a specific activity of 57Ci/mmol

II - Buffers

Dulbecco's phosphate buffered saline (PBS), pH 7.2, was purchased as PBSA from Oxoid, London.

Phosphate buffer (PB), pH 7.2, is 0.01M sodium dihydrogen phosphate adjusted to pH 7.2 with 1.0M sodium hydroxide.

Saline trypsin-versene (STV) containing 0.01% trypsin (1:250 trypsin, Difco, Detroit, U.S.A.) and 0.01% versene (ethylenediamine-tetracetic acid), in PBS.

SSC buffer consisting of 0.15M NaCl

0.015M sodium citrate

pH 7.0

EDTA buffer

0.15M NaCl

0.1M ethylenediaminetetracetic acid,
pH 8.0

HeBs buffer

NaCl, 8g/litre

KCl, 0.37g/litre

Na₂HPO₄ · 2H₂O, 0.125g/litre

Dextrose, 0.1g/litre

N-2-hydroxyethylpiperazine-
N-2-ethane sulphonic acid
(Hepes), 5.0g/litre, pH 7.05

NaCl-Tris consisting of 0.14M NaCl

0.05M tris-HCl,
at pH7.4

III - Cell culture media

Minimal essential medium (MEM), Eagle's Dulbecco Glasgow modification, and basal medium Eagle's (BME) were purchased as 10x concentrates without sodium bicarbonate and glutamine from Gibco-Biocult, Paisley, Renfrewshire.

Pooled calf serum was sterilised by positive filtration through a membrane (APD 0.2 μ m) and inactivated by heat at 56°C for 30 minutes.

Sodium bicarbonate in 4.4% solution was autoclaved and added to the medium to give a final concentration of 0.16%.

L-Glutamine, 200mM, purchased as 100x concentrate from Gibco-Biocult, was added at 1% to all culture fluids.

Antibiotics were incorporated in all media in the following concentrations: penicillin (sodium benzyl penicillin, Glaxo, Greenford, Middlesex), 100 units per ml; and, streptomycin (streptomycin sulphate, Glaxo), 100 μ g per ml.

IV - Other reagents

Schiff's reagent (McGuckin and McKenzie, 1958)

0.8% potassium metabisulphite

1.14% 12N HCl

0.4% basic fuchsin (pararosaniline) in water

The ingredients were slowly stirred in a dark room at room temperature for 2 hours and allowed to settle for a further period of 2 hours.

The colour had then changed to yellow-brown. Activated charcoal was then added, and the mixture stirred briefly and filtered to yield the colourless reagent.

All other chemicals were normal laboratory reagents of the "Analar" grade, unless otherwise specified.

V - Erythrocytes

Fowl red blood cells were obtained by venipuncture from healthy, unvaccinated adult hens and were treated with sterile 3.8% sodium

citrate to prevent coagulation. After three washes with PBS, stock suspensions of 0.3% and 1% were made up in PBS and stored for 3-4 days at 4°C.

Guinea-pig erythrocytes were obtained by heart puncture of anaesthetised, healthy animals and were treated in the same manner as fowl red blood cells. Stock suspensions were made of 0.4% concentrated erythrocytes in PBS and stored as indicated.

VI - Deionized water

The water used for these preparations was either glass distilled followed by deionization, or in some cases was double deionized.

B - CELL CULTURE TECHNIQUES

I - Cells employed

a) Primary cultures

Chick embryo fibroblasts (CEF) were prepared from 10-day-old chicken embryos. The head, limbs and viscera were removed aseptically and discarded, and the remaining tissues were rinsed in warm PBS containing antibiotics. The tissue suspension was minced and subjected to treatment with 0.25% trypsin in PBS at 37°C for 10 minute periods, with intermittent agitation. After the undigested tissue had settled, the supernatant was removed and fresh warmed trypsin solution added. The first two harvests were discarded and subsequent supernatant fluids 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 300xg for 10 minutes and resuspended in growth medium containing 10% calf serum. Viable cell counts were performed using 0.1% trypan blue in a Neubauer counting chamber. Cultures were set up at 5×10^5 cells/ml. In experimental work, secondary cultures were often employed and these were obtained by standard methods following STV treatment of primary monolayers.

b) Continuous cell lines

i) Control cultures

Stoker and MacPherson's (1962) line of baby hamster kidney cells (BHK-21) and a bovine kidney cell culture (MDBK) established by Madin and Darby (1958) were purchased from Flow Laboratories, Irvine, Ayrshire.

ii) Persistent infected cultures

The origin and maintenance in this laboratory of two cell lines persistently infected with NDV, namely MDBK_{pi} and MDEK_{cs}, are fully described in the Results.

II - Techniques of cell cultivation

Cells were propagated in 6" x 5/8" Pyrex test tubes, 2oz. and 4oz. medicinal flats, Brockway "Saniglass" cell culture bottles, one litre Roux flasks, and Winchester bottles in roller cultures. The volume of medium used in each case was 1, 5, 10, 50, 100 and 200ml respectively, and the cells were seeded at 10^5 cells per ml, unless otherwise stated. Generally, the cells were grown in MEM containing 10% calf serum, 0.16% sodium bicarbonate, 0.2mM L-glutamine and antibiotics as indicated, but for maintenance the percentage of serum was reduced to between 2-5%. Occasionally, BME was used in place of MEM but the other supplements were added in the same proportion. Unless otherwise stated, the monolayers were incubated at 37°C and overlaid with maintenance medium 2-4 days after seeding. Some cultures were reseeded every 7-10 days at which time the cells were detached from the glass with STV, pelleted at 300xg, resuspended in growth medium and counted in a Neubauer counting chamber, before seeding. Others were refed every 7-10 days with maintenance medium and held at 37°C for periods varying from 20 to 250 days.

III - Estimation of the rate of growth of cells

The rate of growth in monolayer cultures was estimated by the increase in the number of cells observed at appropriate time intervals after seeding.

IV - Co-cultivation techniques

Suspensions of trypsinised persistently infected cultures containing 10^5 cells per ml and of normal BHK-21 or CEF at 3×10^5 cells/ml were held in growth medium for one hour at 4°C in an attempt to synchronise cell growth. The cell suspensions were then mixed in equal volumes and inoculated into test tubes or medicinal flats. As controls, cultures were also prepared from the individual cell lines used in this experiment.

V - Cloning techniques

Cloning of single cells was attempted by several techniques:

a) Low density seeding

Cell suspensions were diluted in series in order to give 200 to 1000 cells per ml. Aliquots (0.05ml) of each dilution of the cell suspension were then inoculated into microtitre wells. After 3-4 hours of incubation at 37°C the microtitre plates were examined under the microscope, and those wells containing only one attached cell were marked.

b) Use of feeder monolayers

Aliquots of 0.05ml of MDBK control cells at a concentration of 10^4 cells/ml were inoculated in microtitre cell culture plates. After a near-confluent monolayer was achieved cultures were irradiated by means of X-rays in a Stabilipan-250 X-ray Generator (Siemens), with a dose of 97rad/min for periods varying from 10 to 45 minutes, according to the method of Puck et al. (1956). After irradiation, aimed to prevent the viability of MDBK control cells, the low density procedure described above was carried out using persistently infected cell suspensions.

C - VIRUS TECHNIQUES

I - Virus and strains of virus employed

The Herts 33 and B1 strains of NDV were kindly supplied by Dr. J.B. McFerran, Veterinary Research Laboratory, Stormont, Belfast.

Virus obtained from both MDBK_{pi} and MDBK_{cs} cell cultures persistently infected with NDV was collected from the supernatant fluids of the cultured monolayers.

Sendai virus was kindly provided by Mr. P. Eaton, Agricultural Research Council, Institute for Research on Animal Diseases, Compton, Newbury, Berkshire, and a strain of Bovine mammillitis virus (BMV) was obtained by the courtesy of Mr. W.S. Johnston, Veterinary Investigation Centre, Edinburgh.

The M6 virus, which is believed to be a strain of mucosal disease (Huck, 1961) was supplied by Mr. R.A. Huck of the Central Veterinary Laboratories, Weybridge, Surrey.

II - Growth of virus

Sendai virus and both wild-type NDV and NDV isolated from persistently infected cells were grown in 9 to 10-day-old fertile hen's eggs inoculated by the allantoic route with 0.2ml of a viral suspension containing $2 \times 10^3 \text{TCD}_{50}/\text{ml}$. After two days of incubation at 37°C , the eggs were chilled at 4°C for two hours before the allantoic fluids were harvested. The virus was stored overnight at 4°C if not immediately purified, and for longer periods at -70°C .

All viruses grown in eggs, together with BMV and M6 virus, were also propagated in continuous cell lines, as follows.

III - Inoculation of monolayers

NDV and Sendai virus were adsorbed to the monolayers at multiplicities of infection (MOI) varying from 0.01 to 10TCD_{50} per cell, in sufficient maintenance medium (0.2 to 2ml) to cover the cells. After one hour of incubation at 37°C , the viral suspension was removed,

the monolayers washed and fresh medium added. Virus released into the supernatant fluids was harvested after 2-3 days' incubation at 37°C.

In the case of BMV and M6 virus, infection was carried out in a similar manner but 5×10^2 TCD₅₀/ml of virus was adsorbed to the monolayers.

IV - Examination of the monolayers

a) Light microscopy

Unfixed monolayers were observed by direct light employing magnifications of 32x and 80x, and examined for the presence of cytopathic changes and evidence of healthy growth.

Monolayers fixed in Bouin's solution or with methanol were stained with haematoxylin and eosin (HE) or by Giemsa's method and examined for cytopathic effects by direct illumination.

i) Haematoxylin-eosin method

Flying coverslip preparations of cultured cells were removed from the test tubes, washed in PBS, and fixed either in methanol or Bouin's solution. After fixation, they were rehydrated through a series of ethanol-water mixtures, washed in water, stained with 4% Harris' haematoxylin (Gurr, High Wycombe, Buckinghamshire) in 25% ethanol, and rinsed in water. The preparations were decolourised in acid-alcohol, differentiated with saturated lithium carbonate solution and counter-stained with 1% yellowish eosin (Gurr) in 25% ethanol. The stained monolayers were then dehydrated through a series of acetone and acetone-xylol mixtures, cleared in xylol, and mounted in DePex(Gurr)

ii) Giemsa's staining method

Flying coverslips were fixed in methanol, and immersed in a weak solution (1/5) of Giemsa's stain (Hopkin and Williams) for 10 minutes and then differentiated in pH 6.8 buffer for a similar period. The preparations were dehydrated and mounted as above.

b) Ultraviolet light microscopy

i) Fluorescent antibody staining

Cultures on flying coverslips were washed in PBS, lightly blotted, rinsed and fixed in ice-cold acetone for 10 minutes. Dried coverslips were either stained immediately or stored in the presence of silica gel at -20°C . The staining technique was performed according to the standard indirect method, using an appropriated dilution of anti-NDV rabbit serum spread over the coverslip and incubated in a moist atmosphere at 37°C for at least 30 minutes. The monolayers were then washed in PBS with continuous stirring for 20-30 minutes. After being blotted, the coverslip cultures were restained with an appropriate dilution of fluorescein-labelled anti-rabbit globulin, prepared in sheep (Gibco, Santa Clara, California), incubated and washed as before. After a brief rinse in distilled water, the coverslips were mounted in buffered glycerol and examined under ultraviolet illumination. In some experiments, the monolayers were counterstained with a 1/1000 dilution of Evans blue (Gurr), before mounting.

ii) Acridine orange staining method

Coverslip cultures were rinsed in PBS and fixed in 3% acid-alcohol for 5 minutes. The staining technique employed was that of Anderson et al. (1959).

c) Electron microscopy

i) Negative staining

A drop of the specimen to be examined was placed on carbon-coated formvar film on a 400 mesh copper grid. The excess was removed by careful blotting after 30 seconds. When the specimen was obtained from sucrose or tartrate gradients a drop of distilled water was used to remove the excess of gradient material present. A drop of either 2% potassium phosphotungstate (PTA), pH 6.5, or 2% uranyl acetate (UA), pH 5.5, was added as the negative stain. The stain was removed after

10-20 seconds by draining off the excess liquid with filter paper, and the preparation allowed to dry before examination or kept in an evacuated desiccator in the presence of phosphorus pentoxide. The specimens were examined at 60KV with an AEI EM6B electron microscope and photographs taken on Ilford electron microscope plates, EM4.

ii) Ultrathin sections

Following removal of the medium, cell cultures were fixed in situ with ice-cold 3% glutaraldehyde in 0.01M phosphate buffer, pH 7.2. The cells were then scraped off the glass and left in fresh fixative for at least 6 hours at 0°C. After several washes in phosphate buffer, post-fixation was carried out in 1% osmium tetroxide in PB. An improved method of agar pelleted cells were used (Gowans, 1973), and followed by dehydration in a graded series of alcohols, and the cells were finally embedded in Araldite. After sectioning in an OMU3 Reichert microtome (Reichert, Austria), the cells were stained with saturated uranyl acetate in 50% ethanol and lead citrate (Reynolds, 1963), and examined in the electron microscope.

D - EXAMINATION OF THE PROPERTIES OF INFECTED CELLS

I - Calculation of the percentage of cells containing inclusions

Monolayers stained with Giemsa, haematoxylin and eosin or acridine orange were observed under a light or U-V light microscope, employing magnifications varying from 80x to 320x. At least, five hundred cells were examined in each monolayer and the percentage containing inclusions was calculated.

II - Calculation of the extent of cell fusion

Using the method proposed by Reeve and Poste (1971), an estimate of the extent of cell fusion in monolayers stained with Giemsa or haematoxylin and eosin was determined under the light microscope by

counting the number of nuclei present in polykaryocytes and expressing this as a percentage of the total number of nuclei present in the same microscopic field. At least, ten fields were counted in each coverslip preparation.

III - Examination of monolayers by haemadsorption

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

Monolayers were washed with PBS and overlaid with 1ml of either 0.4% guinea-pig erythrocytes for 15 minutes at 37°C, or 0.3% fowl red blood cells for 30 minutes at 4°C. The cell suspension was then decanted and the monolayers washed twice with PBS. Haemadsorption was looked for either by direct observation of the unstained monolayers under the microscope, or after the cell cultures had been fixed in methanol and stained by Giemsa's method. In these, the percentage of haemadsorbing cells was determined by comparing the number of cells showing haemadsorption of at least two erythrocytes, with the total number of cells in at least 10 microscopic fields (at 450x) scoring a minimum of 500 cells. Occasionally, an alternative method was employed using cells previously detached from the glass with STV, pelleted and resuspended in 1ml of 0.3% fowl erythrocytes. After 30 minutes at 4°C, the cells were examined and counted in a Neubauer cell counting chamber.

IV - Estimation of cell-associated haemagglutinin

Cells were detached from the surfaces of the cell culture containers with STV, pelleted at 400xg and resuspended in 1ml of PBS. The number of cells was counted and the cell suspension sonicated for two minutes in a MSE 3000 ultrasonic disintegrator. The haemagglutinin content was assayed by the method described on Section E-V-a (page 69).

V - Production of interfering agents

a) Viral inhibitory factor (VIF)

i) Preparation of cell culture fluids

Culture fluids from both MDBK_{pi} and MDBK_{cs} persistently infected cultures, were collected at different times by low-speed centrifugation (1000xg) to remove cell debris. The supernatant fluids were then harvested and centrifuged for at least 2 hours at 75000xg or 100000xg. Supernatants were then carefully collected, sterilised by filtration, and used to treat indicator cell cultures.

ii) Assay for VIF

Indicator cell monolayers of MDBK and CEF cell types, containing an average of 2×10^5 cells/ml, were treated routinely prior to virus challenge, for at least two hours at 37°C, with volumes of testing fluids ranging from 0.2 to 1ml. Ten-fold dilutions of several viruses (NDV, BMV, Sendai virus, and M6 virus) were used to infect treated and untreated monolayers for 1 hour at 37°C. After the adsorption period was completed, any virus remaining was removed and the monolayers washed and refed with fresh medium. Cell cultures were checked for the presence of cell-associated haemagglutinins or other cytopathic changes 2 to 5 days after infection.

b) Interferon assay

Centrifuged fluids, prepared as above were treated for 2 hours at 56°C, and then used to overlay MDBK or CEF cultures. After overnight treatment, the inoculum was removed and these monolayers, together with similar untreated cultures, were challenged with several viruses as described in ii) above. Interferon activity was assessed by comparing the TCD₅₀ titres obtained in both treated and control cultures.

VI - Ability to support superinfection

a) With NDV

Overnight cultures of persistently infected and control cells grown in test tubes were inoculated with 0.2ml amounts of an egg-grown suspension of NDV containing 10 MOI per each cell of the monolayer. Viral adsorption was carried out for 1 hour at 37°C, when the cell cultures were washed and refed with maintenance medium. Fluids from both test and control cultures were harvested 12 hours post-infection and the amount of the virus released was determined in BHK-21 cells by the method described below (Section E-II-a, page 65).

b) With related and unrelated viruses

i) Superinfection with Sendai virus

Twenty-hour-old monolayers of control MDBK cells and of persistently infected carrier type MDBK_{CS} cells propagated in test tubes, were treated for 30 minutes at 37°C with 1/10 dilution of anti-NDV rabbit serum (HAI 1/128), washed and then inoculated with ten-fold dilutions of Sendai virus, for 1 hour at 37°C. After adsorption was completed, the monolayers were washed and refed with fresh medium, and examined three days later for the presence of haemadsorption.

ii) Superinfection with BMV

Ten-fold dilutions of bovine mammillitis virus were inoculated into 20-hour-old MDBK control and MDBK_{CS} cell cultures for 1 hour at 37°C. When adsorption was completed the monolayers were washed and refed with culture medium. Infected cultures were examined daily for characteristic cytopathic changes up to the 5th day post-infection.

E - EXAMINATION OF THE PROPERTIES OF EXTRACELLULAR AND

CELL-ASSOCIATED VIRUS

I - Electron microscopy

a) Virus morphology

Virus obtained either from the allantois of inoculated hens' eggs or from the supernatant fluids of persistently infected cultures was clarified by centrifugation at 5000xg for 20 minutes and then pelleted at 75000xg for 2 hours. Resuspended viral material was negatively-stained by the method described in Section C-IV-c-i (page 59).

b) NDV-specificity of the components of the persisting virus

In order to detect surface and intracellular viral antigens, the immunoperoxidase method (IP) of Avrameas (1970) was employed. The immunoglobulin fractions of rabbit anti-NDV sera were purified and used in a direct method, by coupling them to activated horse radish peroxidase with glutaraldehyde. The method used was that described by Avrameas and Terrynck (1971).

Overnight, confluent cell monolayers prepared from MDBK_{pi} cultures were grown in 60mm plastic dishes (Nunc U.K., Ltd.) and fixed in situ for 30 minutes in 1% glutaraldehyde in 0.1M phosphate buffered saline. After the monolayers had been rinsed for at least 3 hours with several changes of buffer, the peroxidase-labelled antibody (PLA) was applied to the fixed cells for 1 hour at 37°C. A blocking experiment was performed by treating some of the cultures for 1 hour at 37°C with 1/10 dilution of anti-NDV globulin, prior to PLA treatment. All cell cultures were then washed thoroughly overnight with buffered saline, before being refixed in 2% glutaraldehyde, for 30 minutes at room temperature. Subsequently, the monolayers were incubated for 30 minutes at 20°C in diaminobenzidine. After a further wash, the cells were dislodged from the surface of the glass with a "rubber policeman",

pelleted at low speed centrifugation, post-fixed for 1 hour in 1% osmium tetroxide and processed for ultra-thin sections as described in Section C-IV-c-ii (page 60).

II - Estimation of infectivity of viruses

a) Infectivity of viruses in tissue culture (TCD_{50})

Monolayers grown overnight in test tubes were overlaid with 0.2ml of ten-fold dilutions in maintenance medium of the appropriate virus suspension. After one hour of incubation at 37°C, the unadsorbed virus was removed, and the monolayers washed twice and refed with 1ml of culture fluid. The monolayers were examined for the presence of haemadsorption, cytopathic changes and specific fluorescence after two days of incubation at 37°C. In addition, the supernatant fluids, were tested for the presence of released haemagglutinins and, sometimes, infectious virus. In the case of BMV and M6 virus, in which the presence of the virus was detected only by the occurrence of cytopathic changes, the observations were carried out daily for 4-7 days. The fifty per cent tissue culture infective dose (TCD_{50}) was calculated by the method of Kärber (1931) and expressed as $\log_{10} TCD_{50} / ml$.

b) Enumeration of infected cells or foci by immunofluorescence

Confluent monolayers of the carrier culture MDBK_{CS} were seeded in coverslip test tubes. Two coverslips were removed daily, fixed in acetone and stained by the fluorescent antibody technique described in Section C-IV-b-i (page 59). Under the U-V light microscope, using a 350x magnification, monolayers were examined in at least 10 different fields for individual or groups of fluorescing cells. The results were obtained by comparing the number of infected cells with the total number of cells in the selected fields and expressing the results as a percentage.

III - Virus purification

Virus grown in the allantois of nine-day-old fertile chicken's eggs or released into the supernatant fluids of persistently infected monolayers, was purified by means of potassium tartrate or sucrose gradients. Allantoic fluids were harvested 48 hours after inoculation and the tissue culture supernatants were usually collected 5 to 7 days after seeding the cells. In each case the virus material was immediately used for virus purification. All subsequent operations were carried out at 0°-4°C. Viral fluids were first clarified to remove cellular debris at 5000xg for 20 minutes, and then pelleted at 75000xg for 2 hours. The deposit was resuspended in 1-2ml of cold PBS employing a glass homogenizer and thoroughly mixed for one minute with an equal volume of Halocarbon 113 (trichlorotrifluoroethane, TCTF). After the mixture had been centrifuged at 5000xg for 10 minutes, the upper aqueous phase containing the virus was removed and layered on to 10ml linear gradients of either 15% to 40% (w/w) potassium tartrate in PBS or 25% to 55% (w/w) sucrose in PBS, prepared with a Buchler auto-Densiflow gradient former. The tartrate gradients were centrifuged at 284000xg for 2.5 hours, whereas the sucrose gradients were centrifuged overnight (approximately 16 hours) at 100000xg. The gradients were then fractionated in 0.5 to 1ml fractions, and the haemagglutinin titre, refractive index (in an Abbe "60" refractometer, Bellingham & Stanley Ltd., London), and absorbance at 280nm (in a SP800 spectrophotometer, Pye-Unicam Instruments Ltd., Cambridge) measured for each fraction. The fractions corresponding to the highest concentration of virus were pooled and either dialysed overnight against PB or diluted in a final volume of about 50ml of PBS, pelleted at 75000xg for 2 hours and resuspended in 2ml PBS. This stage of purification is referred to as either TGl or SGl, depending on whether tartrate or sucrose

gradients were used. The purified samples were sometimes subjected to a second cycle of purification by being sedimented in another potassium tartrate gradient and redialysed (TC2). Alternatively they were layered on top of a 10% to 40% (w/w) sucrose velocity gradient, centrifuged at 50000xg for 20 minutes, and processed as described above (this stage of purification is called SG2).

IV - Biochemical studies of viral components

a) Estimation of protein concentration

The amount of protein in the viral samples was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

On some occasions an estimate of protein concentrations was obtained from the absorbances at 260 and 280nm.

b) Disruption of virus

Prior to separation on polyacrylamide gel electrophoresis (PAGE), purified virus was disrupted with sodium dodecyl sulphate (SDS) and urea in the presence of the reducing agent 2-mercaptoethanol. A solution containing 1% SDS, 0.1% 2-mercaptoethanol and 6M urea was added to the virus preparations, incubated for 30 minutes at 70°C and heated on a boiling water bath for one minute.

c) Isolation of viral proteins by polyacrylamide gel electrophoresis

i) Formation and running of gels

Gels of 5mm in diameter and approximately 60mm in length were formed by polymerisation of a solution containing 10% acrylamide (w/v), 0.3% N,N'-methylenebisacrylamide, 0.375M tris-hydrochloric acid buffer (pH 8.9), 0.075% N,N,N',N'-tetramethylethylenediamine, 4.0M urea, and 1% SDS, employing 0.07% ammonium persulphate as a catalyst. The same formula was used to form slab gels in a slab-gel vertical cell, model 220 (BioRad Laboratories Ltd., Bromley, Kent). Approximately 20 to

30 μ g or 5 to 10 μ g of solubilised proteins were layered on top of cylindrical or slab gels, respectively and electrophoresed at either 2mA per gel or 150 volts (slab gel apparatus) for 2 to 3 hours, until the tracker dye (bromophenol blue) had reached the vicinity of the bottom of the gels. A discontinuous buffer system was used with 0.05M tris-glycine (pH 8.9), 0.1% SDS, and 0.5M urea in the upper (cathode) tray, and 0.1M tris-hydrochloric acid (pH 8.1), 0.1% SDS, and 0.5M urea in the lower (anode) tray.

ii) Location of polypeptides in electrophoresed gels

x) Staining with Coomassie blue

Gels were fixed for 30 minutes in a methanol-acetic acid-water (5:1:5) solution, followed by staining for 2-3 hours in 0.25% Coomassie Brilliant Blue R250 (Serva, Heidelberg, Germany) in the same solvent. The destaining process was carried out either overnight in a rotating apparatus with a few changes of the solvent or completed in a few hours by frequent changes of the destain solvent. The gels were scanned at 580nm a Unicam SP500 spectrophotometer with a Gilford 2410-S Linear Transporter attachment. Densitometer tracings were made with the origin to the left in all gel profiles. The method used to estimate molecular weights was that of Weber and Osborn (1969) using bovine serum albumin (fraction V), ovalbumin (grade V) myoglobin (type II) and chymotrypsinogen-A (type II) as markers. The protein standards were obtained from Sigma (London) Ltd., Kingston-upon-Thames, Surrey.

xx) Staining by periodic acid-Schiff

The method used was that described by Zacharius et al. (1969) The gels were fixed for 1 hour in 12.5% (w/v) trichloroacetic acid and washed extensively overnight with 3% acetic acid. After oxidation in a freshly prepared solution of 1% periodic acid in 3% acetic acid,

for 1.5 hours, the gels were again washed extensively (1 to 3 days) with 3% acetic acid. Thereafter, the gels were stained for 1-2 hours with Schiff's reagent, washed twice with freshly made 0.5% sodium metabisulphite, for 0.5 hours in order to differentiate the stained areas, and destained with methanol-acetic acid-water (20:3:17).

d) Estimation of neuraminidase activity

The technique employed is that described by McNulty *et al.* (1975). The substrate used in the neuraminidase assays was α_1 - glycoprotein (orosomucoid) concentrated from human plasma obtained from the Scottish National Blood Transfusion Association and was a gift from Dr. S. McNulty. Triplicate samples of virus diluted in 0.2M sodium acetate buffer (pH 5.1) were added to 0.25ml of orosomucoid (8mg/ml) in the same buffer. The mixture was incubated at 37°C for 15 minutes and the amount of N-acetylneuraminic acid (NANA) liberated was measured by the thiobarbituric acid procedure of Aminoff (1961). The coloured samples were read at 549nm in a Unicam SP800 spectrophotometer and controls containing known quantities of NANA (Sigma) were employed. The specific activity was expressed as nmols of NANA released per minute per μ g protein.

V - Examination of viral haemagglutinin

a) Haemagglutinin assays

These were performed in WHO perspex plates using the technique described by the WHO Expert Committee on Influenza (1953). Doubling dilutions of the test material were prepared in PBS and an equal volume (0.25ml) of a 1% suspension of fowl erythrocytes was added to each well. The results were read after 40 minutes at room temperature or 60 minutes at 4°C. Assays were also performed using one-tenth of these volumes in microtitre plates (Flow Laboratories). Results were expressed in haemagglutinating units per given volume (HAU) corresponding to the reciprocal of the highest dilution showing haemagglutination.

b) Haemagglutination inhibition test

A standard volume of four haemagglutinating units (HAU) of test virus was added to doubling dilutions of antisera prepared from rabbits. The virus-serum mixture was allowed to react for 30 minutes at room temperature. Then, the same volume of a 1% suspension of fowl erythrocytes was added and the test was examined for haemagglutination after 60 minutes at room temperature.

F - EXPERIMENTS PERFORMED WITH RADIOACTIVE ISOTOPES

I - Labelling of cellular and viral RNA with ^3H -uridine

a) Liquid scintillation technique

Twenty-hour-old monolayers propagated in test tubes seeded at 2×10^5 cells/ml, were inoculated with NDV at 0.5 to 5 MOI. Control cultures were refed at the same time with 0.2ml of maintenance medium. Virus was allowed to adsorb for 1 hour at 37°C . At the end of this period, the inoculum and culture fluids were removed and the monolayers washed twice. Half of the tubes of each set (infected and noninfected) were overlaid with 1ml of culture medium containing $1\mu\text{g/ml}$ of actinomycin D, and the remainder fed with medium which did not contain the drug. This state was referred to as time zero. One hour before the time of each harvest the supernatant fluids were removed from four tubes of each set, pooled separately, and held at -70°C for further infectivity assays as described in Section E-II-a (page 65). The monolayer cultures were then refed with media supplemented with $5\mu\text{Ci/ml}$ of ^3H -uridine, and either containing or lacking actinomycin D. After 1 hour pulse the cell monolayers were washed three times with ice-cold PBS, followed by two changes of ice-cold 5% trichloroacetic acid and a final 5 minutes period of fixation in the same solution. The monolayers were rinsed twice with absolute alcohol and then solubilised

overnight with a mixture of 0.1ml of DDW and 0.5ml of Soluene TM 100 (Packard Instruments Ltd., Wembley, Middlesex). Duplicate samples of 0.2ml were transferred from each tube to two glass scintillation vials of 20ml capacity, and 10ml of scintillation fluid (0.1g phenyl-oxazolyl phenyl-oxazolylphenyl (POPOP) and 4g 2-5-diphenyloxazole (PPO) in one litre of toluene) added. The vials were held at 4°C for 16 hours to allow for the decay of any chemiluminescence and counted in an ICN liquid scintillation spectrometer (model CM-2024 BS(L)).

b) Autoradiographic technique

Confluent monolayers of MDBK control and MDBK_{pi} cells were grown in tubes containing coverslips. Some MDBK control cultures were infected previously (16 hours) with NDV at 5 MOI per cell. Some of the normal (infected and noninfected) and persistently infected cell cultures were treated for 1 hour with 5µg/ml actinomycin D. Others were left untreated, but all were pulsed with 5µCi/ml ³H-uridine for 1 hour at 37°C. The coverslip preparations were then harvested, washed with PBS and fixed with formol-saline for 10 minutes at room temperature. After being washed with PBS, the cells were treated with 10% trichloroacetic acid for 10 minutes, rinsed twice in deionized distilled water and allowed to dry overnight. The coverslips were then mounted in DePex with the cell sheet facing upwards on gelatin coated slides. In dark-room conditions, they were covered with a film of fine grain emulsion AR-10 (Kodak Ltd., London), and stored in light-proof boxes containing silica gel, at -20°C. At given intervals, the slides were developed and fixed by standard photographic methods. They were then examined under the light microscope for the presence of dark grains of silver due to the emission of ionizing particles from the radioactive material.

II - Double labelling technique

Twenty-hour-old monolayers prepared from both types of persistently infected MDBK cells and grown in 2oz plastic flats at a concentration of 10^6 cells/ml, were treated with $1\mu\text{g/ml}$ actinomycin D for 6 hours. After discarding the fluids, 1ml of serum-free MEM incorporating $1\mu\text{g/ml}$ actinomycin D and either $100\mu\text{Ci/ml}$ ^3H -leucine or $20\mu\text{Ci/ml}$ ^{14}C -leucine was used to overlay MDBK_{cs} or MDBK_{pi} cultures, respectively. After 16 hours the fluids were harvested separately, clarified at low speed and transferred to 50ml MSE centrifuge buckets where they were diluted with cold PBS. At this stage, 1ml of TCTF treated Herts NDV grown in eggs was added to each tube as a carrier. The fluids were then centrifuged for 1 hour at 75000xg , the pellets resuspended in 1ml cold PBS, layered on top of 25-55% sucrose gradients and centrifuged overnight at 75000xg . The cell sheets remaining in the test bottles were dissolved separately with 1% SDS in PBS at 37°C and stored at -70°C . Gradients were fractionated and each fraction was tested for haemagglutinin and radioactivity. Viral bands were selected and treated as described in Section E-III (page 66).

Samples obtained from both types of persistently infected cells, in the form of purified virus or cell lysates were mixed and run in cylindrical gels together with control NDV Herts samples, as indicated in Section E-IV-c (page 67). Control gels were stained and scanned as previously described. The labelled gels were fixed for 1 hour in 12.5% trichloroacetic acid, and washed overnight with 3% acetic acid. After the gels had been frozen at -20°C they were cut immediately into 1mm slices in a Mickle gel slicer (The Mickle Laboratory Engineering Company, Millworks, Surrey). The slices were put into scintillation vials, dissolved in $100\mu\text{l}$ 30% hydrogen peroxide and incubated at 60°C for 1 hour. Samples were cooled, mixed with 10ml

of scintillation fluid (0.3g POPOP, 16.5g PPO, 1 litre Triton X-100 and 2 litres toluene) and kept overnight at 4°C. Radioactive counts were measured for 10 to 30 minutes in an ICN liquid scintillation spectrophotometer. The tritium and carbon-14 counts were discriminated by the channel ratio method of Hendler (1964).

G - ATTEMPTS TO CHANGE THE CHARACTER OF PERSISTENT INFECTION BY ALTERING THE ENVIRONMENT OF THE CELLS

I - Incubation of cells at 41°C

Monolayers of persistently infected cells were incubated at 37°C and 41°C. The medium was changed every three days and cultures reseeded at weekly intervals. They were examined for haemadsorption and specific immunofluorescence by standard methods (pages 59 and 61).

II - Treatment of cultures with NDV antiserum

Monolayers of MDBK_{cs} cells were cultivated in 4oz bottles, with MEM containing 3% calf serum and incorporating anti-NDV (Herts strain) serum prepared from rabbits, at a final concentration of 25 HAIU/ml. Neutralization assays carried out previously in MDBK_{cs} cells showed that a blocking effect was obtained up to 1/160 dilution of the antiserum (512 HAIU/ml). The cells were examined daily for the presence of haemadsorption and immunofluorescence, after treated cultures have been passaged and some coverslip tube cultures seeded with antiserum-free medium.

III - Treatment of cultures with inhibitors of RNA and protein synthesis

Usually, confluent monolayers of the two persistent infections (MDBK_{pi} and MDBK_{cs}) were grown in test tubes, and overlaid with culture medium containing the appropriate amount of inhibitor. Actinomycin D (Sigma) was added to give final concentrations of 1 or 5µg/ml or cycloheximide (Sigma) was added at 5, 10, 20, 50 and 100µg/ml. The incorporation of ³H-uridine into cellular or viral RNA was detected by the technique described in Section F-I-a (page 70).

The amount of infectious virus released was assayed as described earlier (page 65), and the monolayers examined at the times stated for haemadsorption, immunofluorescence, the quantity of cell-associated haemagglutinin, and the number of cells per ml, by the methods described previously (pages 55, 59 and 61).

IV - Treatment of cell cultures with DNA analogues

Twenty-hour-old monolayers of both types of persistent infections as well as control cultures of MDBK cells were grown in 4oz medicinal flats or in test tubes, and were overlaid with MEM containing 20, 50 or 100ug/ml of 5-iodo-2'-deoxyuridine (Sigma). The treatment was carried out for 24 hours, after which the cultures were washed and overlaid with 10ml normal MEM. Following treatment, 1ml of culture supernatant was collected every second day and was examined for the amount of virus released.

H - ATTEMPTS TO DETECT PROVIRAL INFORMATION

I - Isolation of cellular DNA

The cultures used for the extraction of DNA included normal MDBK, MDBK_{cs}, MDBK_{pi}, and MDBK cells infected with the Herts strain of NDV. The method employed was that of Marmur (1961) as modified by Simpson and Iinuma (1975). A total of about 10^8 cells propagated in Roux flasks were washed with EDTA buffer (pH8.0) and lysed with 15ml per culture of the same buffer containing 1% SDS in a 60°C water bath for 10 minutes. After cooling the lysates to room temperature, they were pooled and combined with an equal volume of 24:1 (v/v) chloroform-isoamyl alcohol and subjected to intermittent agitation for 30 minutes. The aqueous phase was collected following centrifugation at 6000xg for 5 minutes. The nucleic acid fraction

was then precipitated by adding two parts of 95% ethanol to one part of the aqueous phase. The precipitate formed after 10 minutes at room temperature was collected by low speed centrifugation and dissolved over a period of 30 minutes in 15ml of SSC buffer (pH 7.0) by gentle agitation. An equal volume of ribonuclease A, type III (Sigma) in SSC buffer was added to give a final concentration of 50µg/ml and incubation was carried out for 1 hour at 37°C. Thereafter, an equal volume of Pronase (Calbiochem, San Diego, California) dissolved in SSC buffer was added at a concentration of 500µg/ml and a further period of incubation was carried out at 37°C. Deproteinization with chloroform-isoamyl alcohol and subsequent precipitation of the aqueous phase with ethanol, as described above, was repeated. The precipitate obtained in this manner was dissolved in 9ml of SSC buffer within 3 to 5 minutes. After reprecipitation for 10 minutes with one-half the volume of absolute isopropanol, the deposit was collected by low speed centrifugation and sterilized by exposure to 15ml of 75% ethanol for 24 hours at 4°C. The material was pelleted once more and resuspended in 15ml of PBS. Samples were stored at -70°C until required.

II - Estimation of DNA concentration

The amount of DNA present in cell extracts was determined according to the colorimetric reaction of diphenylamine described by Shatkin (1969). In this present work, deoxyribonucleic acid, type I (Sigma) was used as a standard.

III - Transfer of cellular DNA isolates into recipient cell cultures

Recipient cell monolayers of BHK-21 or MDBK cell lines, propagated in 2oz plastic flats, 4oz glass bottles or test tubes carrying coverslips were used throughout these experiments. In a number of instances, cell cultures inoculated with DNA extracts were incubated

at both 35°C and 37°C. In a number of experiments the treated cultures were passaged three times over a period of 60 days. Cultures were examined daily for visible cytopathic changes, haemadsorption and specific immunofluorescence. Three different methods of transfection were attempted, viz:

a) Method of Simpson and Iinuma (1975)

Recipient cell cultures were washed with PBS, and then inoculated with appropriate dilutions of DNA extracts or of DNA samples previously treated with deoxyribonuclease I (Boehringer, Mannheim, Germany), for 1 hour at 37°C. After 10 minutes adsorption at room temperature the inocula were removed by washing the cultures with PBS. They were then overlaid with MEM before further incubation.

b) Method of McCutchan and Pagano (1968)

Monolayers of recipient cell cultures were washed with NaCl-tris buffer (pH 7.4) and treated with 100µg/ml of DEAE-dextran (diethylaminoethyl dextran, Sigma) in the same buffer, at 37°C for 15 minutes. The DEAE-dextran solution was removed and 1ml of NaCl-tris buffer containing an appropriate amount of the DNA extract (treated or untreated with DNAase) was added to each culture. After 15 minutes adsorption at 37°C, the inoculum was removed and replaced with MEM.

c) Method of Graham and van der Eb (1973)

Appropriate dilutions of samples of the DNA extracts (treated or untreated with DNAase) in HeBs buffer (pH 7.05) were made in order to give a final concentration of 10-20µg/ml of the nucleic acid. To these diluted samples 2M CaCl₂ was added to a final concentration of 125mM, and the mixture kept at room temperature for 25 minutes to allow precipitation of the calcium phosphate. Cell monolayers were washed with HeBs buffer and then overlaid with 1ml of the DNA-calcium phosphate mixture. Initially, cultures were incubated at room

temperature for 25 minutes, then 1, 5 or 10ml of growth MEM was added and the cultures incubated at 37°C for a further period of 5 hours. At the end of this period the inoculum was removed, the cultures washed with the same buffer and overlaid with MEM maintenance medium.

RESULTS

SECTION I

INDUCTION AND ESTABLISHMENT OF A CARRIER STATE IN MDBK CELLS INFECTED WITH NEWCASTLE DISEASE VIRUS (NDV)

1 - Establishment of a carrier culture

The first objective in this present work was to attempt to induce a persistent infection with NDV in the Madin line of bovine kidney cells (MDBK). This was achieved successfully in two stages namely: by selective adaptation of the Herts 33 strain of NDV grown in embryonated hen's eggs to MDBK cells for four successive passages, followed by normal propagation of those infected monolayer cultures which did not show evidence of viral cytopathic effects.

Initially, the original virus seed obtained from allantoic fluids of fertile eggs infected with egg-adapted NDV was inoculated at multiplicities of infection (MOI) varying from 0.01 to 10, into one-day-old confluent monolayers of MDBK cells maintained in 4oz glass bottles. This constituted the first passage of NDV in MDBK. After 48 hours' incubation at 37°C, the cultures were examined carefully under the light microscope for cytopathic changes associated with viral activity and for the presence of extracellular haemagglutinating virus in the culture media. Infected culture fluids were collected and inoculated undiluted into freshly prepared MDBK monolayers. These were also incubated at 37°C and the subsequent cycle of viral replication was taken to represent viral passage no.2. After two further passages in MDBK cells no cellular abnormalities were seen nor could haemagglutinins be detected in the fluid phase of the infected cell culture. All inocula, whether of diluted allantoic fluids or undiluted cell culture fluids, were added to the monolayers in 1ml amounts and allowed to adsorb for one hour. The inocula were then removed and the monolayers washed twice and refed with maintenance media. In each instance, the cultures were returned to the 37°C incubator and examined 48 hours later for evidence of virus infection.

As the results in Table 1 show, no CPE was observed at the first passage in monolayers of MDBK cells inoculated with the highest dilution of NDV; but, at the second and third passage level, the virus was capable of destroying no fewer than 75% and 50%, respectively of the cells in the cultures. It is of interest, however, that the cytopathic activity of the virus disappeared abruptly by the fourth passage, and haemagglutinins were no longer detectable in the supernatant fluids.

When these cultures were subsequently propagated at regular 2 to 3-weekly intervals over a period of 18 months they consistently revealed all the characteristics of a persistent viral infection of the carrier state type as originally described by Walker (1964).

Although the principal features of this artificially induced carrier state was the very low proportion of infected cells in the intact monolayers, in some passages a crisis developed and the cultures showed a sudden increase in viral activity. However, the effect was quickly lost during subsequent subcultures and the cells returned to their carrier state.

TABLE 1

INFECTION OF MDEK CELL CULTURES WITH EGG-GROWN AND MDEK-ADAPTED VIRUS

Herts strain of NDV (MOI) ^{a)}	Haemagglutinin titres (HAU/ml) ^{b)}				Evidence of cytopathic effects ^{c)}			
	No. of passages				No. of passages			
	1	2	3	4	1	2	3	4
10	64	8	ND ^{d)}	ND	+++	+	ND	ND
1	64	8	4	ND	++	+	+	ND
0.1	16	16	8	0	+	+++	+	-
0.01	8	32	8	0	-	+++	++	-

a) Multiplicity of infection of the allantoic fluid of egg-grown NDV used as first inoculum.

b) Passage 1 corresponds to the inoculation of MDEK monolayers with indicated egg-grown viral dilutions. Passages 2,3 and 4 correspond to the inoculation of the undiluted MDEK_{cs} infected culture fluids.

c) Cytopathic effect expressed as: ++++100%; +++75%; ++50%; +25% and - negative.

d) Not done.

2 - Characteristics of MDCK_{cs} cultures

a) Proportion of infected cells

Although intact monolayer cultures of carrier cells did not show visible abnormalities nor produce extracellular haemagglutinins, the presence of infected cells could be readily determined by specific immunofluorescent staining techniques which invariably revealed multiple plaques of fluorescing viral antigen in the cytoplasm of affected cells, and by haemadsorption of guinea-pig or fowl red blood cells to the viral antigens on the cell surfaces. Haemadsorption estimation and quantitative determination of cells showing fluorescent viral material were used to assess the proportion of infected cells in the monolayers, as described in Materials and Methods.

The results in Table 2 indicate that a marked reduction in the number of detectable infected cells occurred by the second MDCK_{cs} subculture, and this feature has been typical of the carrier state throughout this present investigation. The appearances of these cells in stained monolayer preparations is shown in Plates 1 and 2; the first represents a low power microscopic view of a culture of MDCK_{cs} cells while the second shows a monolayer of healthy MDCK cells. The photomicrographs clearly show the presence of isolated foci of one or more infected (haemadsorbing) cells in the apparently normal monolayer of MDCK_{cs} cells, and that these are not found in cultured cells of the healthy MDCK line. It is stressed that cytoplasmic accumulations of viral antigen were rarely seen in cultures stained with Giemsa or haematoxylin and eosin although immunofluorescence staining of monolayers of MDCK_{cs} cells invariably revealed aggregates of specifically stained antigen throughout the cytoplasm. In a few instances there was also evidence of the accumulation of fluorescent material on the surface of the cell membranes, in the perinuclear area of the cytoplasm and, even, in the nucleus itself. (Plates 3,4)

TABLE 2

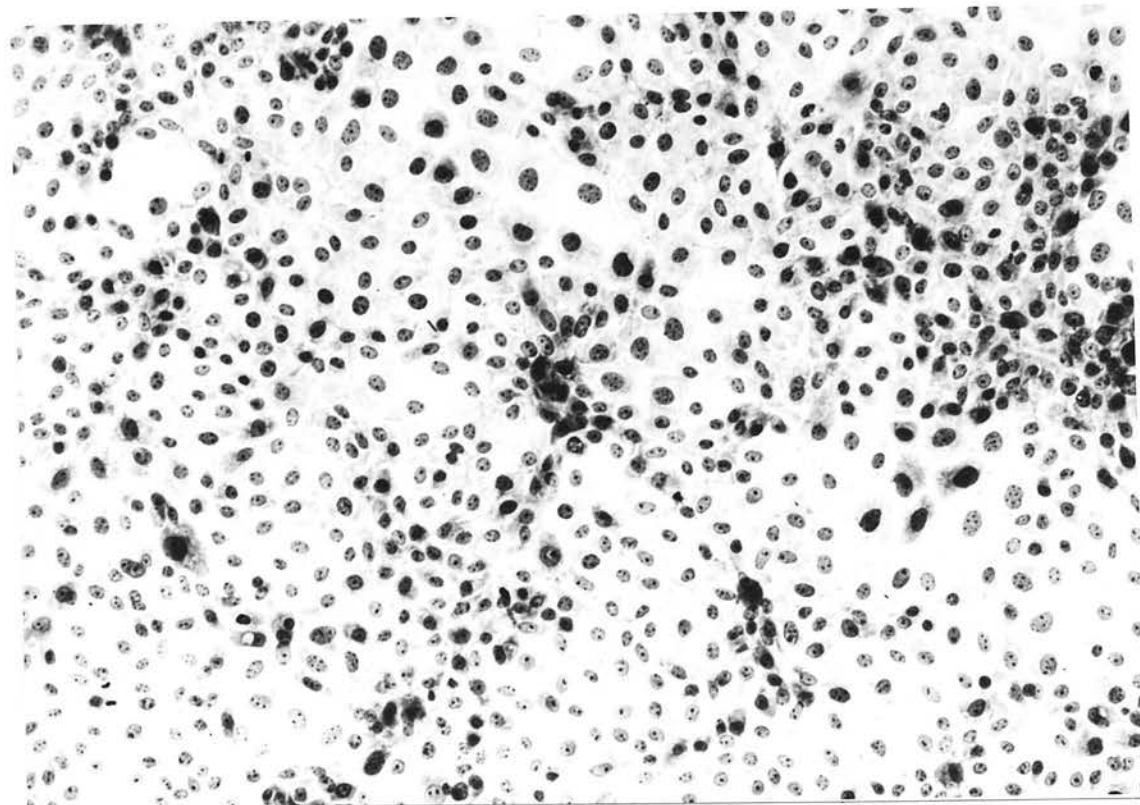
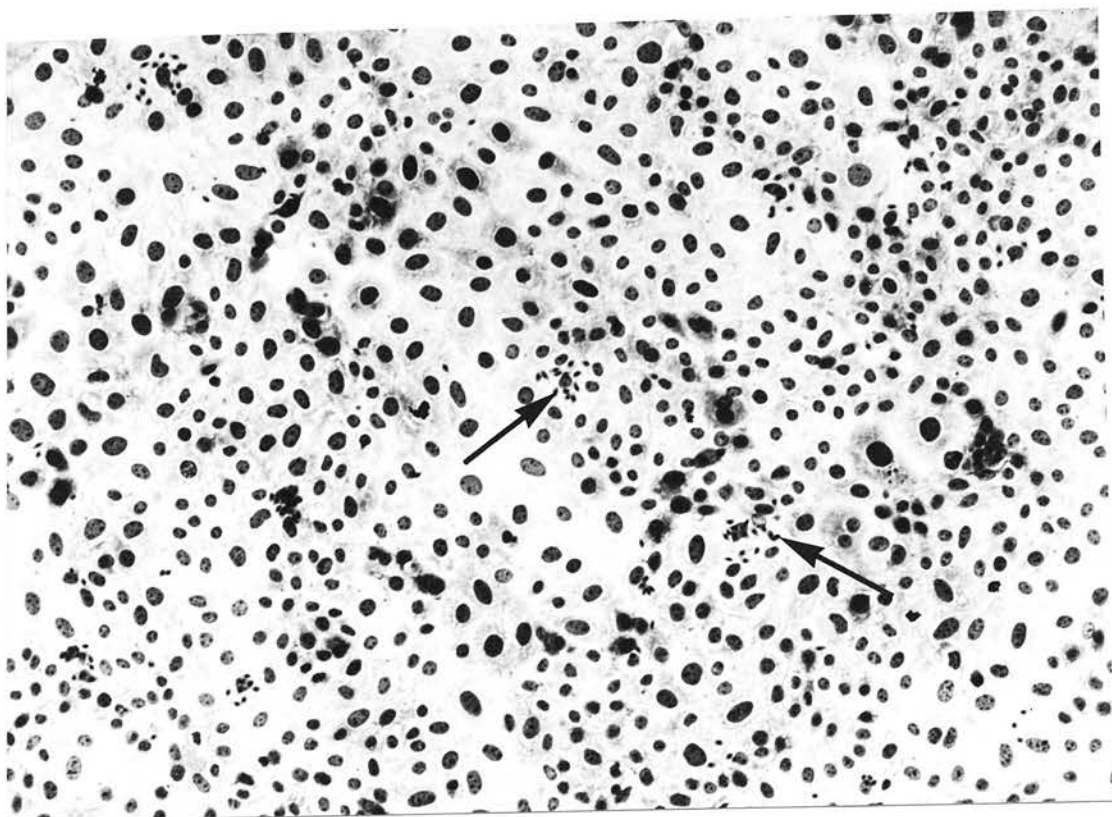
PROPORTION OF INFECTED CELLS AND RELEASED INFECTIONOUS VIRUS
IN MDBK_{CS} MONOLAYERS

Subculture number	% of FA ^{a)} cells	% of Had ^{b)} cells	TCD ₅₀ /ml ^{c)}
1	40	10	5.0x10 ⁴
2	5	5	1.5x10 ⁵
3	1.5	1	1.5x10 ⁵
4	<1	-	1.5x10 ⁵
5	<1	-	7.3x10 ⁴
6	<1	-	1.5x10 ⁵
7	3	-	1.5x10 ⁵
8	<1	-	ND
9	<1	-	ND
10	4	-	ND
11	<1	-	1.5x10 ⁵
12	50	60	7.3x10 ³
13	<1	-	ND
14	<1	-	1.5x10 ⁵
15	<1	-	ND
16	<1	-	3.4x10 ⁴
17	<1	-	5.0x10 ⁴
18	25	25	ND
19	30	25	3.4x10 ⁵
20	<1	-	3.4x10 ⁴
21	40	10	3.4x10 ³
22	<1	-	ND
23	6	-	ND
24	<1	-	ND
25	<1	-	ND

- a) Percentage of fluorescing cells scored in at least 10 microscopic fields
 b) Percentage of haemadsorbing cells scored in at least 10 microscopic fields
 c) 50% of tissue culture infectious doses per millilitre

Plate 1 : Ten-day-old MDEK_{CS} monolayer treated
with healthy avian erythrocytes
showing isolated foci of one or more
infected (haemadsorbing) cells.
Giemsa stain.x190.

Plate 2 : Ten-day-old MDEK control monolayer treated
with healthy avian erythrocytes.
Notice the absence of haemadsorption.
Giemsa stain.x190.



The results (Table 2) also show that all MDBK_{cs} monolayers proved to be infected when stained by the fluorescent antibody technique although, in most cases, the percentage of infected cells was less than 1%. This is in marked contrast to the results obtained by haemadsorption which gave a positive reaction in only 7 of the 25 cell culture passes, although six of these contained a higher proportion of infected cells as revealed by both haemadsorption and immunofluorescent staining methods. There were indications of crisis in five of the cultures in that over 25% of the cells contained viral antigen and showed visible evidence of cytolytic activity. Despite this, however, the proportion of infected cells returned to their previous low levels in the subsequent subcultures. These results clearly emphasise that fluorescent antibody staining methods are much more sensitive than haemadsorption for the detection of viral antigen in persistently infected cells.

b) Infectivity of the released virus

The release of infectious virus into the culture medium of MDBK_{cs} cell monolayers was investigated in the following manner:

Supernatant fluids were harvested from 15 different subcultures of the carrier cells after 5-7 days of incubation at 37°C, centrifuged at low speed (600xg) and tested immediately or stored at -70°C for further examination.

The methods of ascertaining the infectivity titres of the fluid phase material have been described previously (Materials and Methods), and are based on the presence of foci of haemadsorbed chicken erythrocytes in BHK-21 monolayer cultures previously inoculated with MDBK_{cs} fluids.

These investigations revealed a continuous release of infectious virus in all of the culture fluids tested and, as the results in Table 2 show, the infectivity titres varied between 3.4×10^3 and 3.4×10^5 per ml. It should be noted that the two lowest, as well as the highest titres obtained correspond to cultures that showed over 25% of fluorescing cells.

c) Division and growth of infected cells

A number of experiments were designed in order to obtain information concerning the division and growth characteristics of infected cells in carrier cultures. Several recognized cloning techniques were used, ranging from serial dilutions of cell suspensions with a view to obtaining growth of colonies from single cells, to attempts to propagate isolated cells overlaid on "feeder" monolayer cultures of X-ray irradiated cells of the same or different type. Although, unfortunately, none of these procedures was successful, examination of coverslip cultures stained either by Giemsa or fluorescent antibody staining methods suggested that the chronically infected cells were at least capable of undergoing mitosis. Microscopical examination of MDBK_{CS} monolayers haemadsorbed with fowl red blood cells and stained with Giemsa (Plate 5) revealed an isolated cell in mitosis, surrounded by haemadsorbed chicken erythrocytes. As illustrated in Plate 6, immunofluorescence staining of MDBK_{CS} cells frequently showed mitotic cells carrying specifically labelled viral material.

A study of the growth and survival of persistently infected cells was carried out by direct observation of those monolayer cultures which had been haemadsorbed previously. The method used was as follows: Suspensions containing sparsely distributed MDBK_{CS} cells from the 18th and 19th subcultures were propagated in plastic 2oz bottles to each of which a microscopic counter grid was attached on the outside surface. After 12 hours of incubation, red blood cells were added, aseptically, to each of the cultures, and well defined areas containing isolated haemadsorbing cells (or clusters arranged in groups of not more than 4-6 cells), were chosen for further examination. The cultures were then refed with growth medium and returned to the 37°C incubator.

Plate 3 : Fluorescent antibody staining of a
monolayer culture of MDBK_{cs} cells
showing an infected cell with aggregates
of viral antigen dispersed throughout
its cytoplasm. x800.
Counterstained with Evans blue.

Plate 4 : Immunofluorescence staining of monolayers
of MDBK control cells.
Notice the complete absence of
fluorescing particles of viral antigen.
x800.

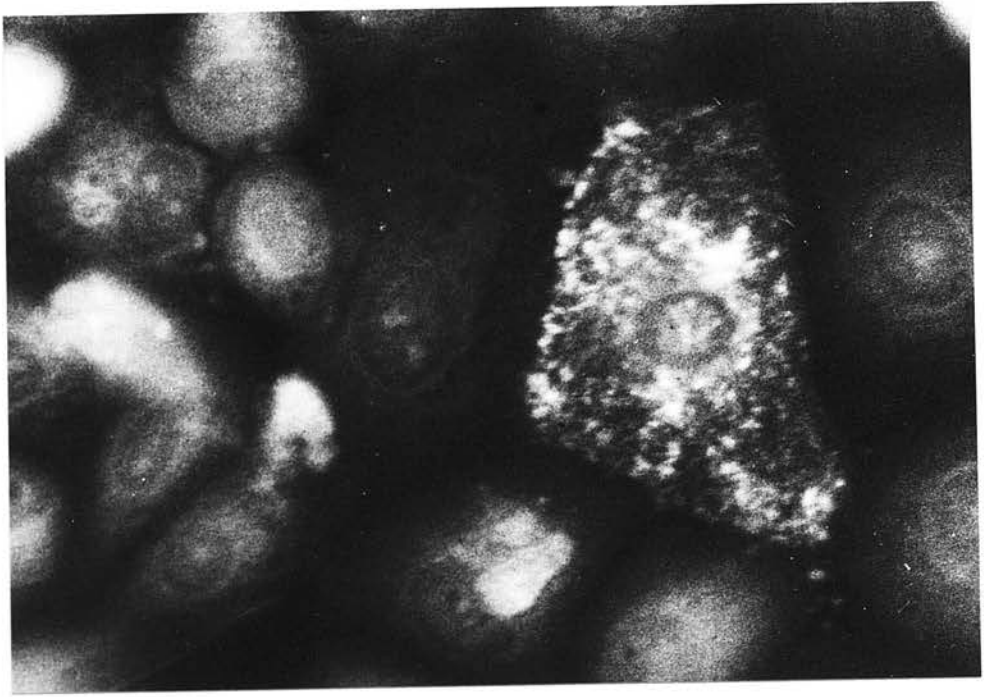
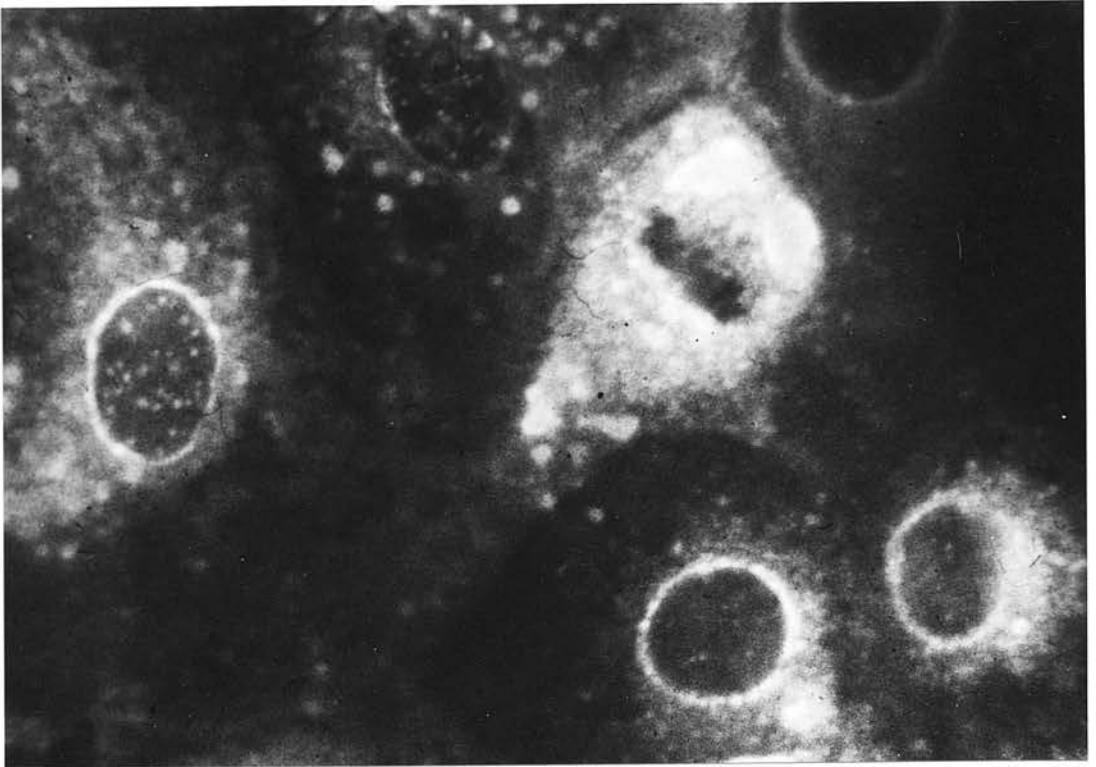
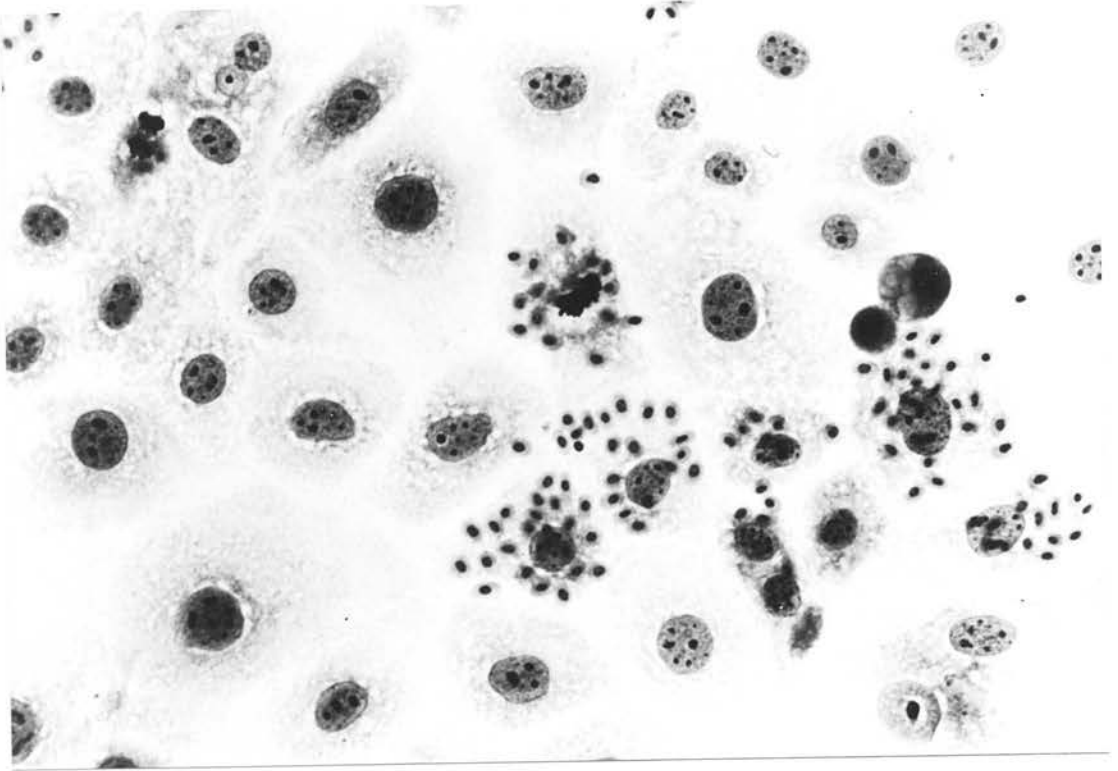


Plate 5 : Preparation of MDBK_{cs} cells showing haemadsorption of fowl erythrocytes. In the centre of the field one of the infected cells is undergoing mitosis (metaphase). Giemsa stain x450.

Plate 6 : Preparation of MDBK_{cs} cells showing marked fluorescence of an infected cell in mitosis (metaphase). x1200.



Observations of the selected areas were carried out every 6 hours for the next two days, using the haemadsorbing technique described above. The results of this experiment showed that about 75% of the infected cells became picnotic and eventually detached from the surface of the glass without dividing or after undergoing multiplication. The remaining cells survived after division, and their progeny continued to show haemadsorbing sites until the end of the test period.

d) Growth rate and cell morphology

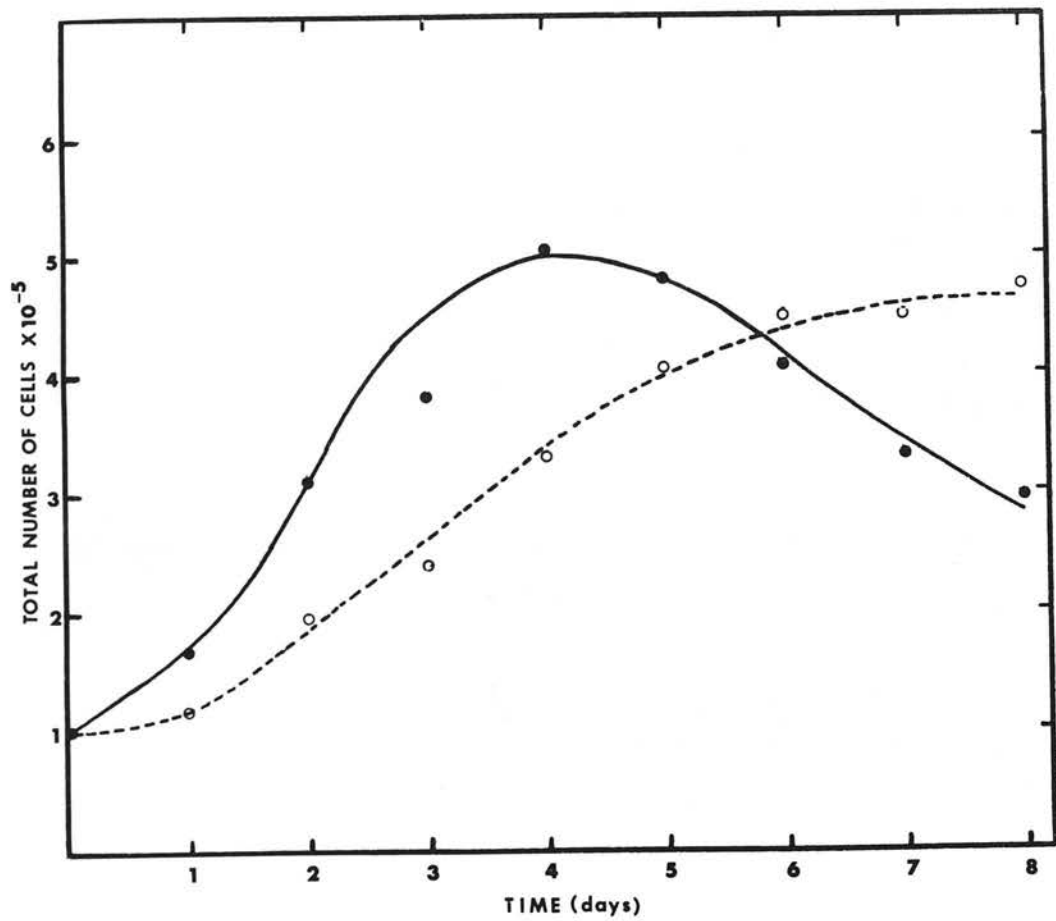
Throughout this study, monolayer cell cultures were propagated at weekly intervals by trypsinization as described in Materials and Methods. As a general rule, the normal "working rate" for the healthy MDBK cell line was approximately 1:3 to 1:4 for cultures maintained in 4oz bottles for 5-7 days at 37°C. In contrast, the rate for carrier cultures maintained under similar conditions was only 1:2 to 1:3.

Although the results obtained in tube cultures were similar to those in medicinal flat bottles, it is emphasised that the medium in the former remained unchanged throughout the experiment whereas the latter cultures were refed with maintenance medium after 2-4 days of incubation. This would suggest, therefore, that the growth cycle of the cells in tube cultures was completed earlier than that in 4oz bottles.

Cells from both of the control and carrier cultures were seeded at concentrations of 1×10^5 cells/ml and incubated at 37°C. Three tube cultures prepared from each cell line were removed at daily intervals during the first 8 days of the experiment and the total number of viable cells per ml was calculated by standard methods. The results, expressed as the mean of four experiments carried out in similar fashion, showed that the total yield of cells from either type of culture was almost identical, although there appeared to be a delay

Graph 1 : Rate of growth of uninfected MDEK and persistently infected MDEK_{cs} cultures. Cells from both types of culture were seeded at concentrations of 1×10^5 cells/ml and incubated at 37°C. Three tube cultures prepared from each cell line were removed at daily intervals during the first 8 days of the experiment and the total number of viable cells per ml was calculated by standard methods.

●—●, MDEK cell cultures ; ⊖---⊖, MDEK_{cs} cell cultures.



of about 4 days before the maximum output of cells was achieved by the carrier cultures (Graph 1). It was also of interest, that the morphology of the carrier cells maintained after the first subcultures was appreciably different from that of the original MDBK cell line. This is clearly illustrated by Plates 7,8 which show, respectively, the masses of MDBK cells piling up to form an irregular network of rope-like structures on the surface of the regular cell monolayer compared with the larger aggregates of MDBK_{cs} cells, without obvious rope-like elements, scattered throughout the monolayer. Although the size of the individual cells in the carrier cultures appeared to be greater than that of the control cells, it is probable that the increase was merely due to their slower rate of multiplication.

e) Treatment of cultures with specific antiserum

The inhibitory effects of NDV antiserum on cells persistently infected with NDV was studied on monolayers of MDBK_{cs} cells at the 11th and 25th passage levels.

Rabbit anti-NDV serum with an haemagglutinating-inhibition titre of 1/256 to 1/512 was diluted 10 to 20 times in maintenance medium. By this means, the antiserum was found to be capable of producing complete neutralization of the virus at dilutions of up to 1/160 when used to overlay the carrier cultures. Treated and untreated cultures were incubated at 37°C and examined daily for the presence of infected cells and for the release of extracellular infectious virus. The tests used included direct haemadsorption with chicken red blood cells for the presence of cell-surface haemagglutinins, and immunofluorescence staining for the detection of specific intracellular viral antigens. Fluids from successive harvests were stored at -70°C and later pooled when examined for the presence of extracellular infectious virus.

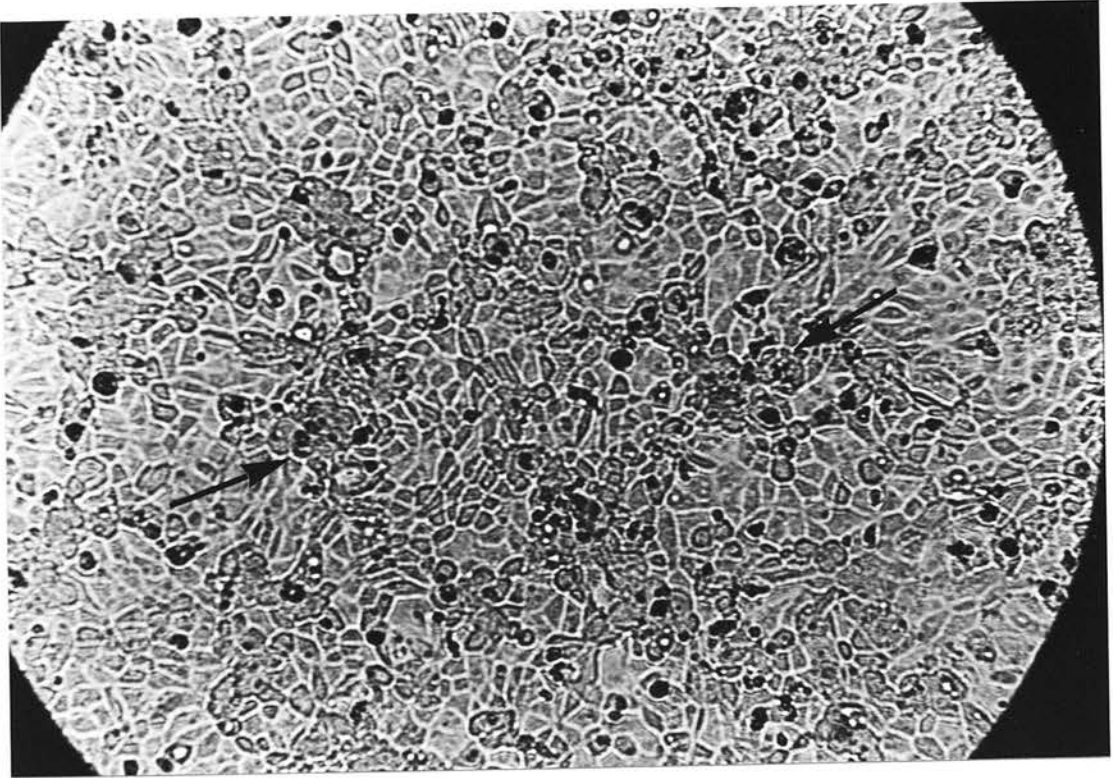
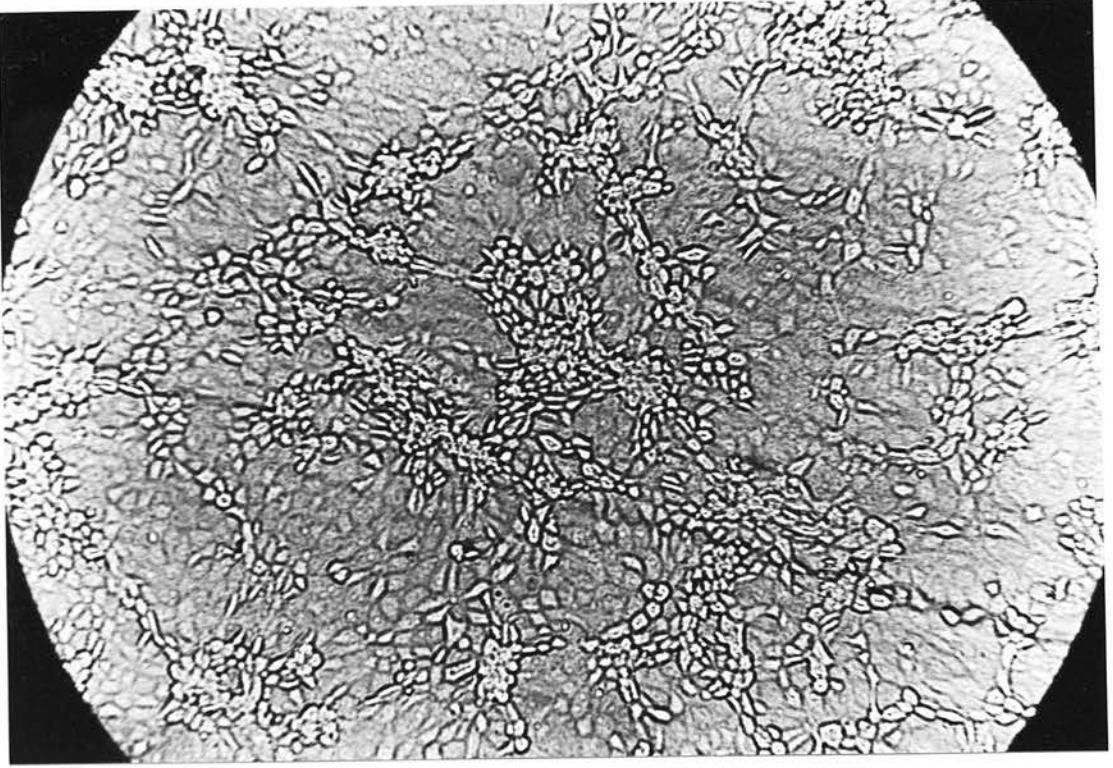
Plate 7 : Seven-day-old MDEK culture, unstained.

Notice the masses of cells piling up to form an irregular network of rope-like structures on the surface of the monolayer. x110.

Plate 8 : Seven-day-old MDBK_{cs} culture, unstained.

There are numerous aggregates of clumped cells. x110

(Arrows) and the individual cells constituting the monolayers are larger than those in Plate 7. x110.



The results of this investigation showed that haemadsorption did not occur in monolayer cultures of persistently infected cells maintained in the presence of NDV antiserum and that the inhibitory effect persisted for up to 6 days after removal of the antiserum from the medium. Although a number of infected cells were seen during the first two subcultivations of carrier cells in the presence of NDV antiserum, the intracellular aggregates of specifically staining viral material had disappeared by the third passage: that is after about a month's treatment with antiserum. The absence of viral material in the 'cured' monolayers was confirmed by fluorescent antibody staining and infectivity tests, as described previously. Further attempts to demonstrate virus material by co-cultivating carrier cultures and BHK-21 cells failed to rescue infective viral particles.

f) Superinfection with related and unrelated viruses

I - Newcastle disease virus

Twenty-hour-old monolayers from MDBK and MDBK_{cs} cultures were infected with 10 MOI of egg-grown NDV. Twelve hours later, fluids from both cultures were harvested and checked for the amount of virus released using "indicator" BHK-21 cells as described in Materials and Methods.

The results shown in Table 3 indicate that there was a significant drop in the infectivity titre of the superinfected carrier cultures as compared with that of the MDBK control. This suggested that the MDBK_{cs} cells persistently infected with NDV are resistant to superinfection with the homologous virus.

II - Bovine mamillitis virus (BMV) and Sendai virus

Both control and carrier cultures were infected with ten-fold dilutions of BMV or Sendai virus by the method used for the estimation of virus TCD₅₀ calculations. Because the parameter used to assess the

TABLE 3

ABILITY OF CONTROL AND CARRIER CULTURES OF MDBK_{cs} CELLS TO
SUPPORT GROWTH OF RELATED AND UNRELATED VIRUSES (TCD₅₀/0.2ml)

Virus	Superinfected carrier cultures of MDBK _{cs}	Control cultures of:	
		Healthy MDBK cells	Carrier MDBK cells cs
NDV ^{a)}	10 ^{3.75}	10 ^{6.75}	10 ^{0.5}
Sendai ^{b)} virus	10 ^{3.5}	10 ^{3.5}	10 ^{0.5}
BMV ^{c)}	10 ^{3.0}	10 ^{3.0}	ND ^{d)}

- a) Twenty-hour-old monolayers of MDBK and MDBK_{cs} were inoculated with egg-grown NDV at 10 MOI/cell, and incubated for one hour at 37°C. After being washed, the cultures were refed with maintenance medium, and the fluids harvested 12 hours later. Infectivity titres were determined in EHK-21 monolayers as described in Materials and Methods.
- b) Twenty-hour-old MDBK and MDBK_{cs} monolayers were infected with ten-fold dilutions of Sendai virus, after being treated for 30 minutes with a 1/10 dilution of anti-NDV rabbit serum (HI 1/128). The TCD₅₀ was determined after 3 days by the presence of foci of haemadsorbing fowl erythrocytes in inoculated cultures.
- c) Control and carrier cultures inoculated with ten-fold dilutions of BMV for one hour at 37°C. Cultures were washed and refed with maintenance medium, and results assessed by the presence of typical CPE after 5 days.
- d) Not done.

presence of virus was by haemadsorption of fowl erythrocytes to infected cells, care was taken to treat the control and test cultures for 30 minutes at 37°C with a 1/10 dilution of anti-NDV rabbit serum (HI 1/128) prior to inoculating them with Sendai virus. In the case of BMV, the presence of the virus was determined by its characteristic herpesvirus-type CPE. (Martin et al., 1966; 1969)

The results of these experiments (Table 3) were read 3-5 days after infection with BMV or Sendai virus and clearly showed that the carrier and control cultures were equally capable of supporting the growth of either type of virus.

g) Temperature sensitivity of the NDV strain in carrier cultures

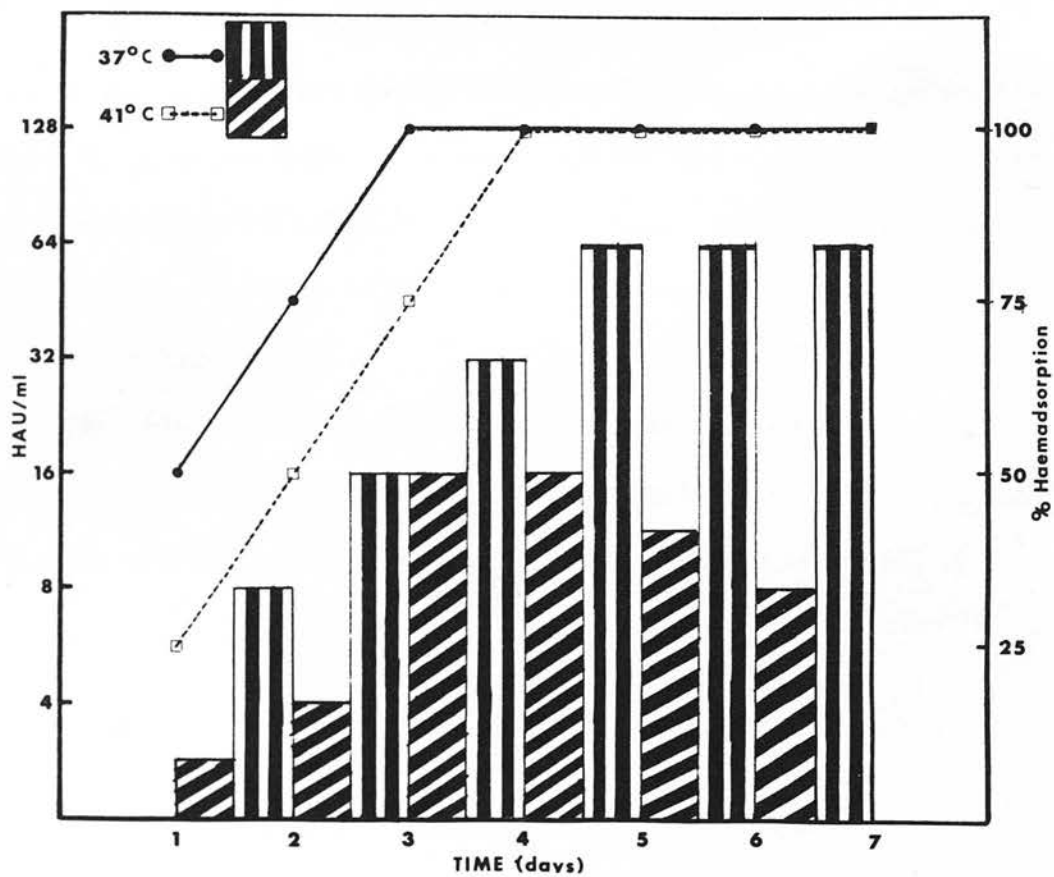
In order to assess the temperature sensitivity of the virus present in MDBK_{cs} cultures, coverslip tubes were inoculated with suspensions of carrier cells dispensed at routine concentrations. These were incubated for periods of up to 7 days at 37°C and 41°C, and checked daily for released haemagglutinins, haemadsorbing cells and the presence of specific intra-cellular fluorescing material.

Cultures prepared simultaneously in 4oz bottles and kept under identical conditions were propagated twice during a period of 7 weeks and checked for the presence of extracellular or cell-associated viral antigens.

Concurrently with these experiments, attempts were made to obtain an enhanced viral response by co-cultivating the carrier and BHK-21 indicator cells in the manner described in Materials and Methods.

The results presented in Graph 2 show that the number of haemadsorbing cells present in the co-cultivated monolayers after 4 days of incubation was the same, whether the cultures were incubated at 37°C or 41°C. Moreover, the degree of haemadsorption was extensive and the infected cells were uniformly distributed throughout the

Graph 2 : Released and cell-associated virus in MDBK_{cs} cells co-cultivated with BHK-21 cells at 37°C and 41°C as described in Materials and Methods. The results refer to daily examinations of the released (histogram) and cell-associated (linear) haemagglutinins both at 37°C and 41°C.



culture. The delayed response of cultures co-cultivated at 41°C to produce as much cell-associated viral material as they do at 37°C might be due, in part, to the non-permissive nature of the metabolic processes of both MDBK and BHK-21 at high temperature. This feature might also account for the reduced output of extracellular haemagglutinins which occurred at 41°C.

In all other experiments, no released haemagglutinins were detected either in tube or bottle cultures, and the results obtained by haemadsorption and fluorescent antibody staining confirmed that less than 1% of cells were infected at either temperature. However, the appearance and distribution of fluorescing viral material in the infected cells at 37°C or 41°C, revealed no differences even after seven weeks of incubation.

Finally, when fluids from the 5th and 6th subcultures of MDBK_{cs} were inoculated in CEF cultures and subsequently incubated for four days at 37°C and 41°C, the infectivity titres were identical.

SECTION II

COMPARISON BETWEEN REGULATED AND CARRIER PERSISTENT INFECTIONS OF MDBK CELLS WITH NDV

1 - General characteristics of the two systems

An accidental persistent infection with NDV appeared in a Madin and Darby bovine kidney cell line (MDBK) several years ago in this laboratory (Edwards, 1972; Fraser et al., 1976).

The infection has been classified as a regulated type of infection (MDBK_{pi}) on the basis of the following criteria: a) over 90% of the cells are infected but continue to divide and grow normally (Plates 9,10); b) the cells cannot be cured by specific antisera; c) the cultures show a high degree of resistance to superinfection with the infecting virus, but not with unrelated viruses; and, d) antibodies are not required in the medium to induce and maintain the carrier state.

Further studies revealed that a very low level of virus particles is released from MDBK_{pi} which are infectious for chick embryo fibroblasts, and the infectivity titres of the cell culture fluids are usually between 10 to 100 TCD₅₀/ml. However, successive passages in embryonated eggs of supernatant fluids or concentrated pellets from such cultures, failed to induce pathogenic effects on the embryos, or even traces of released virus in the allantoic fluids.

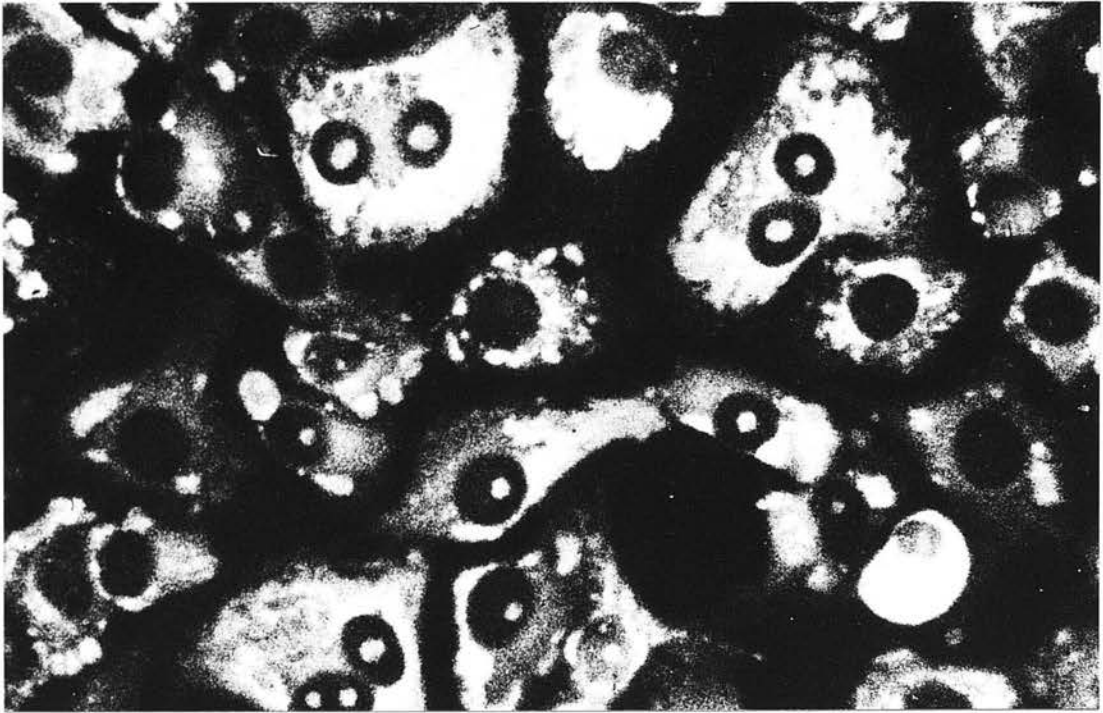
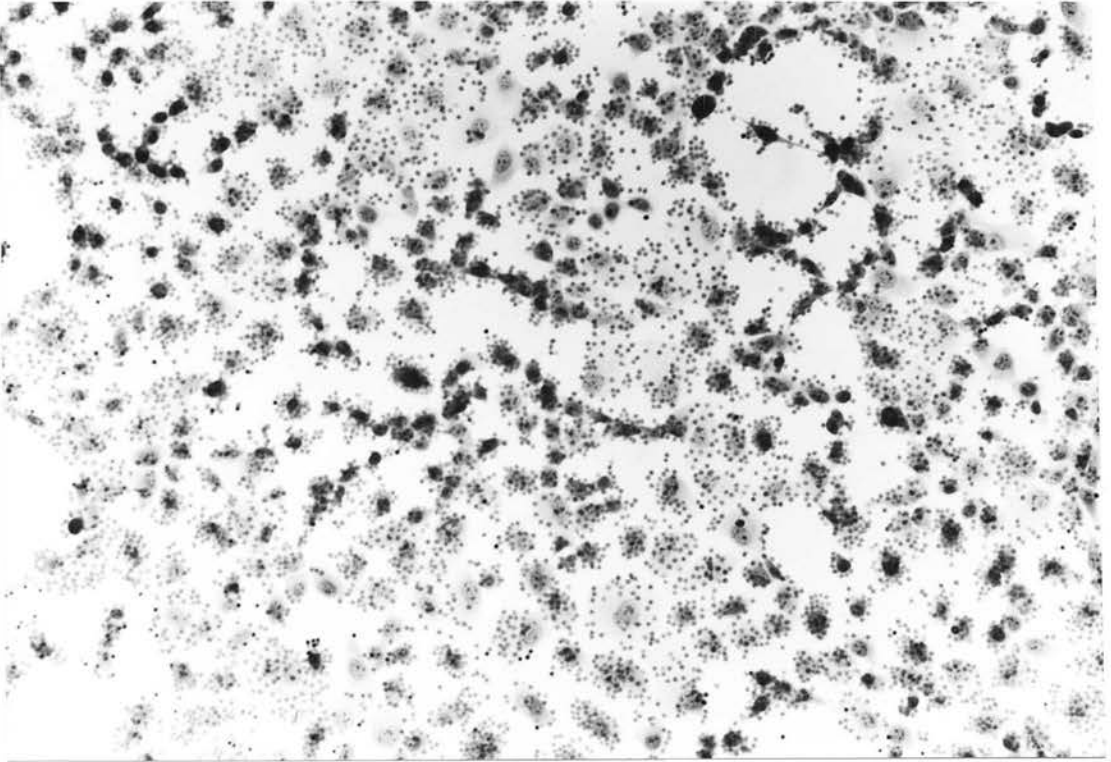
Temperature sensitivity experiments conducted at 41°C showed that the viral material progressively disappears from the surfaces of MDBK_{pi} cells, and that after 4 weeks of incubation at such temperature no haemadsorption or haemagglutinating activity remains. However, intracytoplasmic viral material can still be detected for up to 10 weeks at 41°C. It is interesting to note that all those parameters returned to normal within 2 to 3 days after the cultures were replaced in the 37°C incubator.

Plate 9 : Preparation of MDEK_{pi} cells haemadsorbed
with guinea-pig erythrocytes.

Notice the very high proportion of
infected cells in the monolayer.

H & E x190.

Plate 10 : The same culture as in Plate 9, stained
by fluorescent antibody, showing the
presence and distribution of viral
antigen in infected cells. The
cytoplasm is invariably affected and the
distribution of viral antigen in many
of the nuclei suggests a nucleolar
involvement (see Plate 24). x800.



It has been clearly established that NDV-antiserum was not required to establish the original persistent infection. Also, that propagation of MDBK_{pi} cells for 5 weeks with weekly subcultures using media containing anti-NDV serum, led to the disappearance of surface or released haemagglutinins, but without loss of viral material inside the cells. Furthermore, viral expression was restored almost immediately after the antibody was removed from the surrounding medium.

The ability of MDBK_{pi} cells to support infection with NDV was investigated, and the results confirmed that there was a significant resistance to superinfection with the homologous virus. However, with related viruses of the same genus there was only a partial resistance which was absent when the unrelated herpesvirus (BMV) was tested.

As Table 4 shows, the induced carrier state shares only a few of the characteristics which typify the regulated type of infection. These appear to be closely related to the capacity of the host cell to divide or sustain growth of the infecting and unrelated viruses.

The main features of MDBK_{cs} cultures are in conformity with those of a carrier state infection characterized by the fact that a) only a tiny proportion of the cells show infection, b) variable treatment periods of the cultures with specific antiserum may cure them of the infection, and c) antibodies do not seem to be required to establish and maintain the infection. An additional characteristic of the MDBK_{cs} cultures is the production of an inhibitory factor which is released into the medium but seems to be absent in MDBK_{pi} monolayers. The significance of this observation will be discussed later.

TABLE 4

GENERAL CHARACTERISTICS OF THE TWO MDBK CELL LINES
PERSISTENTLY INFECTED WITH NDV

Character	Persistently infected cultures	
	MDBK pi	MDBK cs
Antibody required to induce and maintain the persistent infection	-	-
Presence of dividing mitotic cells	+	+
Percentage of cells showing infection	>90	<1
Infection cured by specific antiserum	-	+
Resistance to superinfection with NDV Sendai virus EMV	High Moderate None	High None None
Temperature sensitivity at 41°C	+	-
Infectivity for embryonated eggs	-	+
Inhibitory factor in culture media	-	+

2 - Comparative biochemical studies

a) Viral purification

In order to study the biochemical and biophysical properties of the virus released from the two types of persistent infection (MDBK_{pi} and MDBK_{cs}), purification was undertaken as described in Materials and Methods. Viruses were purified either from cell culture fluids or after passage in embryonated eggs. Typical yields achieved in the various systems are set out in Table 5. Two different types of equilibrium gradients were tested, namely potassium tartrate and sucrose, as described in Material and Methods, but the results showed no significant difference in the performance of both systems.

i - Of virus grown on cell cultures

Volumes varying from 0.5 to 2 litres of supernatant fluids of MDBK_{pi} and MDBK_{cs} cell cultures, or from "mixed cultures" of these cell lines with BHK-21 as indicator cells, were used in these experiments. Cell cultures were propagated either in stationary cultures maintained in Roux bottles or in rolling cultures using Winchester bottles. After pelleting and purification in the sucrose gradients, the haemagglutination titres for any of the fluids tested did not exceed 256 HAU/0.05ml (Table 5).

Electron microscopic studies on concentrated and purified material from both persistent infections disclosed only very few complete particles of round or elongated shape containing typical herring-bone nucleocapsid structures, and limited by a viral membrane with regular projections, similar to a typical paramyxovirus (Plate 11).

II - Of virus grown in embryonated hen's eggs

Fluids from MDBK_{cs} cultures were centrifuged at low speed to sediment the cell debris, and were inoculated in nine-day-old embryonated eggs with 2×10^3 TCD₅₀/egg. Within 36 to 48 hours of

TABLE 5

YIELD OF VIRUS DURING PURIFICATION STAGES

Source of virus	Start volume (ml)	Stages of purification (HAU/0.05ml)					
		Fluid ^{a)}	Clarified fluid ^{b)}	Pellet ^{c)}	TCTF ^{d)} treatment	SG1/ TG1 ^{e)}	SG2/ TG2 ^{f)}
MDBK _{cs}	1000	-	-	4	8	16	ND ^{g)}
MDBK _{cs} in BHK-21 co-culture	600	2	2	128	128	256	ND
MDBK _{cs} egg-grown	200	256	256	4096	4096	16384	8192
MDBK _{pi}	2000	1	1	16	16	256	ND
MDBK _{pi} in BHK-21 coculture	800	2	2	8	8	128	ND
NDV Herts egg-grown	200	256	256	8192	8192	16384	8192

a) Allantoic or cell culture fluids

b) Fluids were clarified by centrifugation at 500xg for 15 minutes at 4°C.

c) Supernatants from b) were pelleted at 75000xg for 2 hours at 4°C, and resuspended in 1.5ml of PBS.

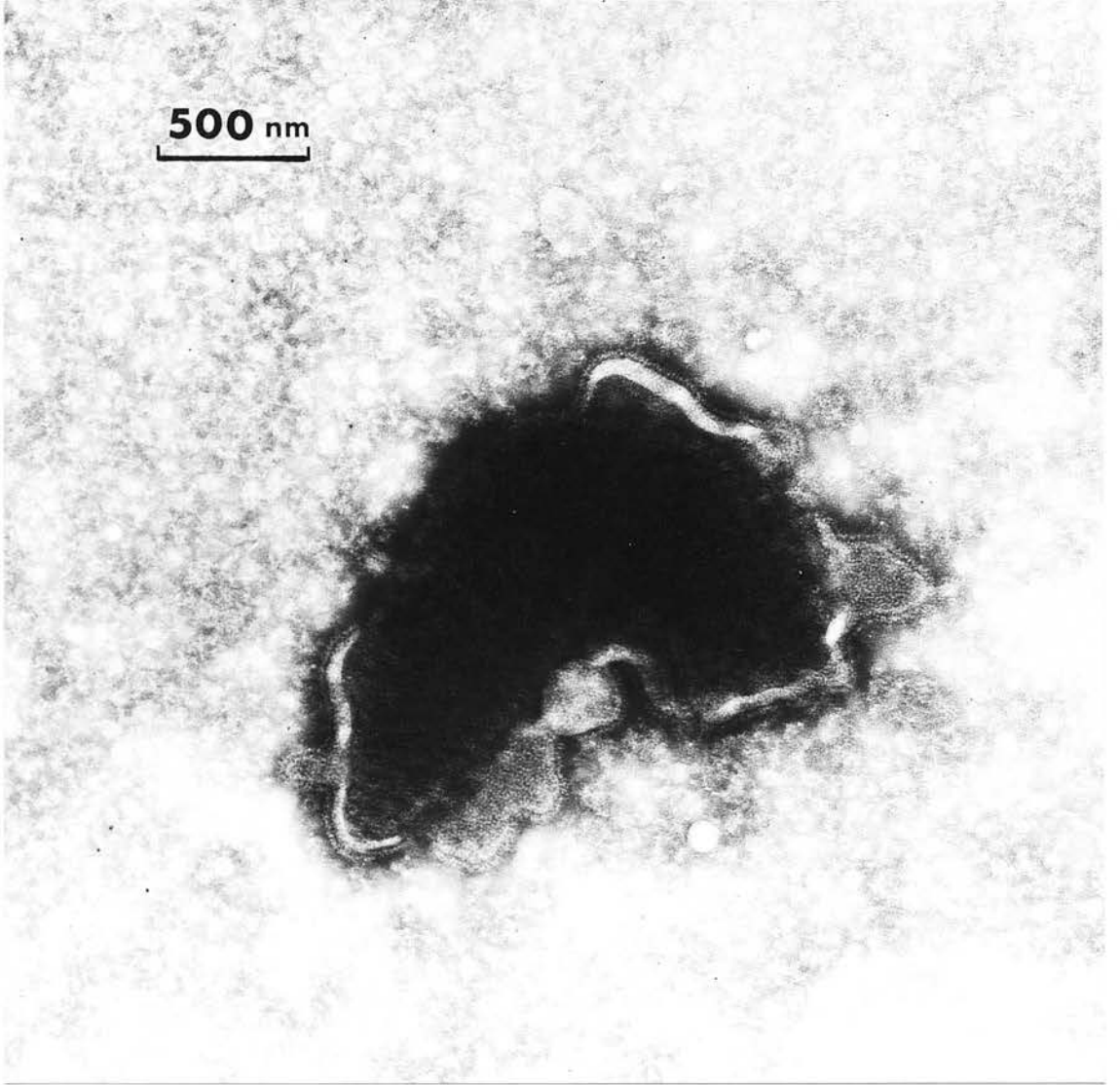
d) Resuspended pellets were treated with equal volumes of trichlorotrifluorethane (TCTF), and separated by low speed centrifugation for 10 minutes at 4°C.

e) TCTF treated pellets, made up to 1.5ml, were layered on top of 25% to 55% (w/w) sucrose or 15% to 40% (w/w) tartrate gradients and sedimented for over 15 hours at 100000xg, at 4°C.

f) Sucrose-free purified virus were overlaid on top of 10% to 30% (w/w) sucrose gradients and centrifuged at 75000xg for 20 minutes, at 4°C. Dialysed virus from tartrate gradients were reprocessed as in e). All purified pellets were resuspended in 1.5ml of PBS.

g) Not done.

Plate 11 : Electron micrograph of MDBK_{pi} cell culture fluid after concentration, showing a paramyxovirus-like particle. Notice the thick envelope bears a characteristic fringe of radially arranged peplomers. The RNP is not well defined. x40000.



incubation all embryos died showing punctuated or extensive haemorrhagic lesions. The haemagglutinins released could be detected up to titres of 256 HAU/0.05ml. the infectivity titres of the allantoic fluids reached similar levels to those obtained with the original Herts strain of NDV, that is 10^8 to 10^9 TCD₅₀ per ml.

On the other hand, when embryonated eggs were inoculated with either undiluted or concentrated MDBK_{pi} culture fluids, no viral infectivity was demonstrable even after four egg passages.

The distribution of haemagglutinin and protein in the first sucrose gradient of the purification of both MDBK_{cs} and Herts strain of NDV grown in embryonated eggs, is shown in Graph 3 A and B respectively. It can be seen that the distribution of the two viruses in equilibrium is very similar. Usually, when the gradients were examined with the unaided eye, two opalescent bands were visible. The top band was thin and indistinct, and was about one quarter of the distance from the top of the tube, whereas the lower band was thick and well defined, and was just below the middle of the gradient. When the gradient were fractionated, the two bands described corresponded with the two distinct peaks of haemagglutinin.

Electron microscopic examination of negative contrast stained preparations of those areas of the gradient have shown that particles with paramyxovirus-like morphology are present in large quantities in the lower band, but are sparse in the upper band (Plates 12,13).

Infectivity determinations of fractions 4 and 8 of MDBK_{cs} gave values of 7.3×10^6 and 3.4×10^{10} TCD₅₀/ml, respectively.

b) Studies on the polypeptide composition of the viruses

The polypeptide composition of the various purified viruses was studied by polyacrylamide gel electrophoresis. The purified viral isolates were disrupted with sodium dodecyl sulphate, and subsequently

Graph 3 : Distribution of haemagglutinin and protein in first sucrose gradient of purification of both MDEK_{CS} virus (A) and the Herts strain of NDV (B) grown in embryonated eggs.

○—○, HA titres; ○---○, concentration of protein (μg/ml); , density of sucrose in fractions, estimated by refractometry.

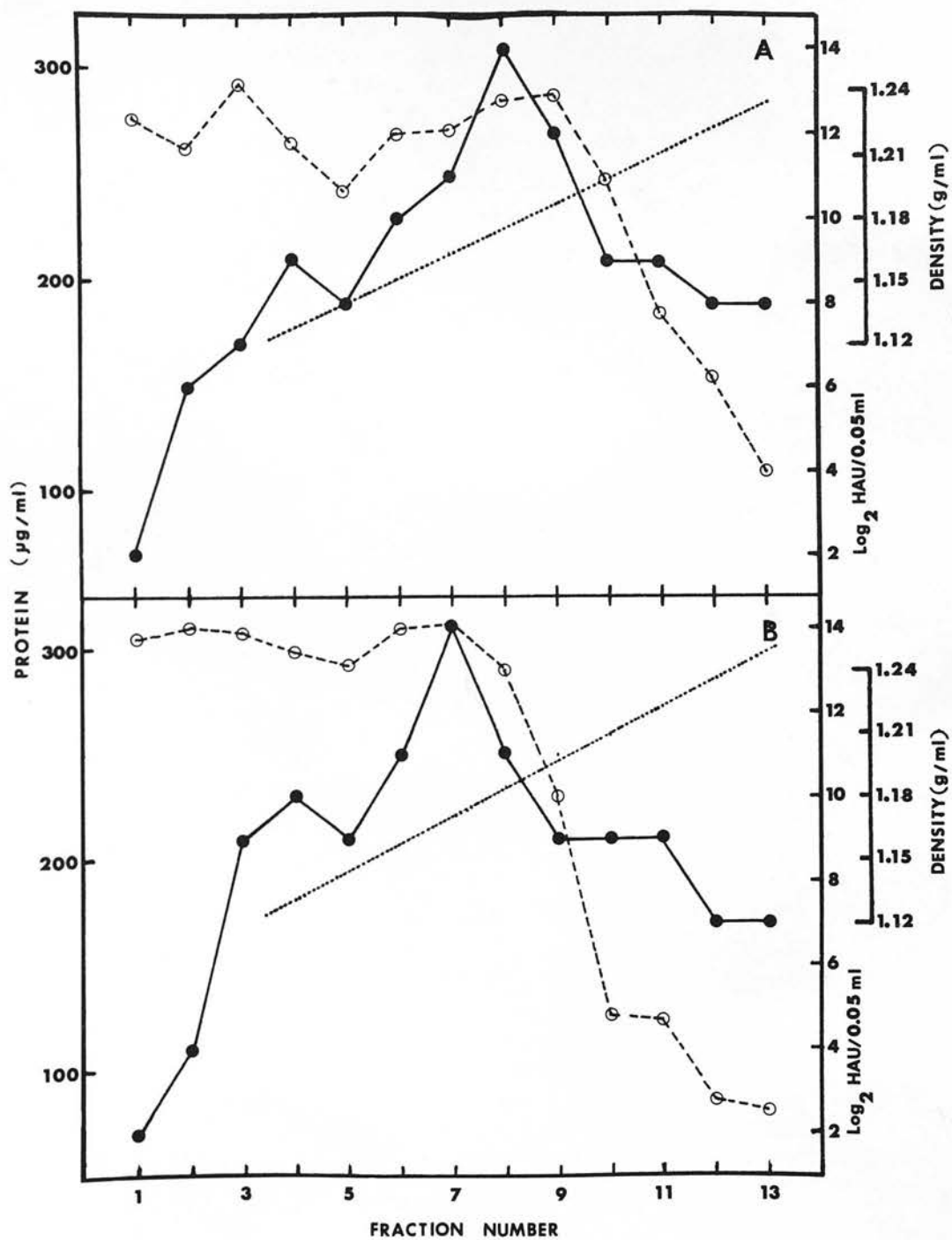
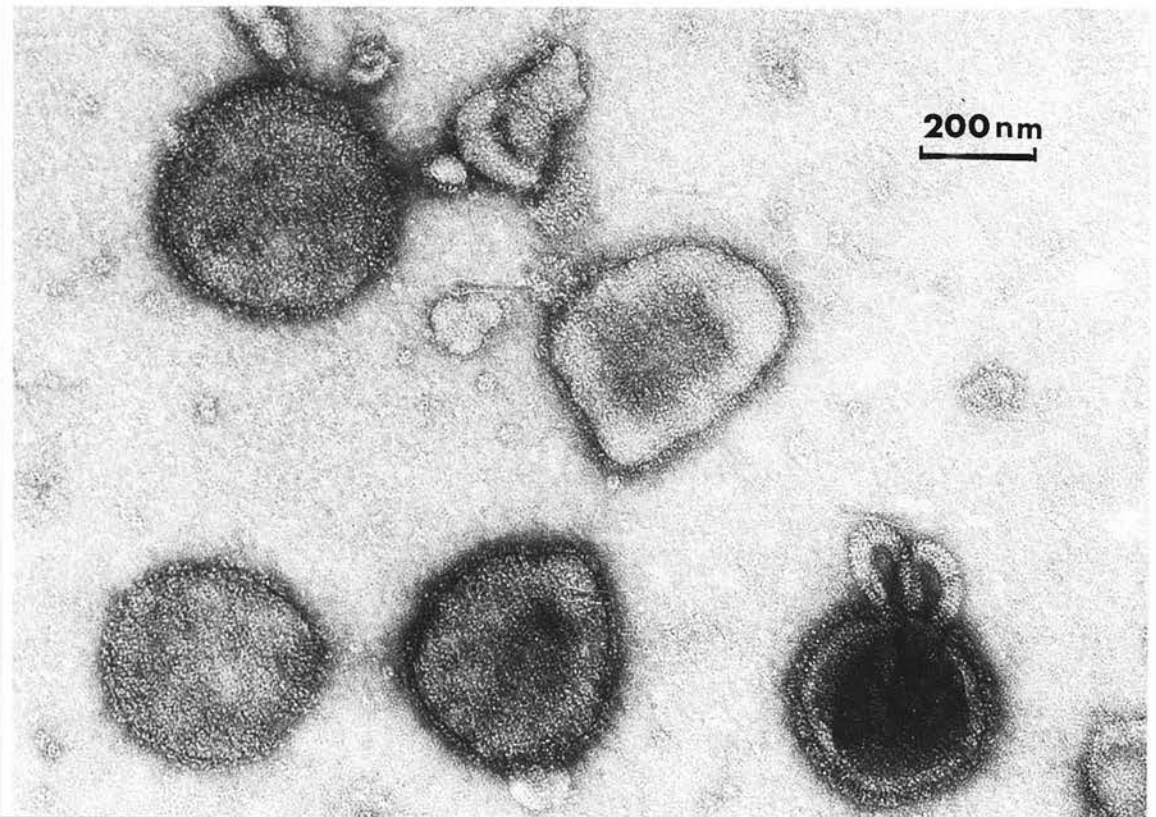
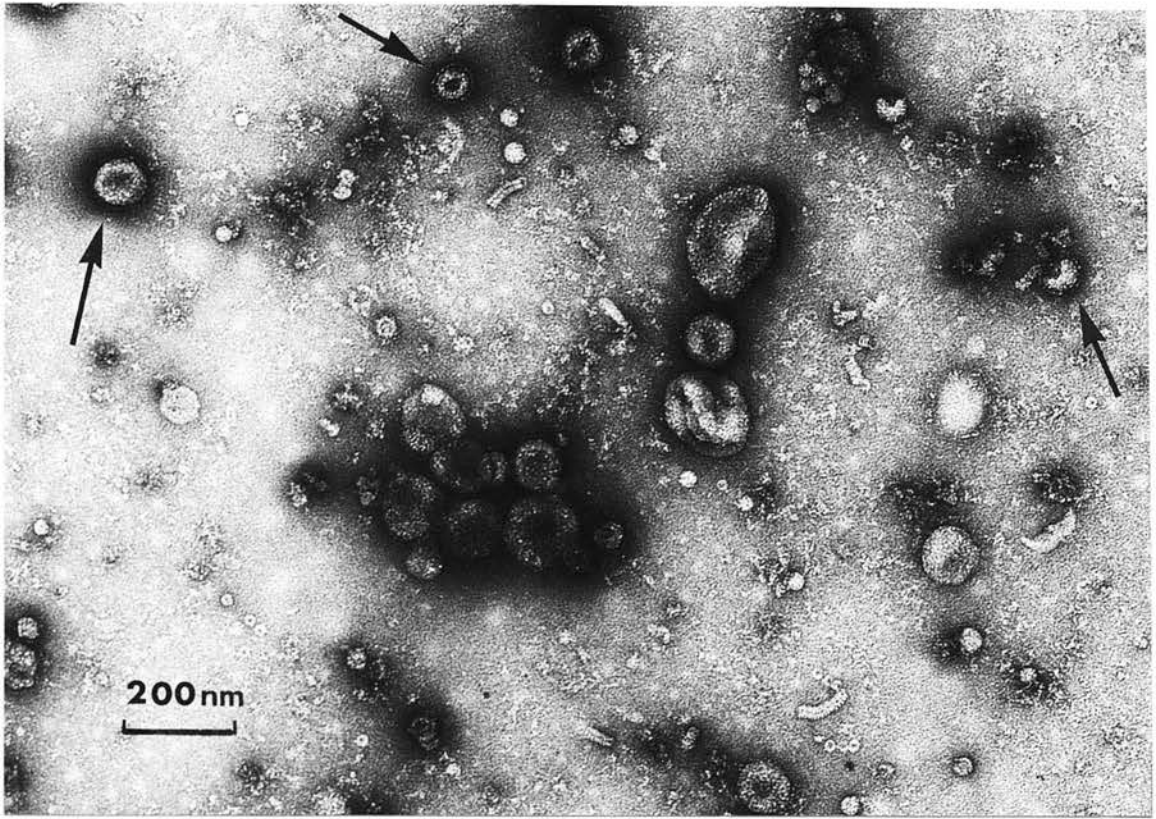


Plate 12 : Electron micrograph of viral band 1 from a sucrose gradient, showing only a few virus-like particles. (arrows) x60000

Plate 13 : Electron micrograph of viral band 2 from a sucrose gradient. Notice several virus particles with well-defined surface projections and a typical herring-bone RNP structure protruding from one of the particles. x60000



were subjected to electrophoresis, as described in Materials and Methods. Both cylindrical or slab gels were used and the viral polypeptides were stained with Coomassie Blue. Similarly, glycoproteins known to be present in NDV virions were stained in the slab gels by the Schiff's-periodate method.

Stained cylindrical gels were scanned as described, and gel profiles compared in order to detect differences in composition or distribution of the viral proteins. In order to determine the size of the viral polypeptides, four reference proteins of known molecular weight, namely bovine serum albumin (68000), ovalbumin (43000), chymotrypsinogen (25700), and myoglobin (17200), were subjected to electrophoresis in parallel gels. The molecular weights of viral proteins were calculated by the method described by Shapiro et al. (1967).

At least seven viral structural polypeptides were found in the original Herts strain of NDV as well as in egg-passaged MDBK_{cs} purified viruses (Plates 14,15). Such findings are in agreement with the results of several authors working with virulent or avirulent strains of NDV (Mountcastle et al., 1971; Scheid and Choppin, 1973; Moore and Burke, 1974; Morrison et al., 1975; Nagai et al., 1976b). In Table 6, the sizes of the viral polypeptides of NDV reported by some of these authors are compared with our present findings.

Three major peaks HN (mol.wt. 76000), NP (mol.wt. 53000) and M (mol.wt. 39000) were easily identified in Coomassie Blue stained gels. At least three other discrete peptide bands could be seen with the unaided eye, namely L (mol.wt. 177000), U1 (mol.wt. 45000) and U2 (mol.wt. 41000). However, on scanning the gels, the U1 could not be clearly resolved, and was evident as a shoulder on the NP/F peak (Plates 14,15). The nomenclature adopted in the present study is that initially proposed

TABLE 6
MOLECULAR WEIGHT OF NDV PROTEINS

Protein ^{a)}	Results of present study	Results observed by other authors				Biological function
		Mountcastle et al. (1971)	Scheid and Choppin (1973)	Moore and Burke (1974)	Nagai et al. (1976)	
L	177000	-	-	180000	b)	Unknown
HN ₀	-	-	-	110000	82000	HN precursor
HN	77000	74000	76000	75000	74000	Haemagglutinin & neuraminidase
F ₀	-	62000	-	-	68000	F precursor
F	53000	56000	62000	55000	56000	Haemolysis & cell fusion
NP	53000	56000	62000	55000	56000	Nucleocapsid subunits
U1	45000	53000	b)	52/ 53000	-	Unknown
U2	41000	46000	b)	49000	-	Unknown
M	39000	41000	b)	42000	41000	Matrix protein

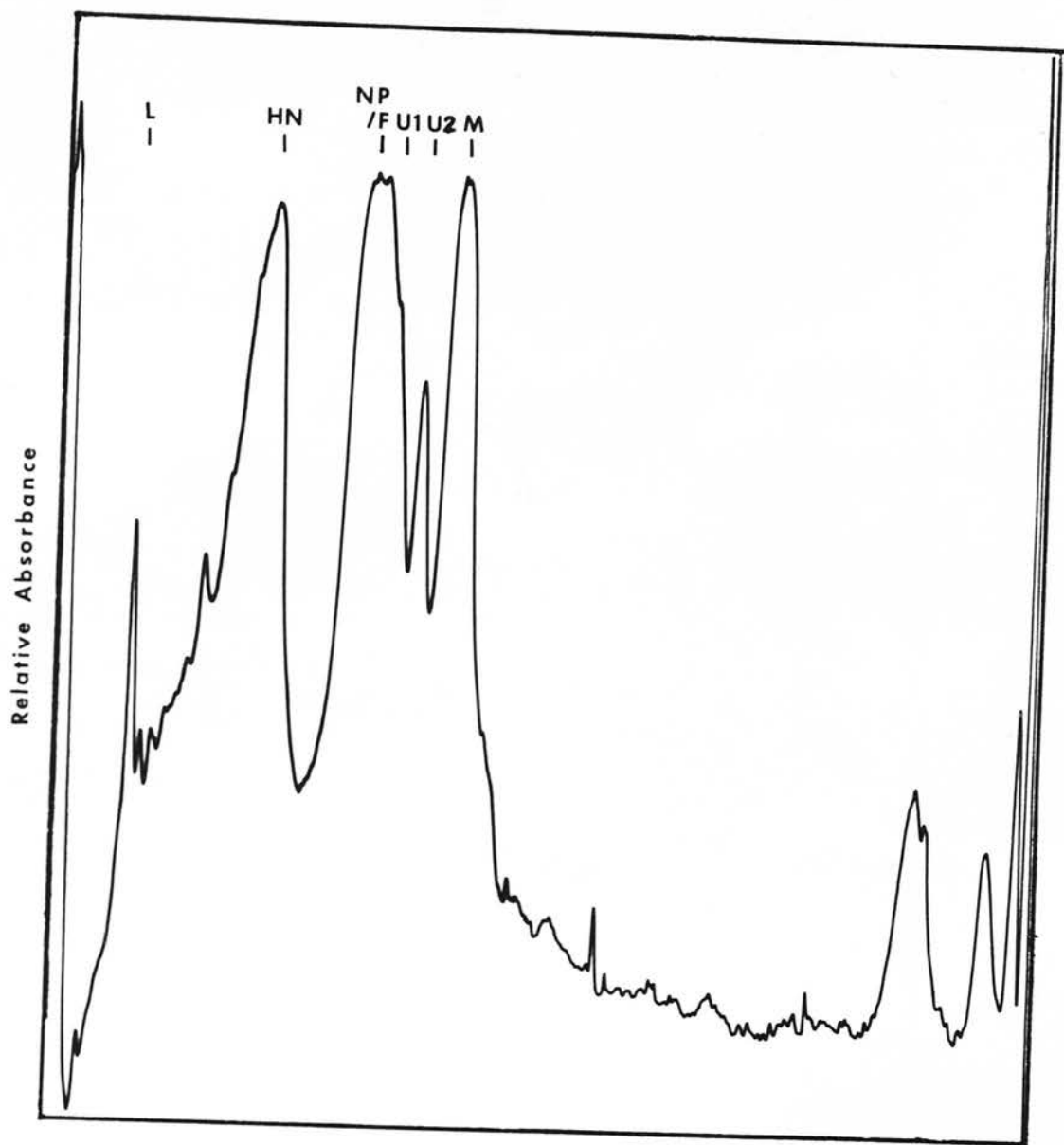
a) The nomenclature is that proposed by Scheid and Choppin (1974).

b) Mentioned but molecular weight not stated.

Plate 14 : Distribution of viral polypeptides of
Herts NDV in PAGE preparation.

The scanned profile (upper) should be
compared with the photographed gel
(lower).

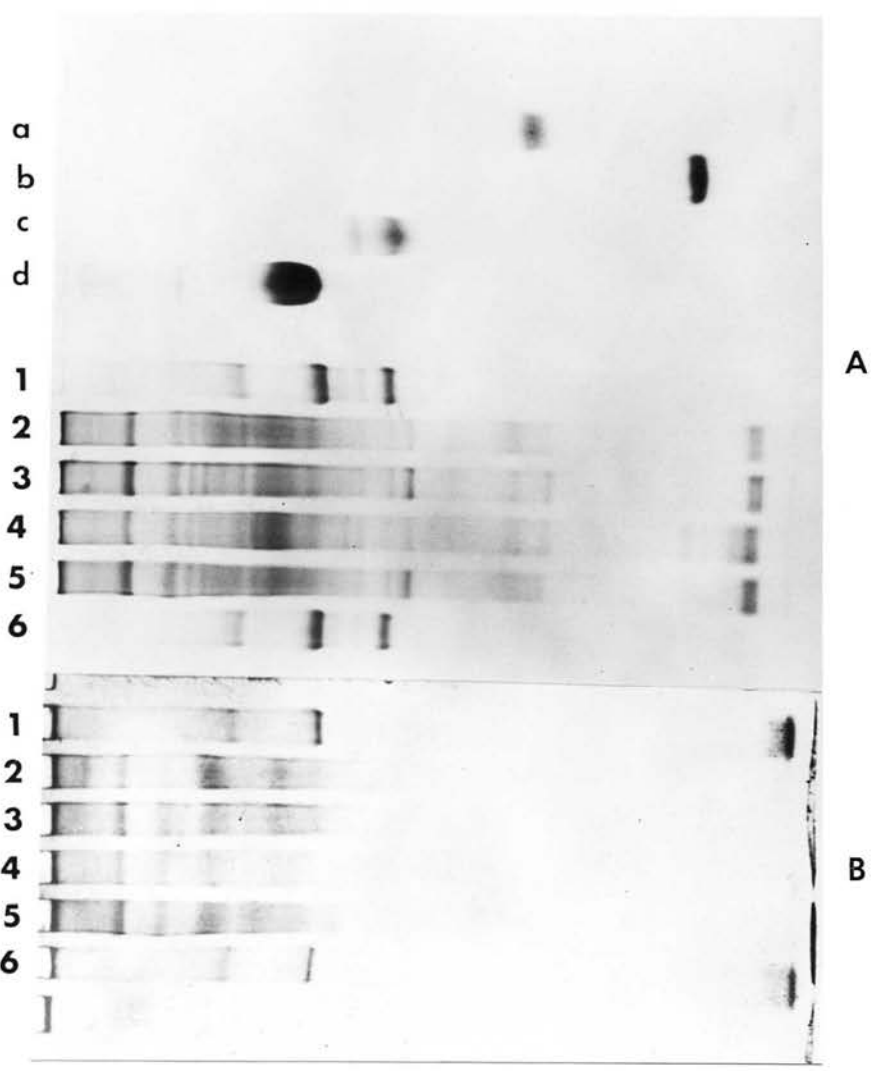
Note the seven viral proteins referred
as L, HN, NP/F, U1, U2 and M.



migration →

Plate 15 : Comparison of the various types of NDV by electrophoresis in a polyacrylamide gel slab. Conditions of electrophoresis as defined in Materials and Methods. Same series of viral samples stained with A) Coomassie blue method, and B) periodic acid-Schiff's method: 1 - MDBK_{cs} egg-grown; 2 - MDBK_{cs} cell culture grown; 3 - Herts strain of NDV from cell culture; 4 - MDBK_{cs} in co-culture with BHK-21 cells; 5 - MDBK_{pi} in cell culture; 6 - Herts strain of NDV egg-grown.

Four reference proteins: a) chymotrypsinogen; b) myoglobin; c) ovalbumin; d) bovine serum albumin.



by Scheid and Choppin (1974) for Sendai virus polypeptides and which later became widely used with other viruses.

Herts strain of NDV grown in cell culture and virus released from both types of persistent infection gave similar gel patterns (Plate 15). These are characterized by the presence of a great number of bands in which it is very difficult to distinguish the three major viral structural proteins. However, viral proteins L, U2 and M appear to be well defined in both cylindrical and slab gel preparations. The multiplicity of bands appearing in the purified isolates of cell culture grown viruses may be due to the presence of cellular or bovine serum proteins, and unsuccessful attempts were made in this laboratory to improve the resolution of the viral proteins present in cell culture isolates (Ruben, unpublished observations). The glycoprotein pattern of cell culture grown viruses gave no indication that any glycosylated component was associated with HN or NP/F areas. However, some of the glycoprotein material was in areas adjacent to the bands staining for carbohydrate in the egg grown viruses.

c) Haemagglutinin and neuraminidase activities in Herts NDV and
MDBK_{cs} viruses

Tartrate and sucrose density gradients containing either Herts NDV or egg-grown MDBK_{cs} virus were harvested in 0.5 to 1ml fractions, as described in Materials and Methods. The haemagglutinin titre, refractive index and optical densities at 280 and 260nm of each fraction were measured. The two viral bands, corresponding to the haemagglutinin peak activity, also showed distinct sedimentation densities, of 1.12 and 1.18 for the top and the lower band respectively. The fractions related to these areas were pooled separately and treated in order to remove the tartrate or sucrose present, as described in Materials and Methods.

The pooled fractions related to each viral band were then subjected to protein determination, neuraminidase assay and haemagglutinin titration. The results showed that the specific activity of both haemagglutinin and neuraminidase per μg protein was lower in band 1 than band 2 (Table 7). There is a difference in that activities obtained in the neuraminidase assays were about five times higher when compared with haemagglutinin activity. The reason is probably due to the inherent inaccuracy of the technique of measuring haemagglutination.

When material from those viral bands was subjected to acrylamide gel electrophoresis, the gel profiles showed that the top band (band 1) consists mainly of heavy polypeptides stained near the HN peak area. The levels of polypeptides in the NP/F and M bands were reduced in comparison with the normal viral profile. The lower band (band 2) gel profile corresponded to the typical pattern of NDV (Plate 16).

3 - Studies on RNA and protein synthesis

Experiments were conducted in order to investigate the mechanisms of persistent viral synthesis. Actinomycin D and cycloheximide, transcription and protein synthesis inhibitors, respectively, were used to ascertain whether there was a fault in the replication cycle where viral synthesis was affected.

Preliminary studies were also made in primary infections of MDBK cells with the Herts strain of NDV to enable a comparison to be drawn between primary and persistent infections.

a) Effect of actinomycin D on primary infection of MDBK cells with NDV

One-day-old monolayers of the control MDBK cell line, seeded in culture tubes at 2×10^5 cells/ml were inoculated with 0.5 MOI of NDV in 0.2ml. of diluted allantoic fluid. Control cultures were overlaid with 0.2ml. of maintenance medium. At the end of the adsorption time, a

TABLE 7

HAEMAGGLUTININ AND NEURAMINIDASE ACTIVITIES
OF DIFFERENT GRADIENT VIRAL BANDS

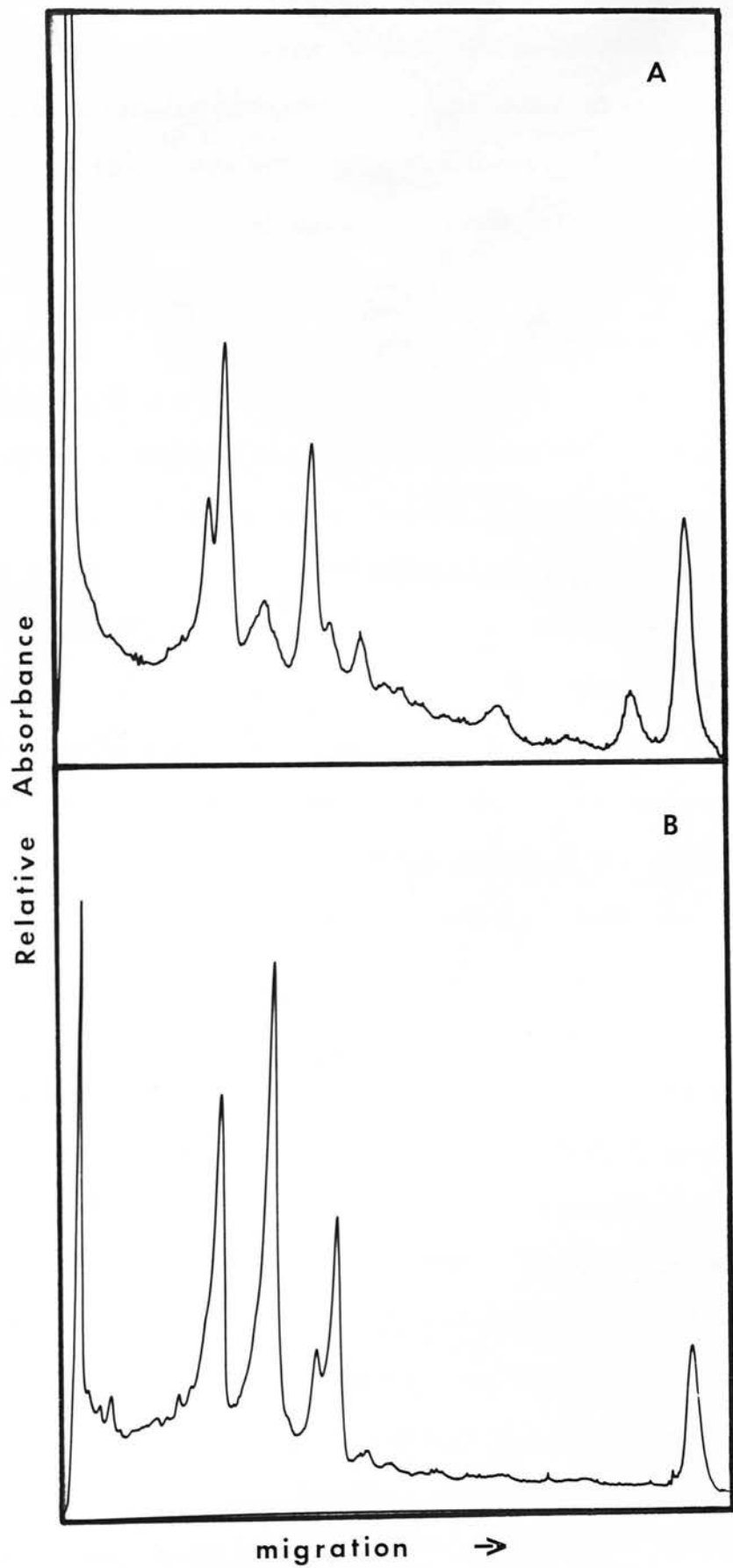
Virus ^{a)}	Haemag. ^{b)} specific activity HAU/ μ g protein	Nase ^{c)} specific activity Units/ μ g protein	Protein μ g/ml	Density at 20°C	
NDV	band 1	0.023	0.195	70	1.124
	band 2	0.034	0.863	377	1.182
MDEK cs	band 1	0.089	0.192	18	1.122
	band 2	0.105	0.898	243	1.184
NDV	band 1	0.021	0.052	75	1.120
	band 2	0.069	0.521	185	1.186
MDEK cs	band 1	0.024	0.080	33	1.126
	band 2	0.043	0.933	590	1.180
MDEK cs	band 1	0.020	0.119	79	1.128
	band 2	0.049	0.685	1035	1.184

a) Concentrated virus was laid on top of 15 to 40% (w/w) potassium tartrate gradients and centrifuged for 2.5 hours at 184000xg. Gradients were fractionated and the fractions analysed as described in Materials and Methods.

b) Haemagglutinin specific activity expressed in HAU/ μ g protein.

c) Neuraminidase specific activity expressed as units of enzyme per μ g of protein.

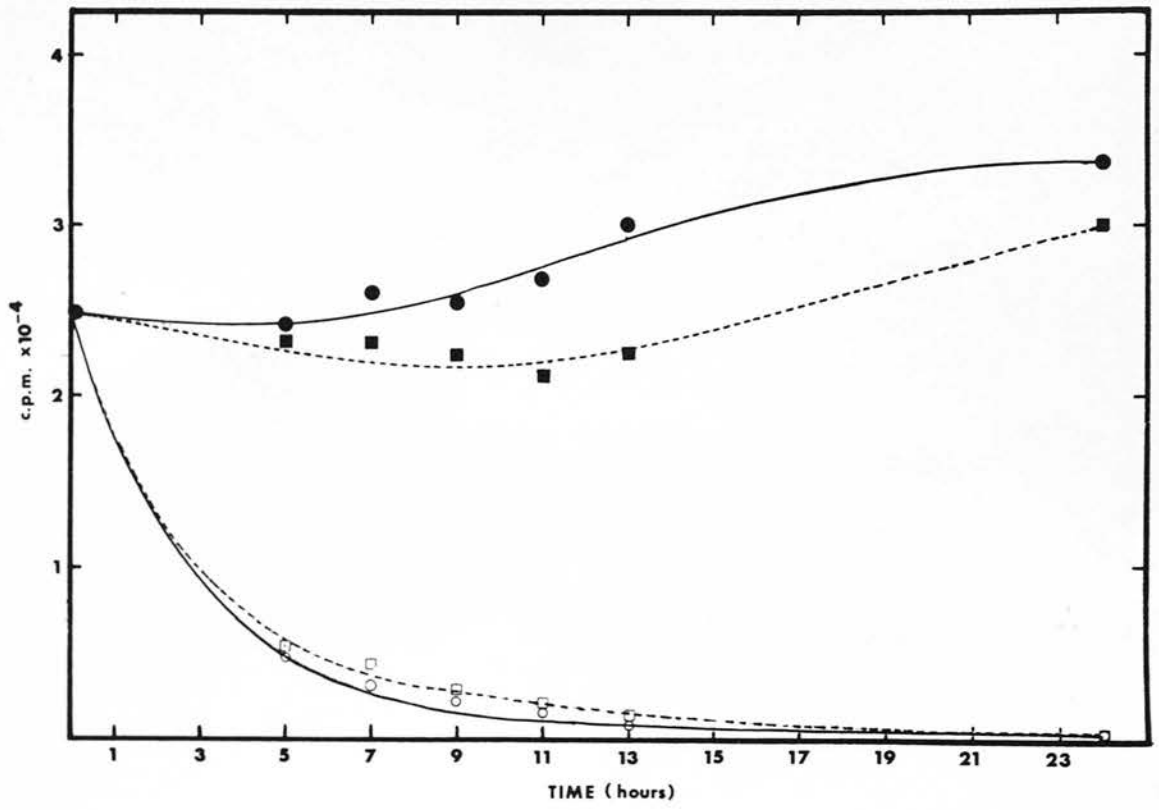
Plate 16 : PAGE profiles of MDBK_{cs} egg-grown virus with different equilibrium sedimentation coefficients. (A) showing PAGE profile of virus obtained from band 1. Note the atypical distribution and concentration of the main viral proteins. (B) PAGE profile of typical Herts NDV obtained from band 2 on tartrate gradient.



series of procedures with pre-treatment of some of the infected and uninfected cultures with actinomycin D, and the pulse-labelling of all monolayers with tritiated uridine, were carried out as described in Material and Methods. Samples consisting of four tubes in each set, were harvested every 2 to 3 hours after drug treatment. The fluids were pooled and used for infectivity assays, and the cell sheets solubilized and the radioactivity determined.

The results (Graph 4) indicated that synthesis of cellular RNA in MDBK cells steadily increased six hours after the start of the experiment. However, in NDV infected cultures the overall RNA production slightly decreased during the first 9 to 11 hours, but the rate of synthesis after that period was parallel to that of the controls. The presence of actinomycin D greatly depressed the synthesis of cellular RNA, which showed a reduction of over 95% compared with the untreated controls by 8 hours after the addition of the drug. On the other hand, no detectable rise in the number of counts was seen during the experiments in NDV infected cultures treated with the drug, although the virus is believed to be actinomycin D insensitive (Scholtissek, 1969).

However, when harvested fluids were examined for the presence of infectious virus a different picture emerged. In the first 9 to 10 hours post-inoculation, no significant differences were observed in both treated and untreated monolayers. After that period, a gradual increase of released virus was detected which was more pronounced in the actinomycin D treated cultures infected with NDV. At 24 hours, these fluids showed about ten-fold more infectious virus than those of control monolayers. These results not only confirmed the known insensitivity of NDV to actinomycin D, but also suggested that the enhancement of viral release was probably due to a derepression effect on MDBK cells by the presence of the drug (Graph 5).



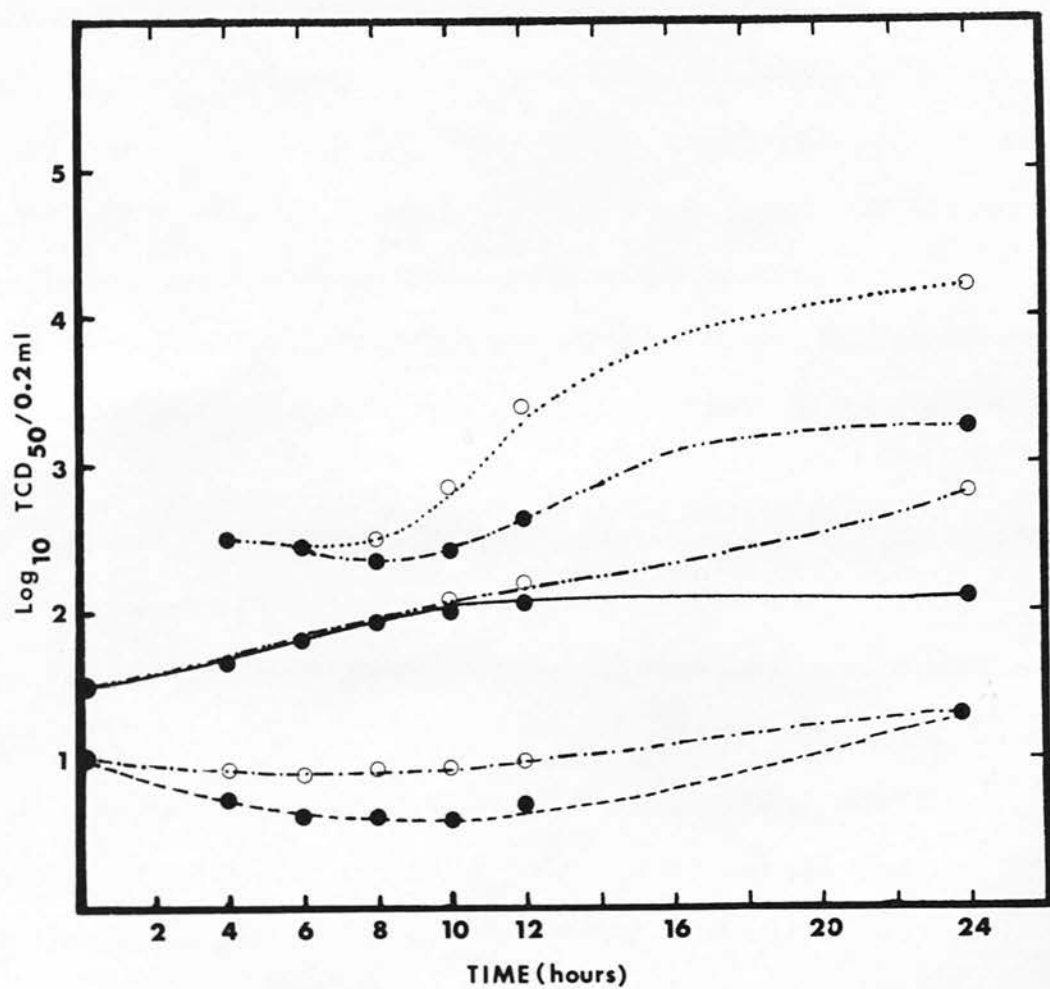
Graph 4 : RNA synthesis in MDBK cells primarily infected with Herts strain of NDV.

One-day-old monolayers of MDBK control cell line were inoculated with 0.5 MOI of NDV in 0.2ml of diluted allantoic fluid. Control cultures were overlaid with the same volume of culture medium. At the end of the adsorption period, a series of procedures were employed with pre-treatment of some of the infected and uninfected cultures with actinomycin D, and the ~~pulse-labelling and uninfected cultures with actinomycin D, and the~~ pulse-labelling of all monolayers with $1\mu\text{Ci/ml}$ of ^3H -uridine, as described in Materials and Methods. Samples consisting of 4 tubes in each set, were harvested every 2 hours starting 5 hours after drug treatment. The fluids were pooled and used for infectivity assays (see Graph 5). The cell sheets were then solubilized and the radioactivity determined.

●—●, MDBK uninfected cells; ■---■, MDBK infected cells; ○—○ MDBK uninfected cells treated with $1\mu\text{g/ml}$ of actinomycin D; □---□, MDBK cells infected with NDV and treated with 1 g/ml of actinomycin D.

Graph 5 : Effect of actinomycin D on levels of released virus in primary and persistent infections. The results shown that infectivity titres of fluids harvested every two hours from the experiments described in Graph 4 and 6.

- , MDBK_{pi} control cultures; ⊙-----⊙, MDBK_{pi} cultures treated with 1 μ g/ml actinomycin D;
- , MDBK_{cs} control cultures; ⊙-----⊙, MDBK_{cs} cultures treated with 1 μ g/ml actinomycin D;
- , MDBK cultures infected with NDV;
- ⊙-----⊙, MDBK cultures infected with NDV and treated with 1 μ g/ml of actinomycin D.



b) Effect of actinomycin D on persistently infected cultures

Parallel experiments were conducted using both persistently infected cell cultures, MDBK_{pi} and MDBK_{cs}. The incorporation of ³H-uridine into the trichloroacetic acid insoluble fraction of the cells (Graph 6) followed a similar pattern to that observed in the primary infection described above.

Initially, the rate of RNA synthesis showed an increase in both types of persistent infection up to 9 hours after the start of the experiment. After this time, the rate of RNA synthesis was fairly constant. The curves for both types of persistent infection were remarkably similar. When the levels of counts were taken at 24 hours in either primary infection of MDBK cells by NDV or persistent infections, it was noted that the latter had slightly higher values than those of the MDBK cell monolayers.

In both types of culture the depression of the overall RNA synthesis caused by actinomycin D was over 90% of that of the controls after 8 hours, and was identical to that of the primary infection of MDBK cells with NDV.

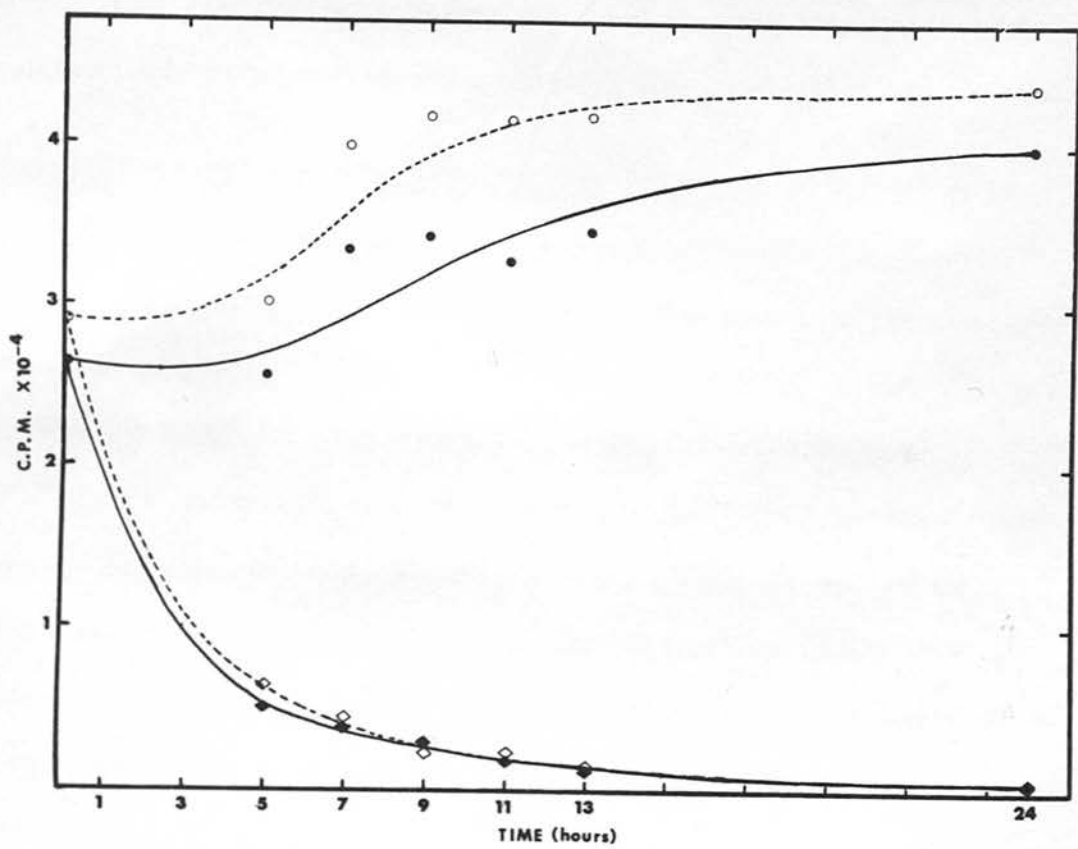
When culture fluids were tested for the presence of infectious virus in an indicator cell system, a clear difference was observed. As Graph 5 indicates, NDV carrier cultures (MDBK_{cs}) showed a similar pattern to that of primary NDV infection. After 24 hours of treatment with actinomycin D there was approximately a ten-fold increase in the titre of released virus compared with the untreated controls. In earlier stages, the MDBK_{pi} cells treated with actinomycin D showed slightly higher titres of extracellular virus than untreated cultures. However, after 24 hours there was no difference in the virus titres from treated or untreated MDBK_{pi} cultures.

Graph 6 : RNA synthesis in MDEK persistent infections.

Both MDEK_{cs} and MDEK_{pi} cell cultures were treated as described earlier (Graph 4) and the incorporation of ³H-uridine into the trichloroacetic acid insoluble fraction of the cells was determined.

○-----○, MDEK_{pi} control cultures; ◇-----◇, MDEK_{pi} cultures treated with 1μg/ml actinomycin D;

●-----●, MDEK_{cs} control cultures; ◆-----◆ MDEK_{cs} cultures treated with 1μg/ml actinomycin D.



An autoradiographic assay was designed as described in Materials and Methods, in order to compare the RNA synthesis in MDBK cultures primarily infected with NDV, with that in MDBK cells. In Plates 17 to 20, it may be seen that the number of grains corresponding to labelled RNA molecules is greatly reduced by the presence of actinomycin D. However, no difference in the amount of silver grains was noted between MDBK or MDBK_{pi} monolayers (treated or untreated). The localization of the grains in both drug treated preparations is clearly cytoplasmic and the number of cells showing RNA production is similar.

c) Viral protein synthesis on persistently infected cultures.

In order to study the synthesis of viral polypeptides in the two types of systems (MDBK_{pi} and MDBK_{cs}), cells were grown in the presence of radioactive leucine, ³H-leucine in the case of MDBK_{cs}, and ¹⁴C-leucine in the case of MDBK_{pi}. The cells were then subjected to gel electrophoresis to determine whether any labelled viral proteins were present. In order to keep cellular protein synthesis as low as possible, cells were treated with actinomycin D prior to and during labelling. The duration of the drug treatment and of the pulse-label used, as well as the technique employed, are described in Material and Methods.

A suitable amount of concentrated Herts NDV suspension from egg-grown stocks was added as a carrier to each cell culture clarified fluid, and a viral purification procedure, using continuous sucrose gradients followed, as described in Materials and Methods. Sucrose gradients showing a well defined band at the middle of the tube, were fractionated and the fractions resulting were studied for refractive index, haemagglutinin activity, and the presence of radioactive material. The results revealed that only a tiny quantity of labelled viral material, in both persistent infections, seemed to be present at the

Plate 17 : Autoradiographic preparation of MDEK cells primarily infected with NDV at 5 MOI/cell, and labelled with ³H-uridine. Grains are distributed throughout the cytoplasm and are particularly abundant in the nucleus.
x 450

Plate 18 : Autoradiographic preparation of MDEK_{pi} cells. The effect is similar to that shown in Plate 17 . x450

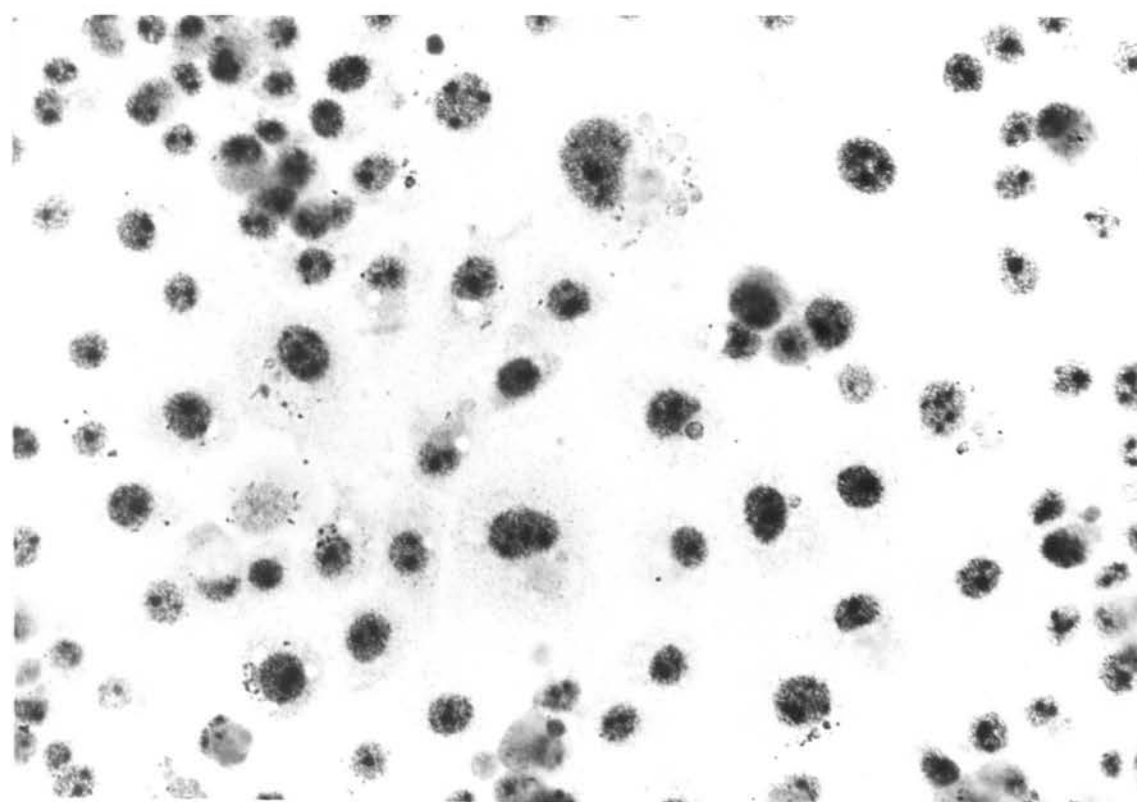
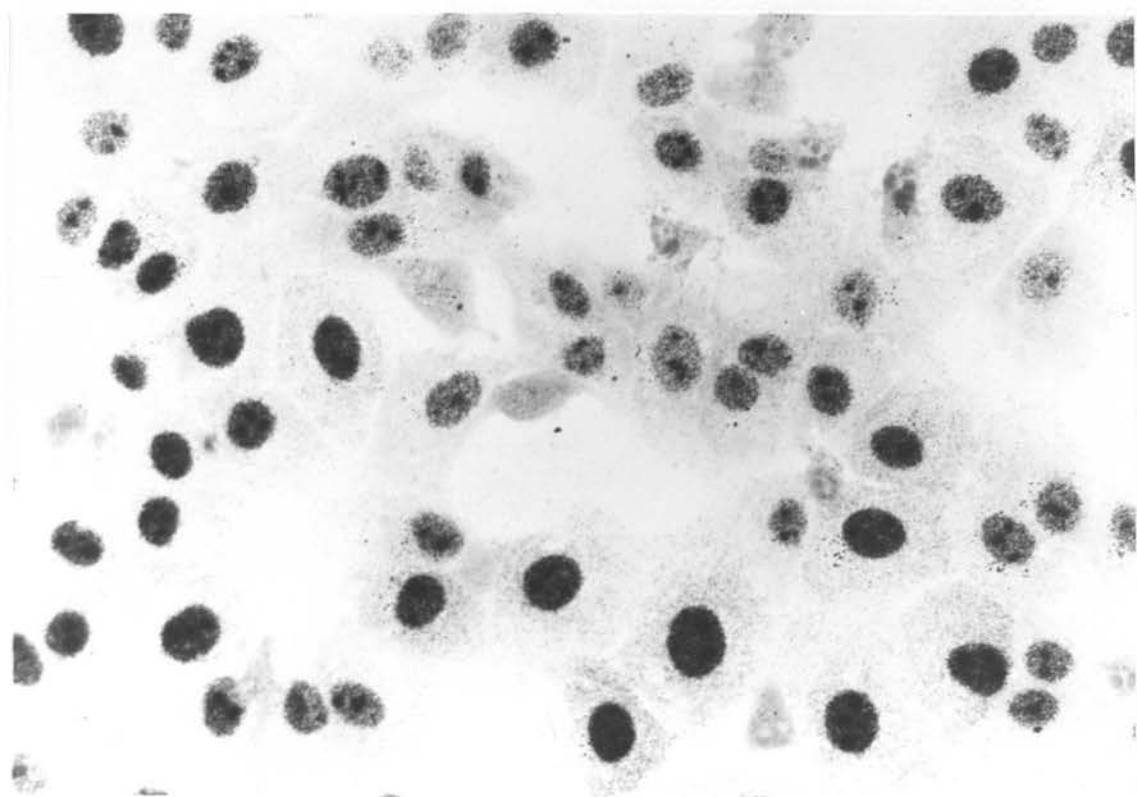
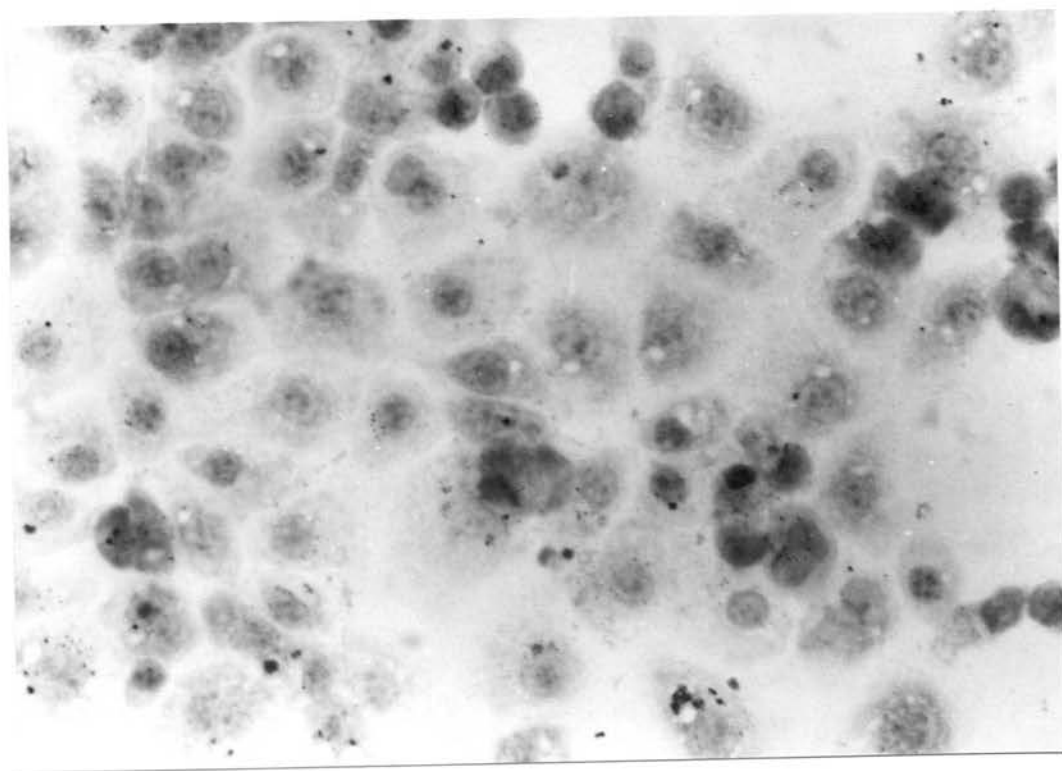
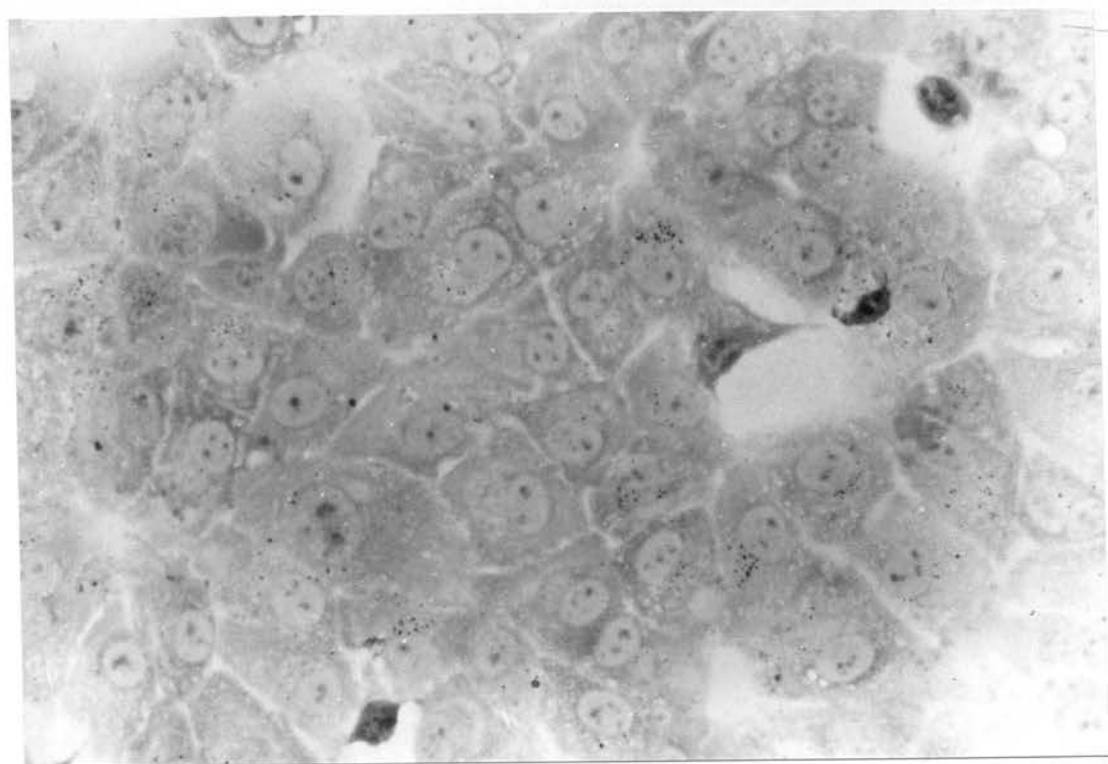


Plate 19 : Autoradiographic preparation of MDBK cells infected as in Plate 17 and treated for one hour with $5\mu\text{g/ml}$ of actinomycin D and labelled as above. In this preparation the grains are markedly reduced and are confined to the cytoplasm. x450

Plate 20 : Autoradiographic preparation of MDBK_{pi} cells treated with actinomycin D and labelled as above. Note the similarity of the results shown in Plates 19 and 20.
x 450



sedimentation density of NDV (Graph 7). No other areas than the top of the gradient showed any particular concentration of the radioactive label. The fractions corresponding to the NDV band were pooled and treated, as described, to be used later in polyacrylamide gel electrophoresis.

Cell monolayers of labelled cultures were washed and dissolved with 1ml of PBS containing 1% SDS, at 37°C. Cell lysates were kept at -70°C for further testing.

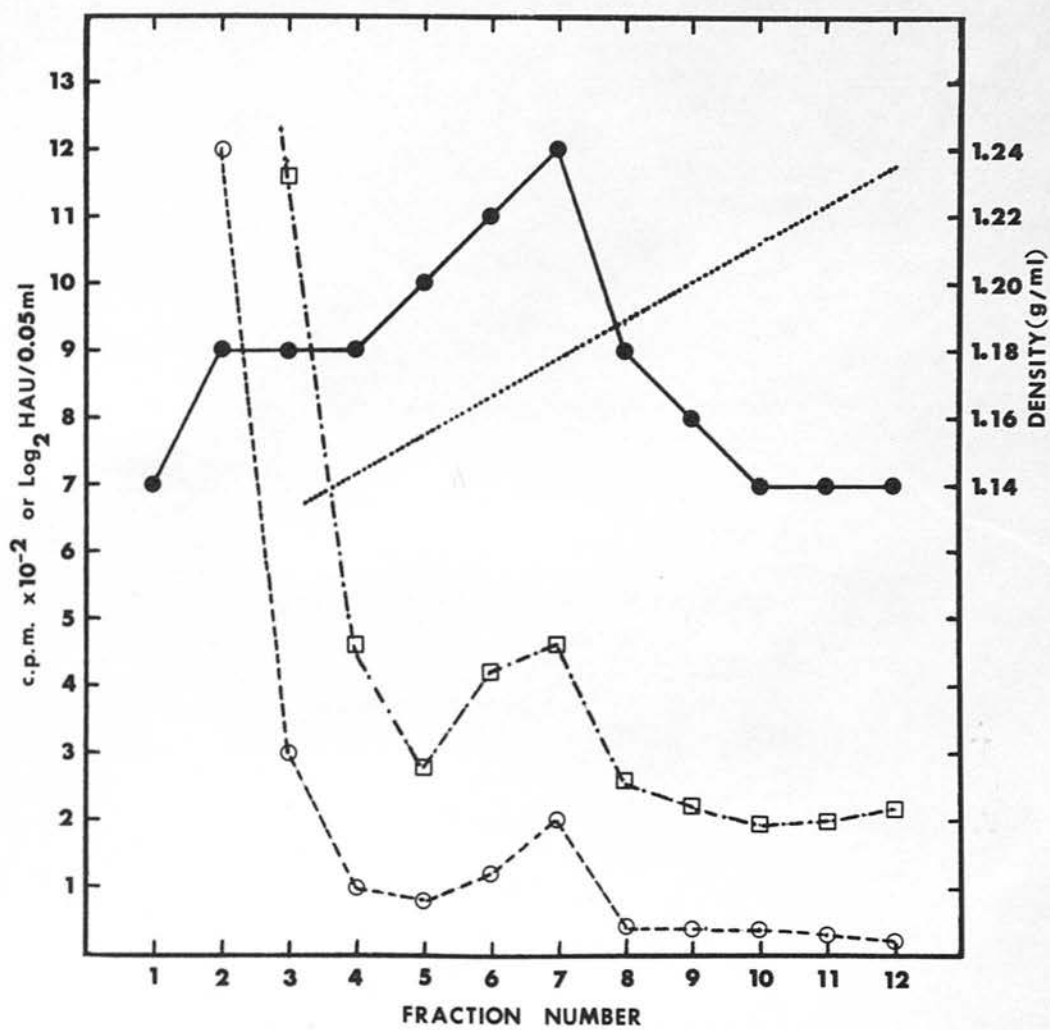
In order to compare the two viral preparations directly, aliquots of both purified agents obtained from cell culture fluids or cell lysates were mixed, disrupted with SDS, and separated under reduced conditions in PAGE cylindrical gels. This meant that MDBK_{cs} and MDBK_{pi} were being compared directly in the same gels. These were frozen and cut in 1mm slices with a "Mickle" slicing machine. Radioactivity in the slices was determined as described in Materials and Methods.

Graph 8 shows a comparison of purified Herts NDV with the labelled polypeptides found in the cell lysates. The upper figure (A) shows the profile of a stained gel containing Herts NDV, and it may be seen that the distribution of polypeptides is directly comparable with the gel depicted in the lower half (B), which contained the intracellular labelled polypeptides. The seven main viral structural proteins described previously and seen in the upper profile can also be detected clearly in the trace of the intracellular labelled polypeptides. However, there is a clear difference in the amounts of the individual polypeptide present.

The MDBK_{cs} preparation showed a characteristic polypeptide profile with the three main peaks (HN, NP/F, and M) and the L protein present. However, U2 is only seen as a shoulder of protein M. Several minor peaks of lower molecular weight were seen which were not found in egg-grown NDV preparations.

Graph 7 : Study of the fractions from equilibrium sucrose gradients loaded with MDBK_{cs} or MDBK_{pi} labelled virus grown in cell culture and Herts strain of NDV propagated in embryonated hen's eggs. After overnight centrifugation at 75000xg, the sucrose gradients were fractionated and the resulting fractions studied for refractive index, haemagglutinin activity and the presence of radioactive material.

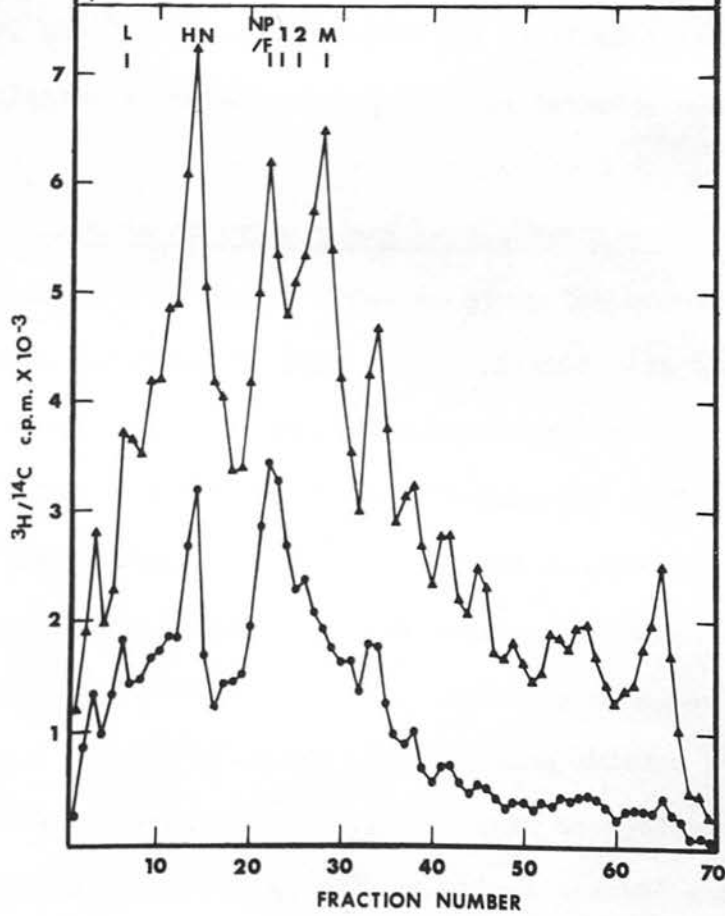
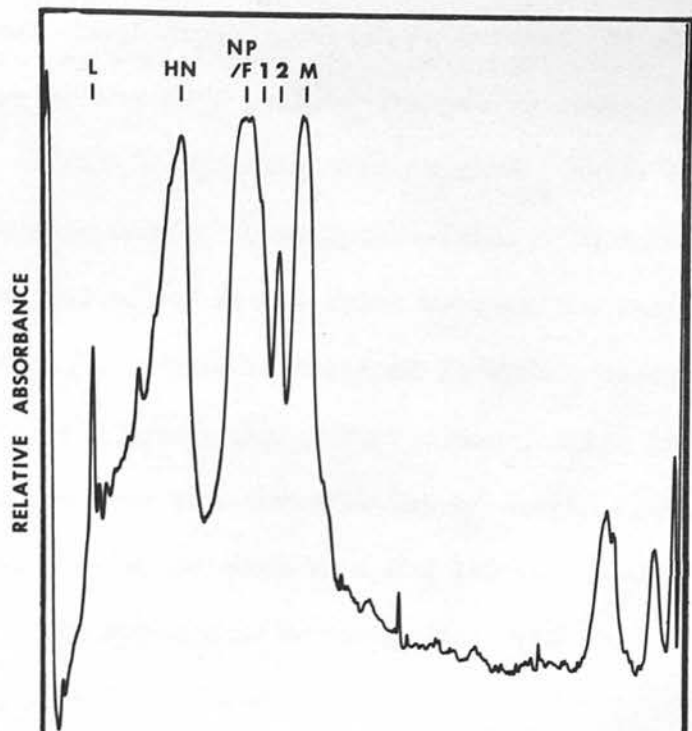
●—● Herts NDV; □—□—□ ^{14}C MDBK_{pi} ; ○—○—○ ^3H MDBK_{cs}



Graph 8 : Comparison of purified Herts NDV (A) with the labelled polypeptides of the two persistent infection agents found in cell lysates (B).

Aliquots from both cell lysates were mixed, disrupted with SDS, and separated in PAGE cylindrical gels. This meant that MDBK_{cs} and MDBK_{pi} were being directly compared in the same gels. These were frozen and cut in 1mm slices. Radioactivity in the slices were determined as described in Materials and Methods. Herts NDV purified virus was disrupted and separated in parallel PAGE gels stained by Coomassie blue and scanned as described. Plate A shows a profile of a stained gel containing Herts NDV. Plate B shows the profiles of the intracellularly labelled polypeptides of MDBK_{cs} and MDBK_{pi}.

●—●, MDBK_{pi} cell-associated virus;
▲—▲, MDBK_{cs} cell-associated virus.



On the other hand, the profile of regulated infection (MDBK_{pi}) shows a peculiar feature. Only two of the major peaks (HN and NP/F) are present. Besides the L protein, which is evident, U1 and U2 appeared as shoulders of the NP/F peak. The matrix protein (M) is absent from the gel profile suggesting that in MDBK_{pi} cells this particular polypeptide is unable to be synthesized. This finding suggests that a close relationship may exist between the fact that only a tiny amount of infectious virus is released in MDBK_{pi} cells and the defectiveness in M protein synthesis. Furthermore, these facts could also account for the presence and accumulation of smooth viral nucleocapsid intracellularly, as will be shown later. Similarly as with MDEK_{cs}, at least two recognizable peaks were seen in the region of proteins weighing less than 35000.

Unfortunately, the levels of radioactivity obtained in the preparations of released virus were too low to be detected in the gels after electrophoresis.

d) Effect of cycloheximide on primary and persistent infections

Experiments were carried out in order to study the effect of inhibition of protein synthesis in MDBK cells infected with NDV.

First, an assay was set up using a primary infection of NDV at low multiplicity (5 MOI of NDV/cell) in twenty-hour-old MDEK monolayers. After one hour of incubation at 37°C, the residual inoculum was discarded, and monolayers washed twice with culture medium. All inoculated cultures were then treated with anti-NDV serum for 15 minutes. Monolayers were again washed twice and all but three culture tubes, which were overlaid with medium containing 20 µg/ml of cycloheximide, received normal culture fluid. The media of three treated and of the untreated cultures were collected separately 8 hours after treatment began, and the respective fluids were pooled and stored at -70°C. At

each time indicated in Graph 9, three previously untreated infected cultures were now treated with the same concentration of the drug, (and the remaining cultures given fresh medium). The fluids of the treated and of another three of the untreated cell cultures were harvested 8 hours after the time of the treatment.

The results indicate that cycloheximide treatment reduces the release of virus in early infection stages. However, 24 hours after infection, viral titres were nearly identical and, later, the situation was reversed with higher titres in the treated cultures. These findings suggest that NDV production is more sensitive to cycloheximide early in the infection than in later stages.

One-day-old monolayers of MDBK_{cs} and MDBK_{pi} were treated with the same drug to determine whether inhibition of protein synthesis had any effect on viral production and release.

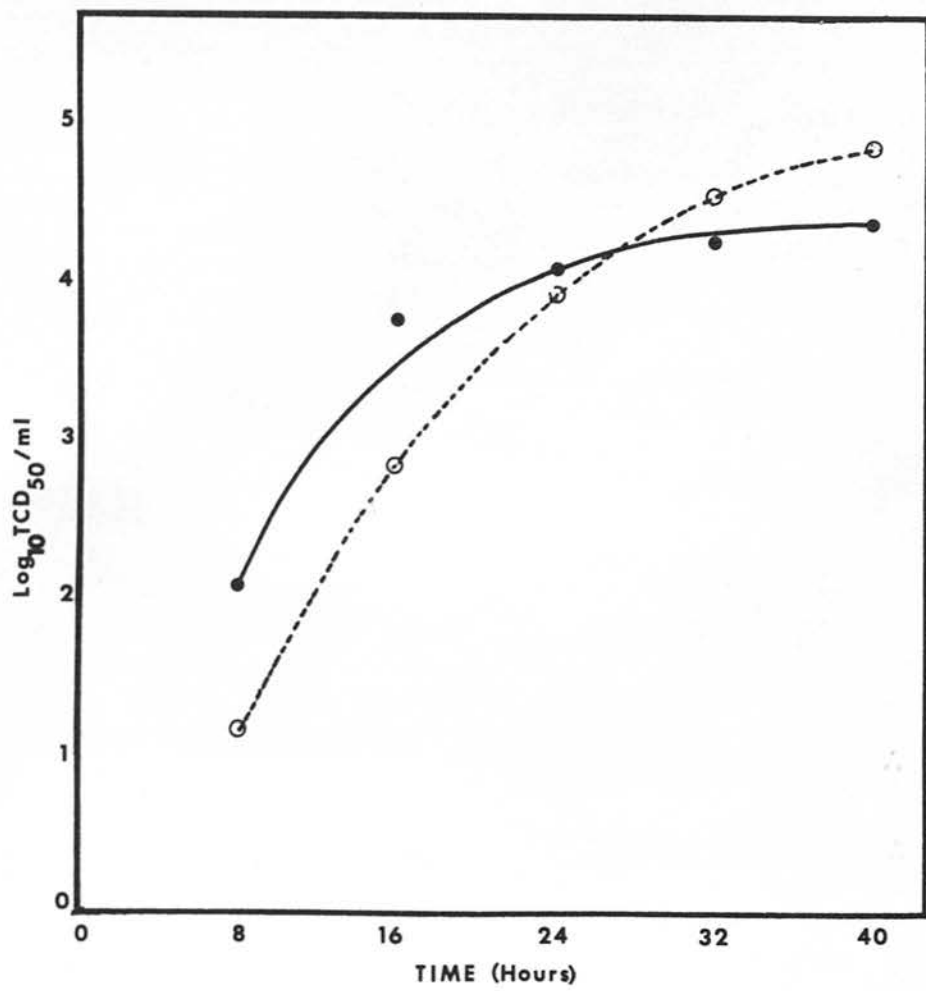
A preliminary assay was set up by treating carrier cultures with several different concentrations of cycloheximide (5, 10, 20, 50 and 100 $\mu\text{g/ml}$) for 8 hours, and checking the amount of infectious virus released in a suitable indicator cell system, as described. The results were rather different to those obtained in the primary infection. It can be seen (Graph 10) that at all concentrations of cycloheximide employed there is an enhancement of the levels of extracellular virus compared with the controls. However, this enhancement was greatest at 5 $\mu\text{g/ml}$, with a steady reduction at increasing levels of cycloheximide. In further experiments, the amount of cycloheximide used was 20 $\mu\text{g/ml}$, which corresponded to a tenfold increase in the titre of released virus compared with the controls.

In another set of experiments, MDBK_{cs} cells were treated with cycloheximide for a period of 12 hours. Samples were examined at three-hourly intervals and revealed that a steady increase in the amount

Graph 9 : Effect of cycloheximide on primary infection of

MDBK cells with Herts strain of NDV

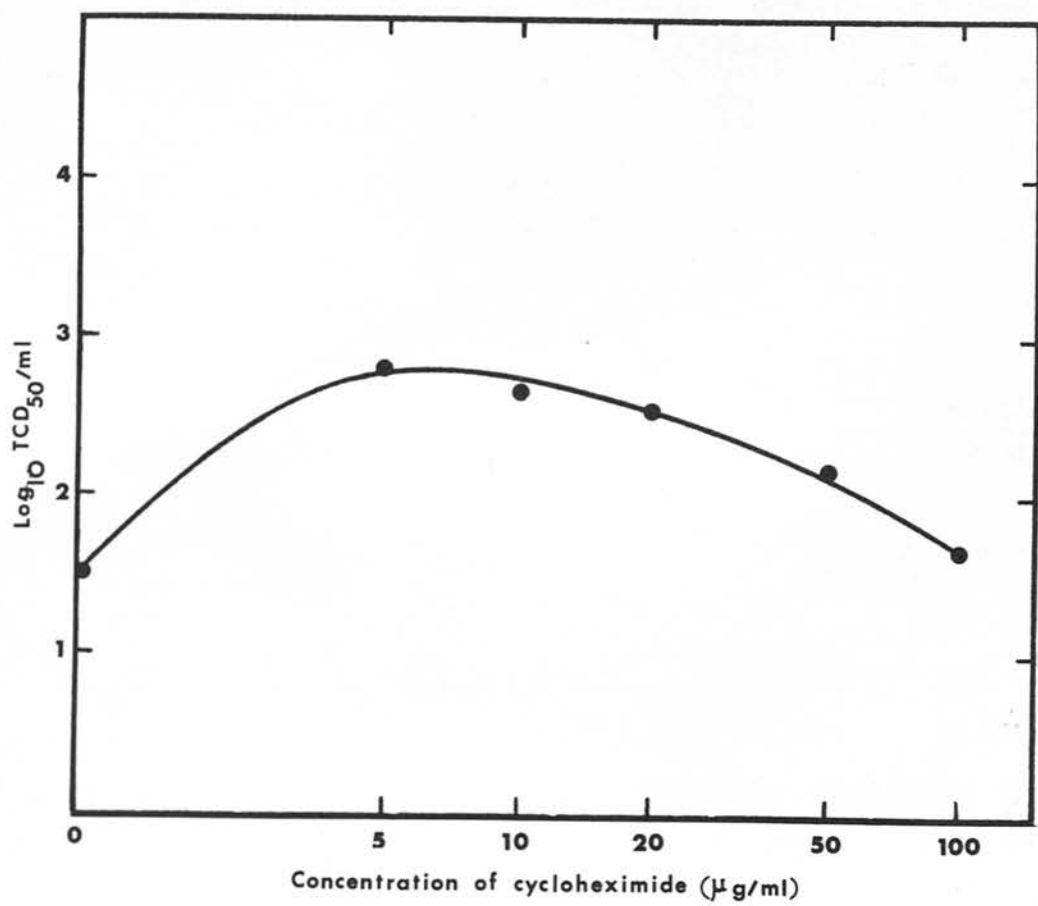
One-day-old MDBK cultures were infected with NDV at 5 NOI/cell. After adsorption was completed, cultures were then treated with anti-NDV rabbit serum for 15 minutes. All but three cultures, which were overlaid with medium containing 20 $\mu\text{g}/\text{ml}$ of cycloheximide, received normal culture medium. The media obtained from three treated (O) and untreated (●) cultures were collected separately 8 hours after treatment began and titrated for infectivity. At the times indicated, three previously untreated cultures were now treated with the same concentration of cycloheximide. The fluids of the treated and of another three of the untreated cell cultures were harvested 8 hours later and were examined as above.



Graph 10 : Effect of different concentrations of cycloheximide
on MDBK_{cs} cells.

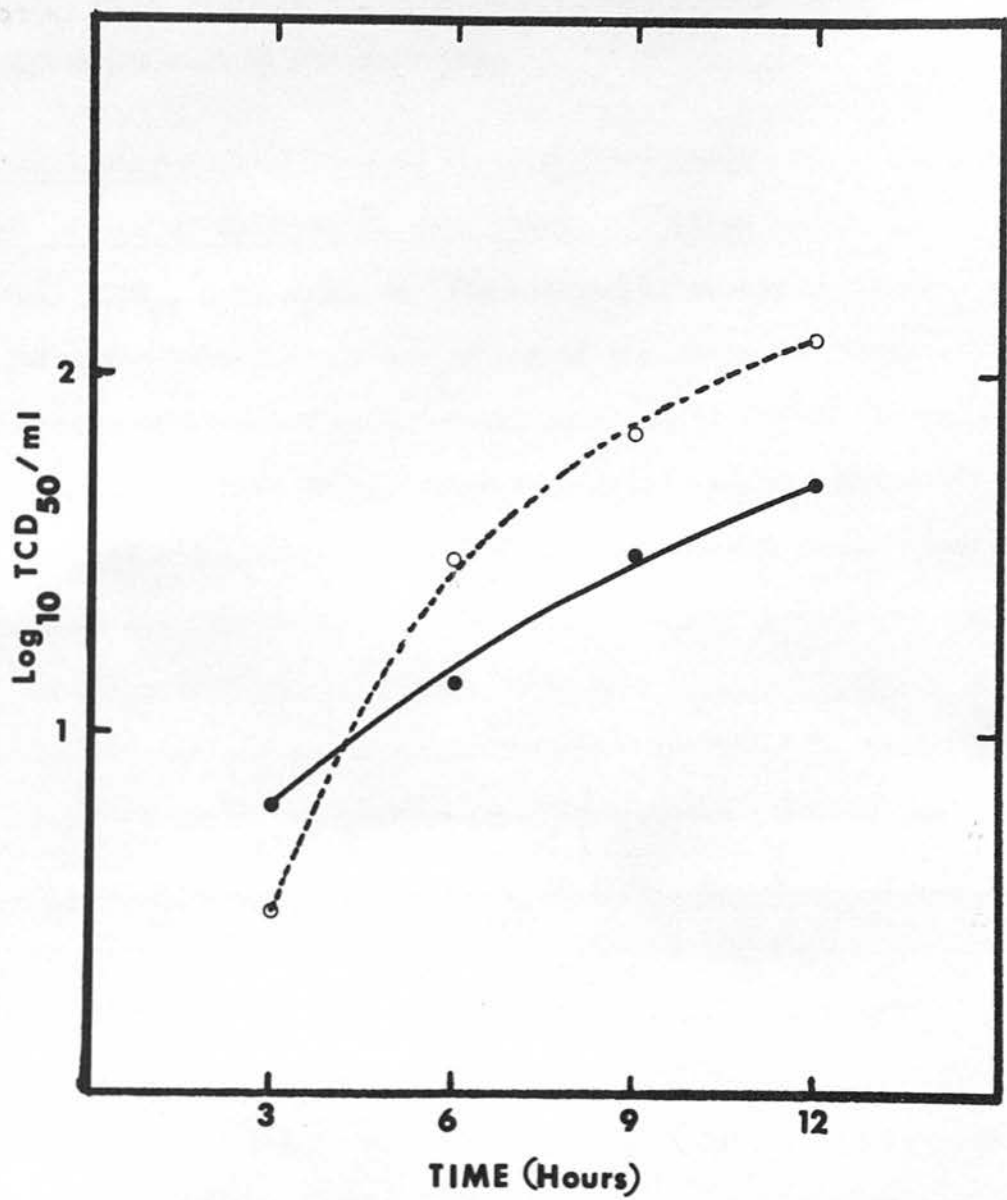
One-day-old MDBK_{cs} cell cultures were treated with
several different concentrations of cycloheximide
(5, 10, 20, 50 and 100 μ g/ml) for 8 hours.

Infectivity titres of the released virus were
determined in BHK-21 indicator cells as described
in Materials and Methods.



Graph 11 : Effect of 20 μ g/ml of cycloheximide on MDBK_{cs}
viral release.

One-day-old MDBK_{cs} monolayers were treated (○) or not treated (●) with 20 μ g/ml cycloheximide for 12 hours. Samples were harvested every 3 hours and the fluids from both sets (treated and untreated) checked for the amount of released virus.



of released virus was obtained, suggesting that a cellular protein(s) may be involved in the prevention of release of infectious virus from MDBK_{cs} cells (Graph 11). However, when MDBK_{pi} monolayers were treated for a period of 8 hours with cycloheximide, the percentage of haemadsorbing cells only showed an increase of 4% compared with the percentage of haemadsorption showed by the controls.

4 - Intracellular accumulation of viral nucleocapsids

a) Effect of age of cultures on persistent infections

Normal MDBK_{pi} cell cultures, subcultured at weekly intervals, showed intracytoplasmic areas consisting of aggregates of viral material. The proportion of affected cells varied from 40% to 90% of the total number examined. These observations were confirmed by routine staining with haematoxylin-eosin and by immunofluorescence, and showed that the viral antigen was frequently arranged in perinuclear plaques or, more rarely, was dispersed throughout the cytoplasm (Plates 21, 22). Sometimes in fluorescence antibody preparation, as shown in Plate 10, the viral antigen appear also in the nucleus possibly related with the nucleolus. In contrast, eosinophilic inclusions were less frequent in routinely subcultured MDBK_{cs} cells, and immunofluorescence staining showed that the viral antigen occurred as densely packed granules throughout the cytoplasm rather than perinuclear plaques as in MDBK_{pi} cells (Plate 3).

However, when a number of MDBK_{pi} cultures, referred to as aged cultures, were refed with maintenance medium and held without trypsinisation for periods of 40 to 250 days, a lower proportion of intracytoplasmic inclusions developed. Instead, 5% to 10% of the total number of cells in the monolayers contained virus-specific intranuclear aggregations which stained acidophilically with haematoxylin-eosin (Plates 23, 24). Cells showing intranuclear inclusions always carried intracytoplasmic aggregates, but not the converse. Furthermore,

Plate 21 : Preparation of MDEK_{pi} cells subcultured at weekly intervals. Note the intracytoplasmic inclusions present in some cells. Haematoxylin-eosin stain. x300

Plate 22 : The same as in Plate 21, showing fluorescing virus-specific material arranged in perinuclear plaques fluorescent antibody stain. x800

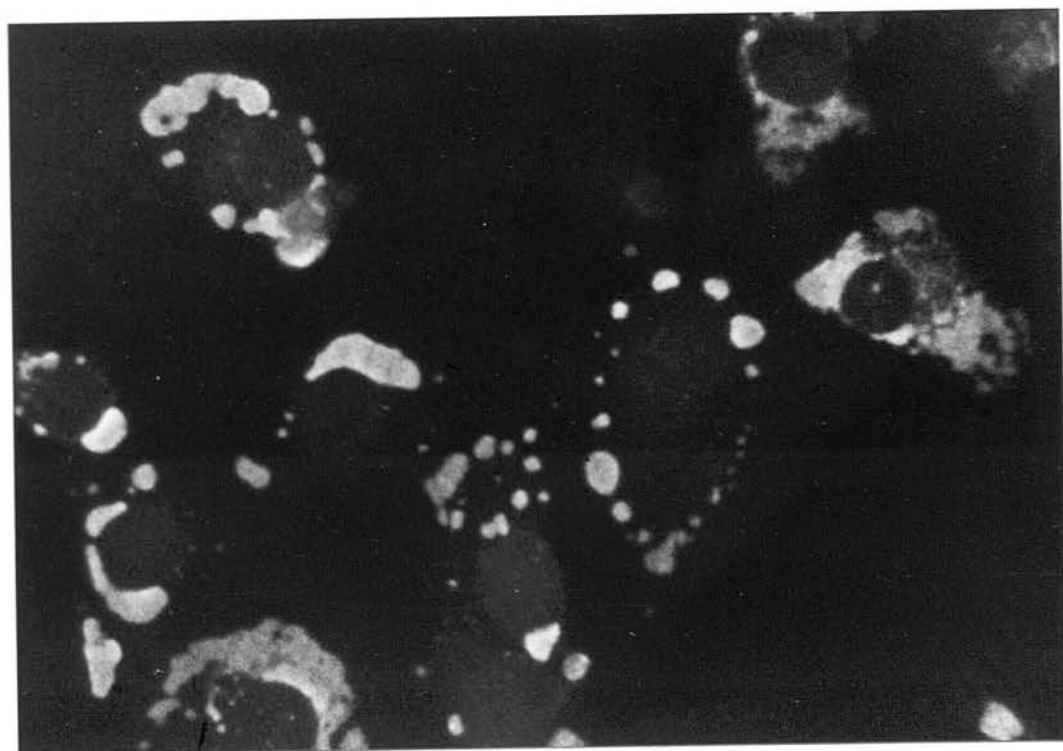
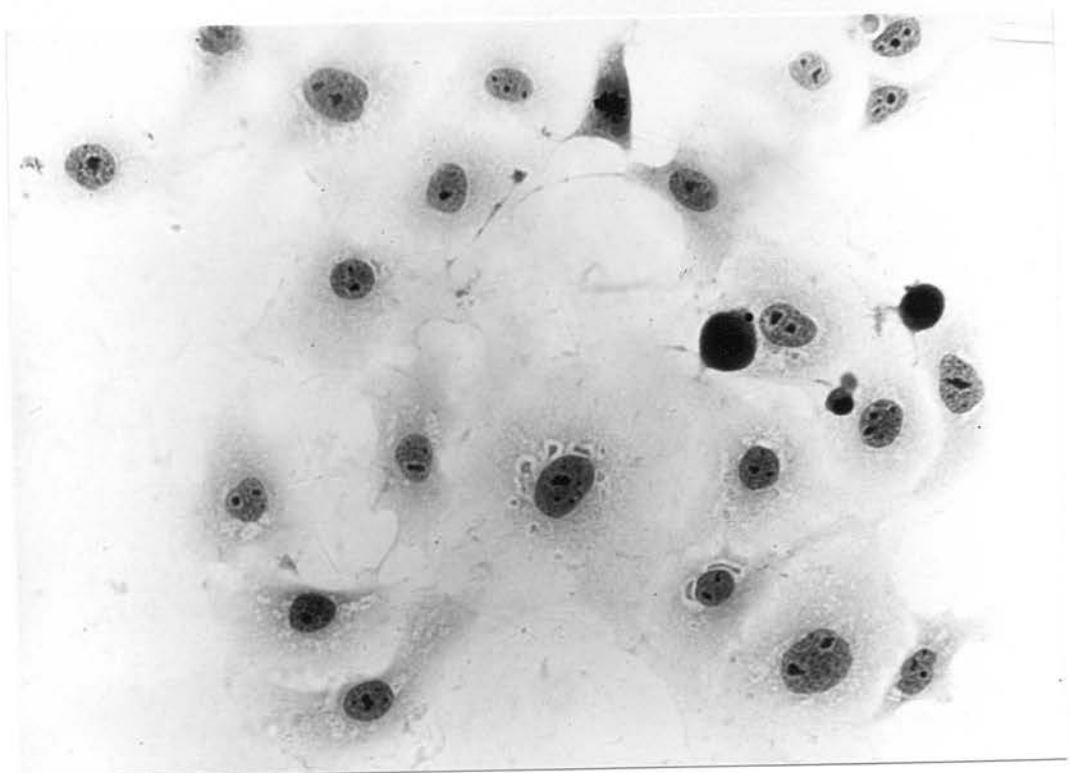


Plate 23 : Monolayer of aged MDEK_{pi} cells. Some of the cells contain intracytoplasmic and intranuclear inclusions. Haematoxylin-eosin stain. x300

Plate 24 : The same as in Plate 23. A number of cells contain both nuclear and cytoplasmic aggregates of specific labelled viral material, but compare the fact with that shown in Plate 10. Fluorescent antibody stain. x1000

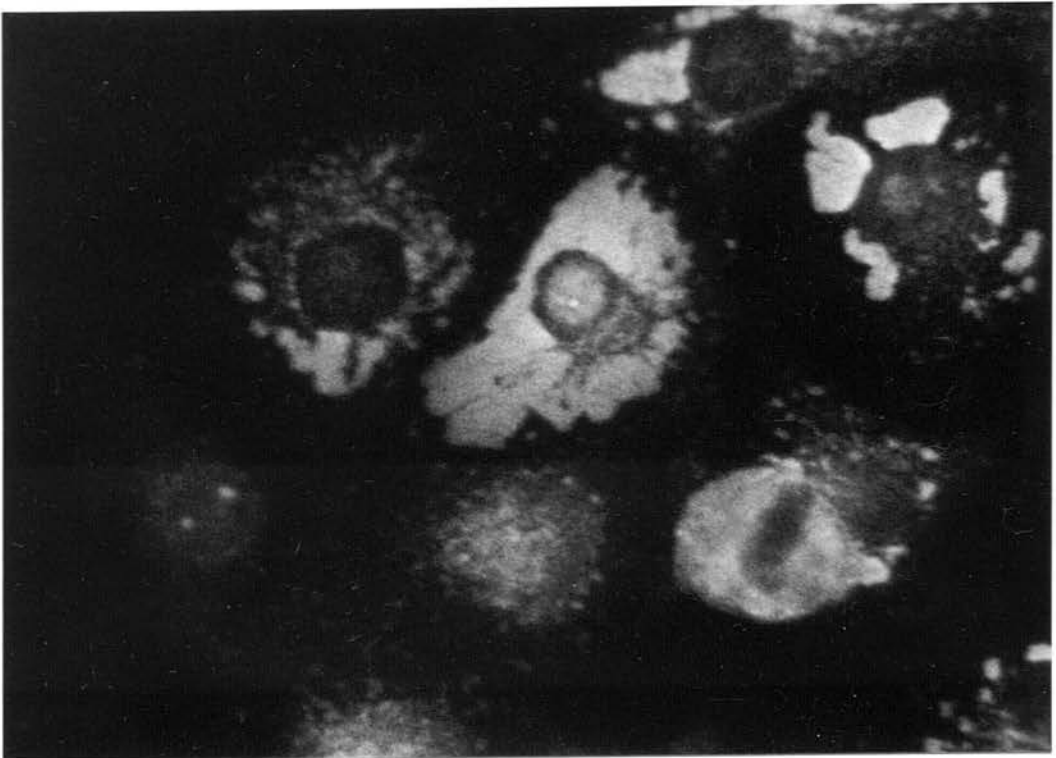
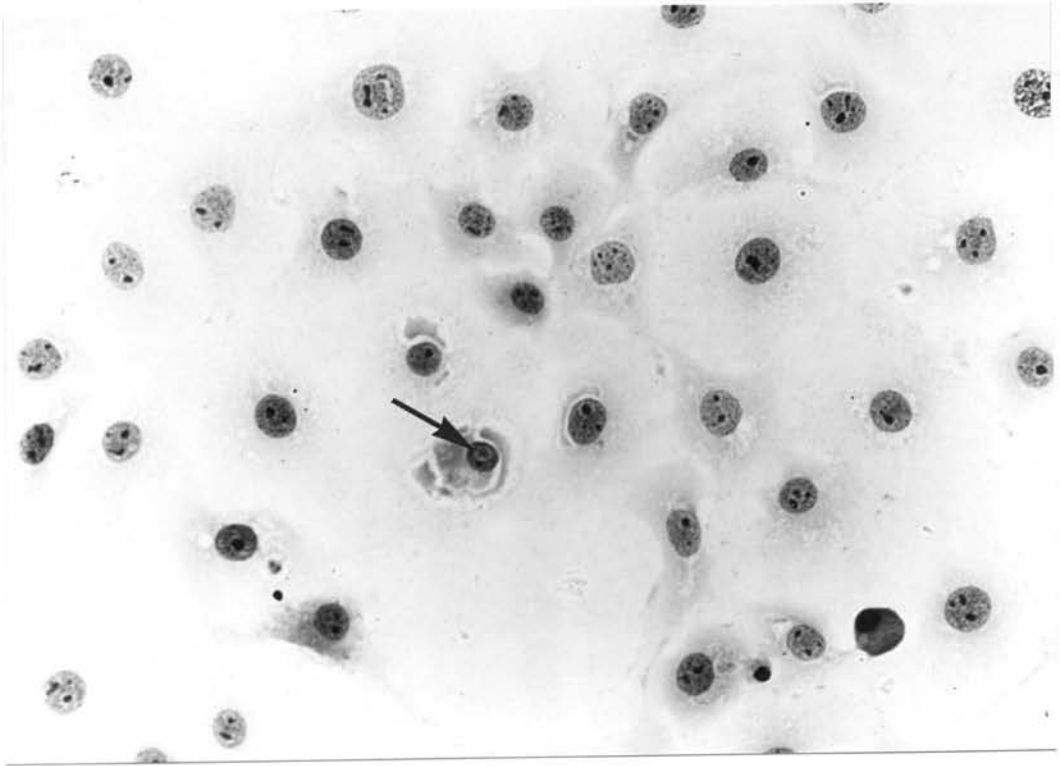


Plate 25 : Preparation of aged MDBK_{pi} cells
showing only two infected cells.
Fluorescent antibody stain. x750

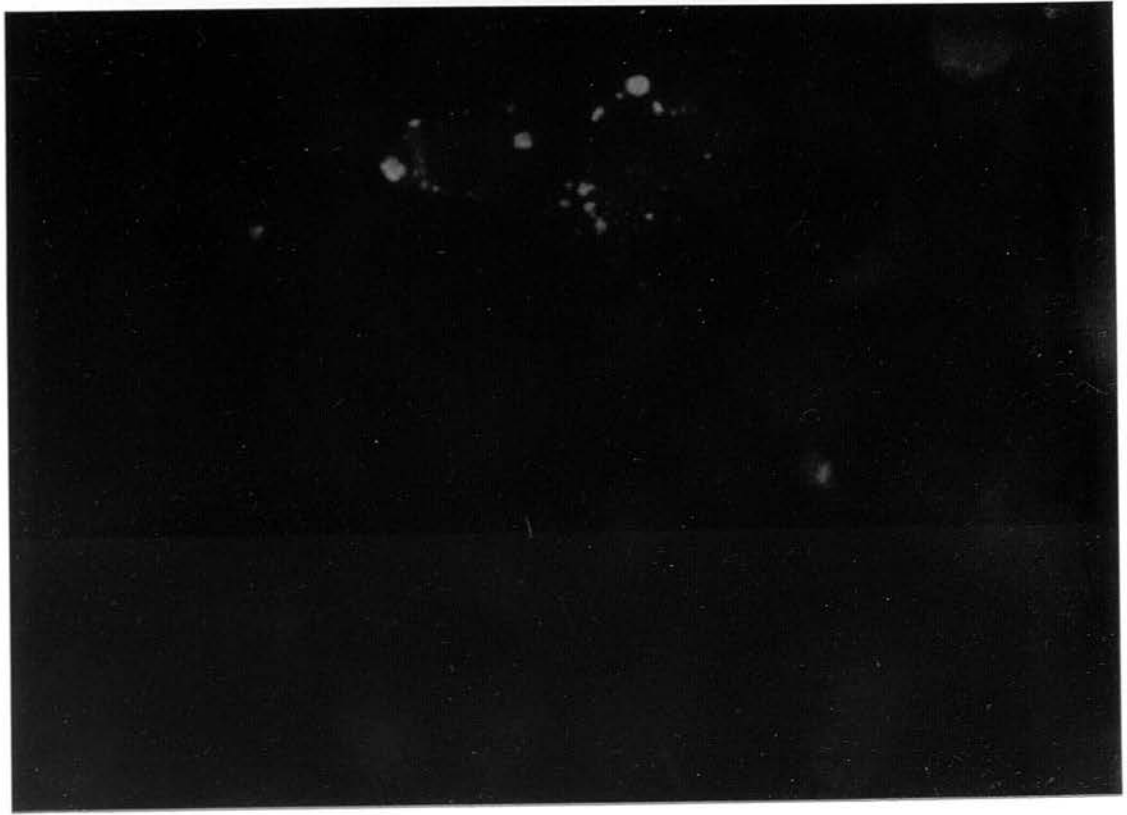
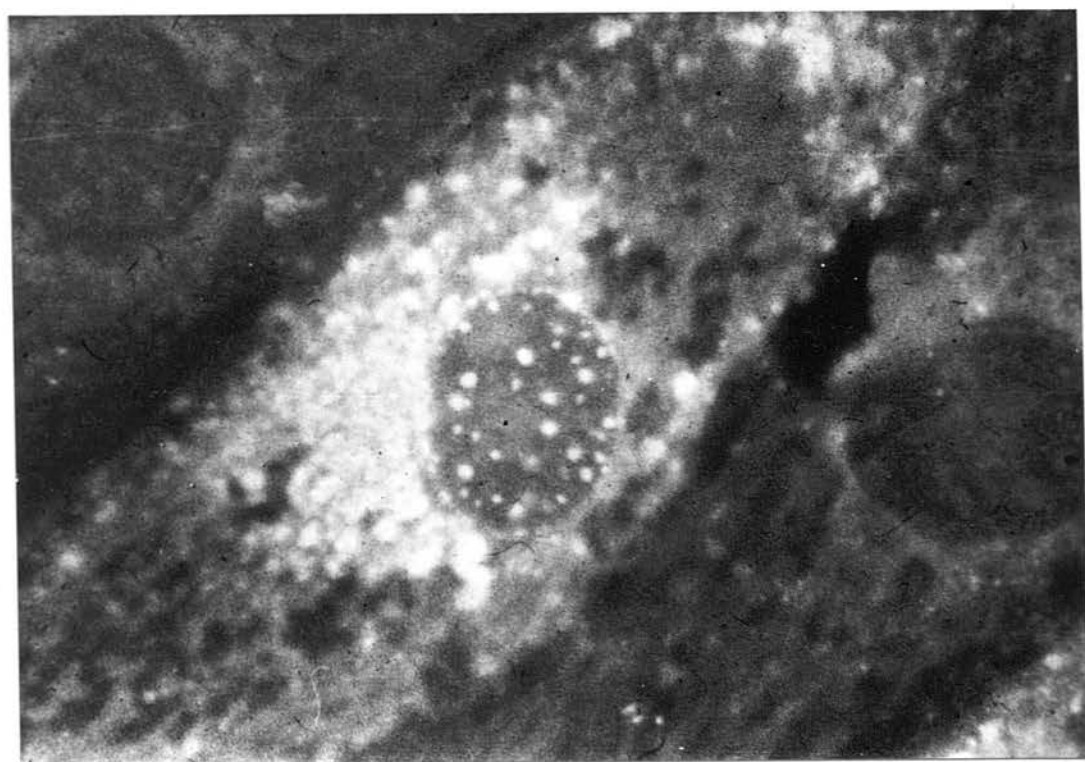
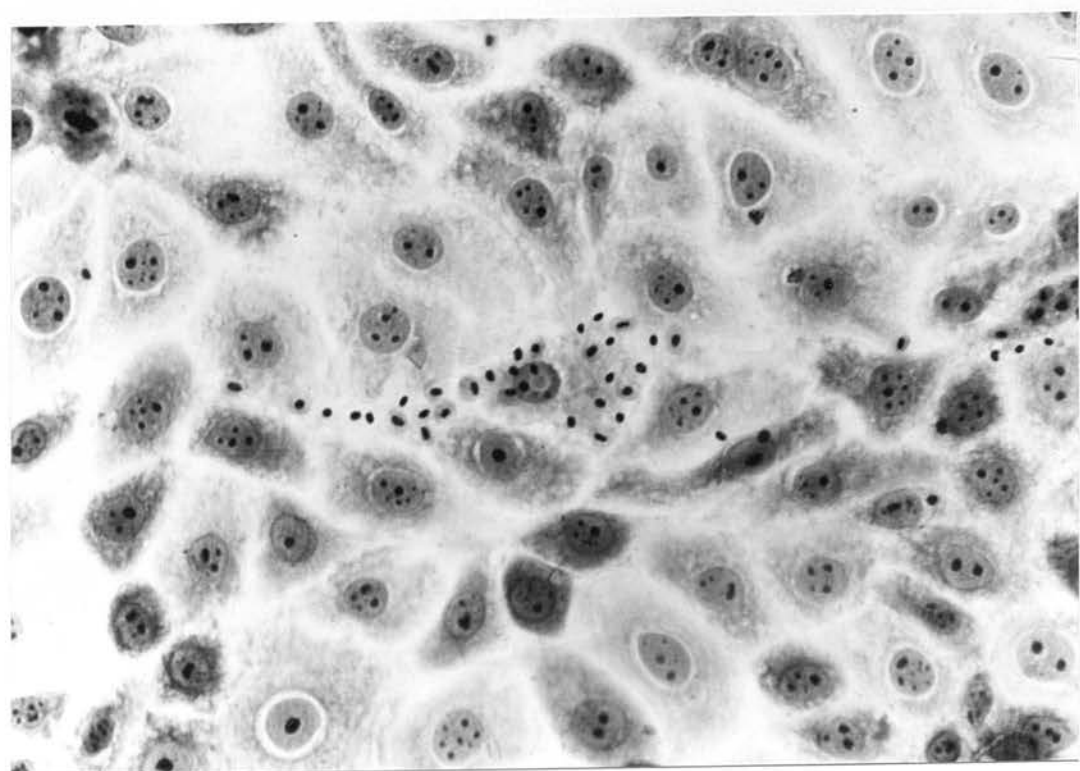


Plate 26 : Aged MDCK_{CS} cells haemadsorb fowl erythrocytes. Note also the presence of intranuclear inclusion in the haemadsorbing cell in the centre of the field. Giemsa stain x450

Plate 27 : The same as Plate 26 showing an infected cell with multiple intranuclear particles and numerous granular intracytoplasmic aggregates. Fluorescent antibody stain x2000



participation by the nucleolus in the development of these aggregates seemed unlikely since cells with nuclear inclusions invariably showed normal nucleoli. Occasionally, the number of infected cells in aged cultures was surprisingly low, and levels of only 1% to 10% of positive tissue cells were observed when the monolayers were examined by fluorescent antibody staining methods (Plate 25).

Initially, the MDBK_{cs} cultures did not appear to contain viral material within the nuclei of infected cells kept under similar conditions as described for MDBK_{pi} aged cultures. However, subcultures allowed to age for periods over 90 days showed intranuclear aggregates, with eosinophilic characteristics, which fluoresced when stained with NDV antiserum labelled with fluorescein isothiocyanate (Plates 26,27).

In both MDBK_{pi} and MDBK_{cs} aged cultures, the development and proportion of intranuclear inclusions, as well as the occasional disappearance of most of the viral material from MDBK_{pi} cells, were sustained and followed by prompt reappearance of the normal characteristics after 1 to 3 subcultures at weekly intervals.

b) Ultrastructure of MDBK_{pi} cells subcultured at weekly intervals

Electron micrographs of ultrathin sections of normal MDBK_{pi} cells showed the presence of ultrastructural changes that are characteristic of paramyxovirus infections. These included cytoplasmic accumulation of aggregates of viral nucleocapsids, alignment of nucleocapsids beneath modified thickened areas of the plasma membrane, and release of virus particles by budding of the modified cell surface (Plate 28).

Two types of nucleocapsid were observed in MDBK_{pi} cells according to their ultrastructure, namely smooth and granular. The smooth nucleocapsids consisted of cylindrical filaments 15-18nm in diameter (Plate 29). Granular nucleocapsids, on the other hand, were composed of similar filaments but these were surrounded by an irregular coating of granular material (Plate 30).

Plate 28 : Electron micrograph of an MDBK_{pi} cell
showing cytoplasmic aggregates of viral
nucleocapsids and alignment of
nucleocapsids beneath modified thickened
areas of the plasma membrane (arrows).

x30000

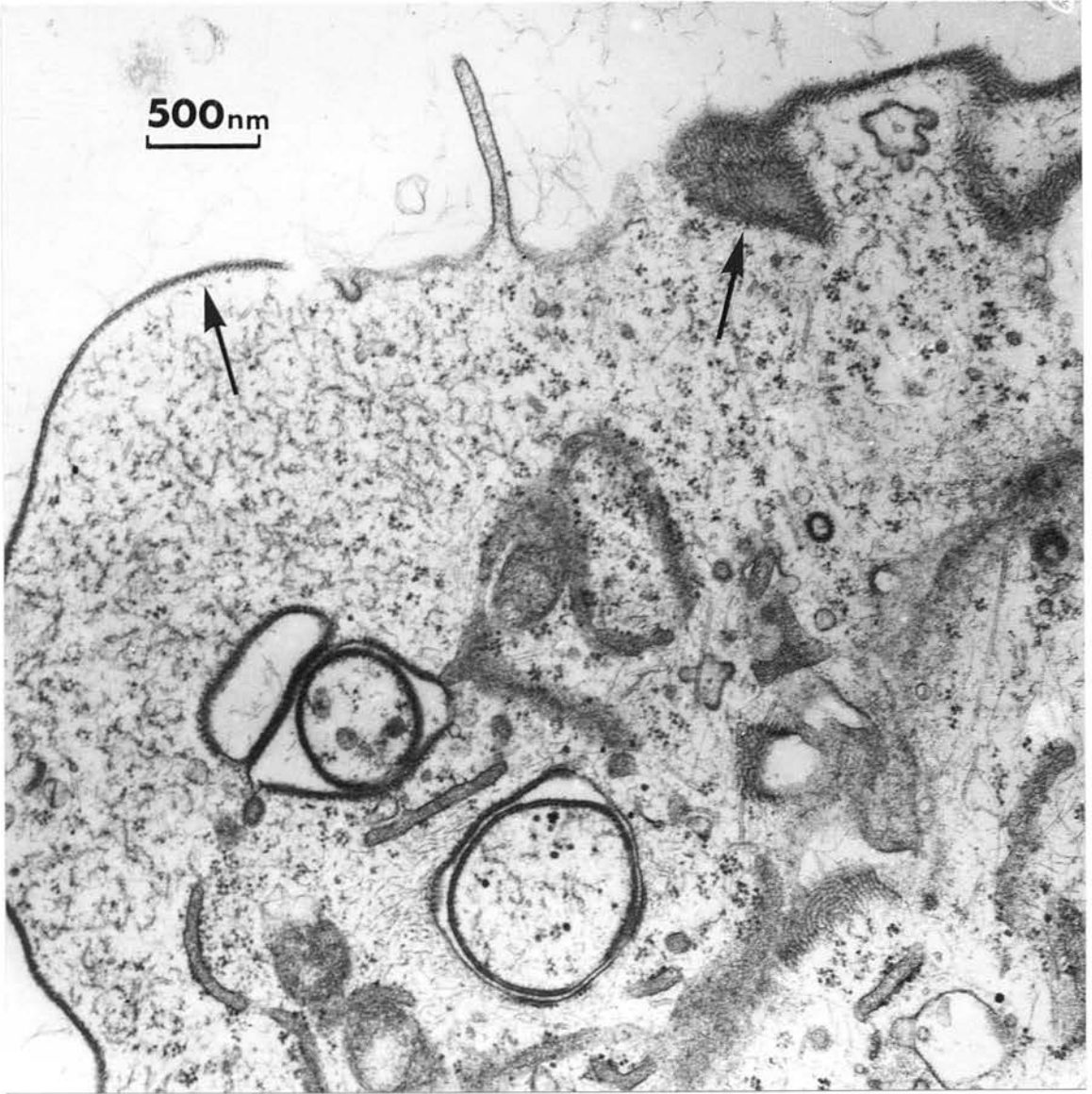
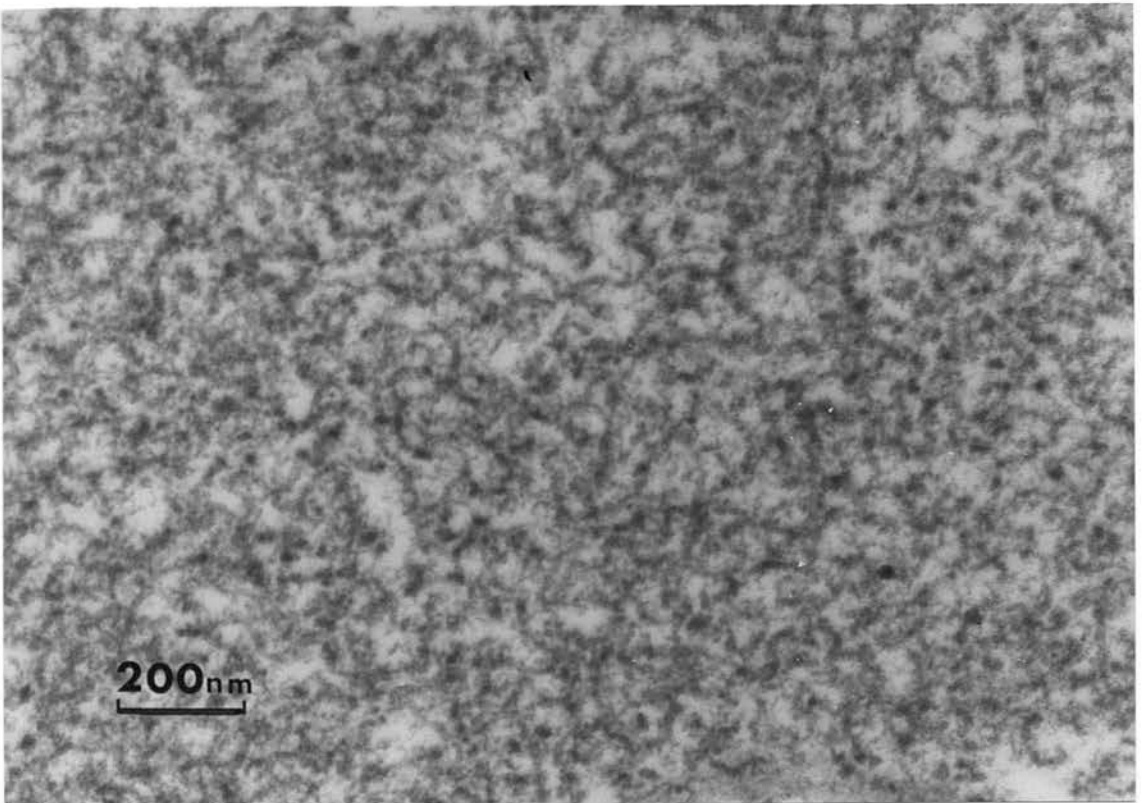
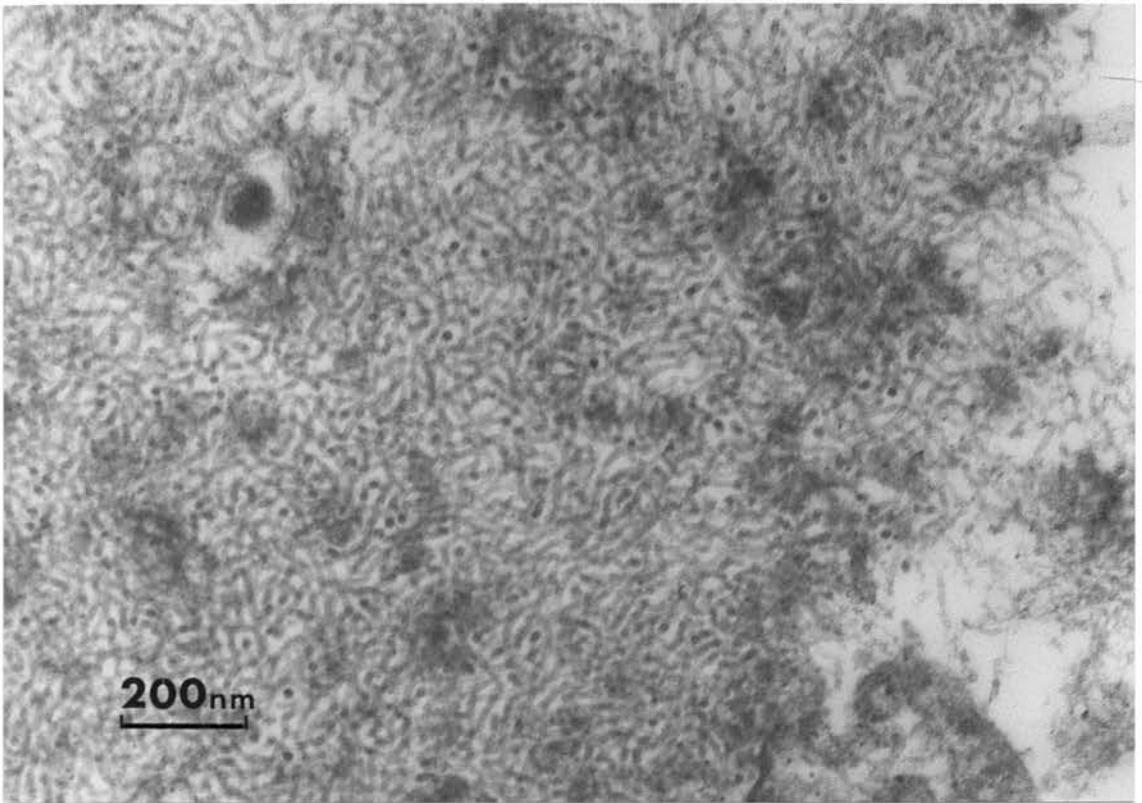


Plate 29 : Electron micrograph of an MDBK_{pi} cell
cytoplasm. Detail of an aggregate of
smooth nucleocapsids consisting of
cylindrical filaments 15-18nm in
diameter. x80000

Plate 30 : As in Plate 29 the cytoplasm contains
granular nucleocapsids composed of
filaments surrounded by an irregular
coating of granular material. x80000



Granular nucleocapsids were observed in the cytoplasm of the majority of the cells, and it was this type of nucleocapsid which was aligned beneath the modified plasma membranes and was incorporated into the virus particles (Plate 31).

Smooth nucleocapsids were present in only about 10% of the cells examined. They were usually found in those areas of the cytoplasm which contained large numbers of vacuoles as well as masses of electron-dense material (Plate 33). Smooth nucleocapsids were never seen aligned under modified plasma membranes or in virus particles. Cells which contained smooth nucleocapsids always had granular filaments, but the converse was not true.

Virus particles released from MDBK_{pi} cells were round or oval in shape and contained nucleocapsid material in amounts comparable to those observed by other workers in productive NDV infections (Yunis and Donnelly, 1969; Feller et al., 1969; Donnelly and Yunis, 1971; Loni, 1971). (Plate 32)

Electron microscopic studies of MDBK_{cs} infected cells proved unsuccessful due, probably, to the fact that the number of infected cells in the samples examined was very limited.

c) Ultrastructure of aged MDBK_{pi} cells

Aged MDBK_{pi} cells contained much larger cytoplasmic aggregates of nucleocapsids than did normal, regulated cultures subcultured at weekly intervals (Plate 34). Furthermore, intranuclear inclusions of viral nucleocapsids were observed in about 10% of the cells examined, which is in agreement with the results obtained by immunofluorescence. Both nuclear and cytoplasmic nucleocapsids were of the smooth type and were not coated by granular material (Plate 35). Modified areas of the plasma membrane with associated alignment of nucleocapsids were not present, and neither budding of virus particles nor extracellular virus particles were observed.

Plate 31 : Electron micrograph of a highly magnified area of MDEK_{pi} cell surface, showing granular nucleocapsids aligned beneath modified plasma membrane in a nearly budded virus particle. x80000

Plate 32 : Electron micrograph showing virus particles released from MDEK_{pi} cells.
x30000

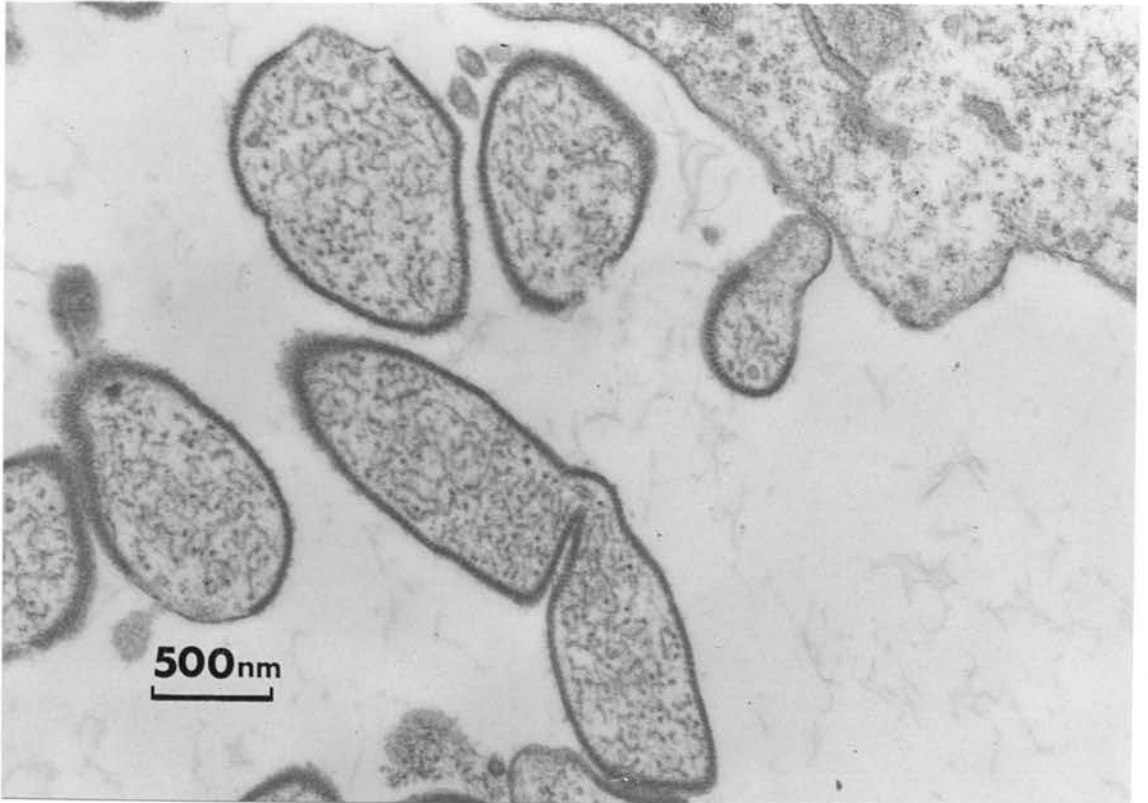
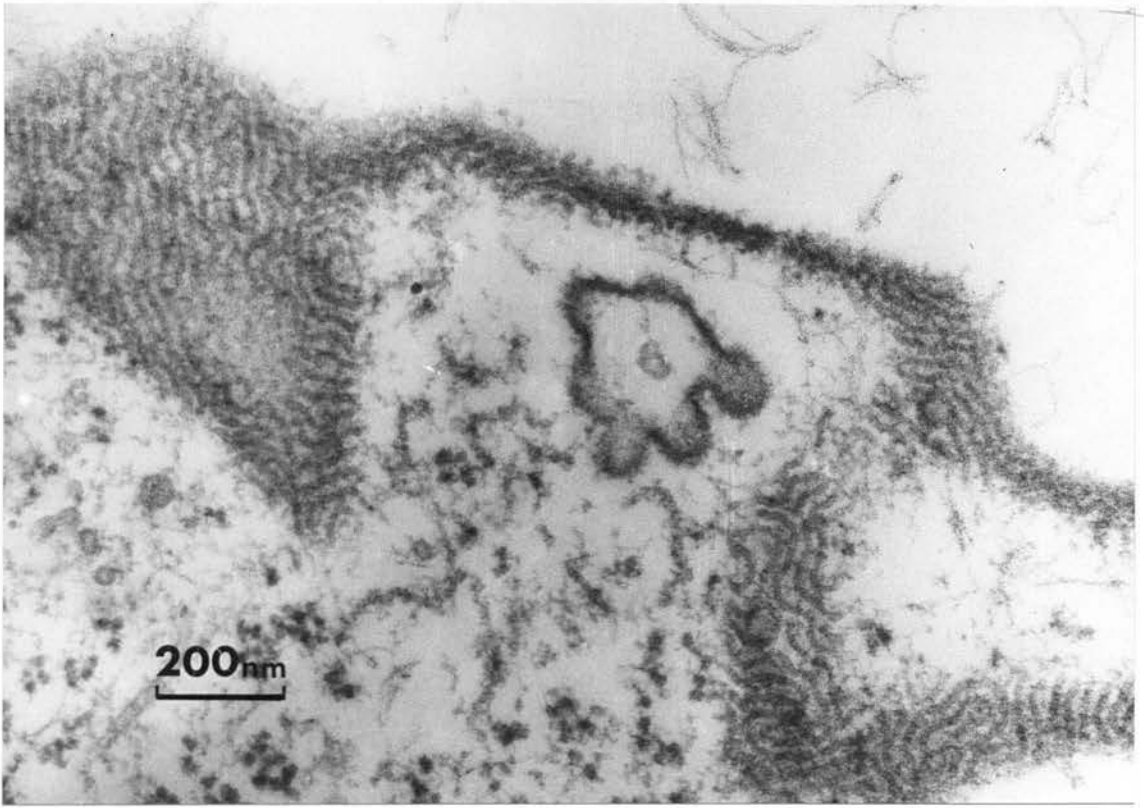


Plate 33 : Electron micrograph of MDEK_{pi} cells
with a detail of the areas where smooth
nucleocapsids usually appear (arrows).
x30000

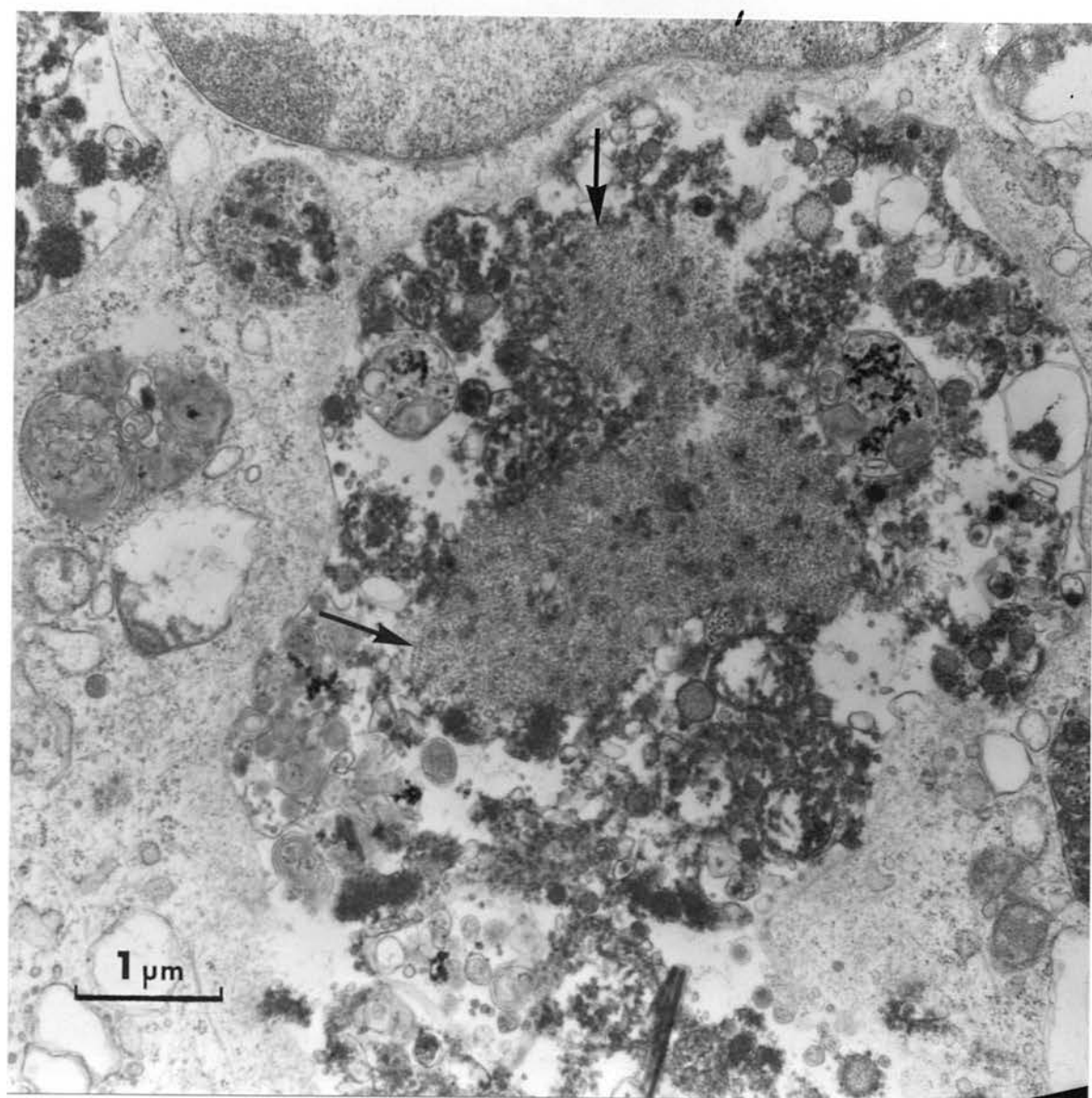
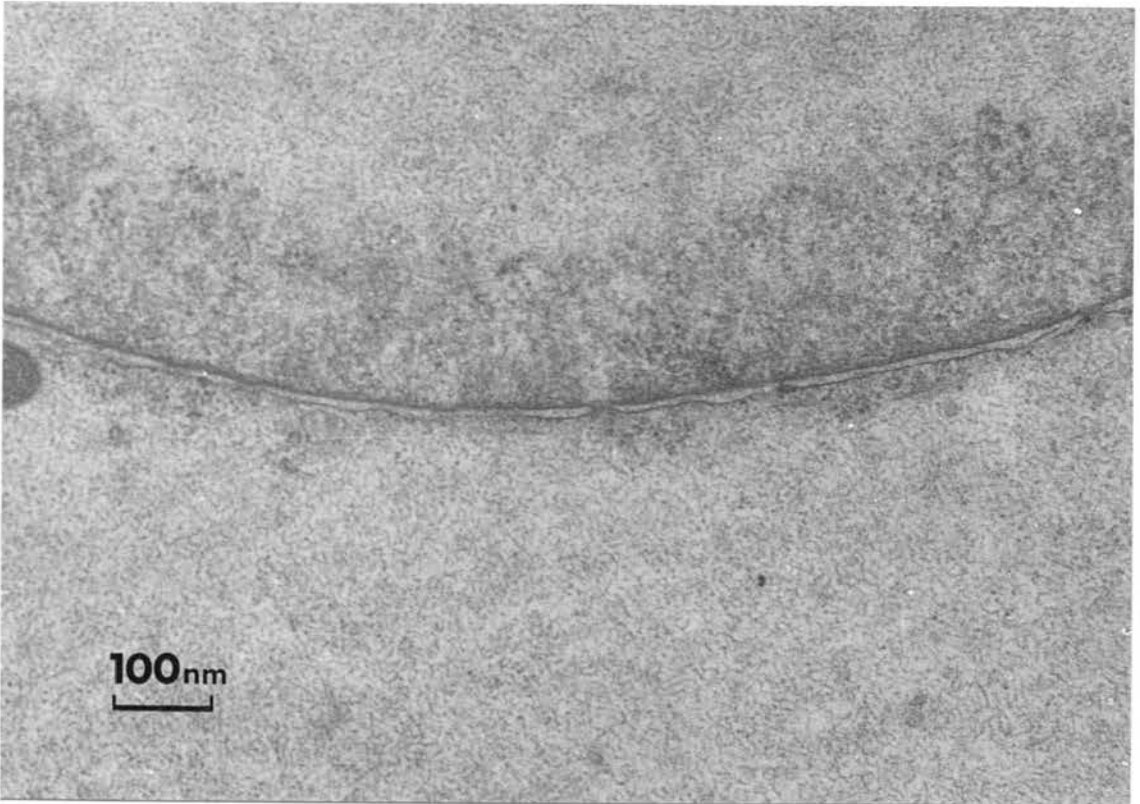
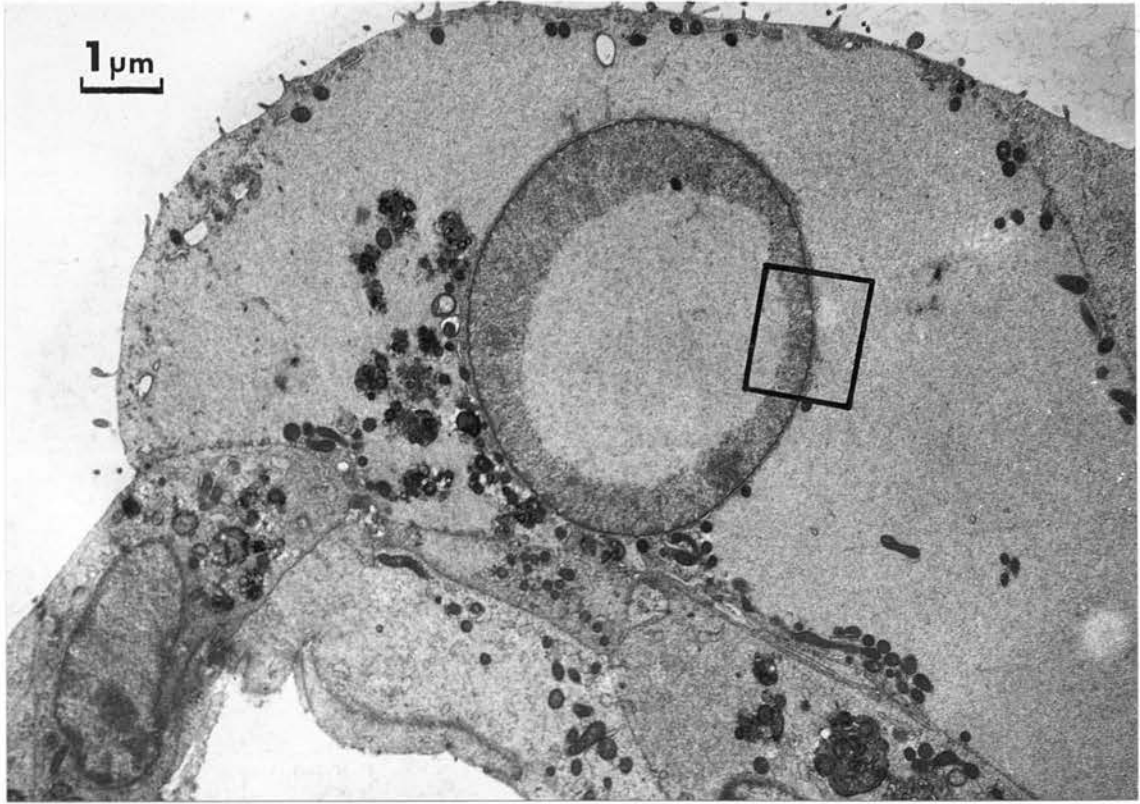


Plate 34 : Electron micrograph showing large
accumulations of viral nucleocapsids
^{aged}
in the cytoplasm of [MDBK_{pi} cells.
x10000

Plate 35 : Electron micrograph of a detail of the
viral nucleocapsids present in nucleus
and cytoplasm of an ^{aged} [MDBK_{pi} cell.
x 120000

Note alignment of residual
chromatin under the nuclear
membrane.



d) NDV-specificity of different types of MDBK_{pi} nucleocapsid

The specificity of the two nucleocapsid types found in MDBK_{pi} cell cultures was investigated by the immunoperoxidase method (IP), followed by observations under the electron microscope of the respective ultrathin sections as described in Materials and Methods.

A conjugate of the enzyme peroxidase with purified immunoglobulin fractions of rabbit anti-NDV serum (PLA) was prepared by the method of Avrameas (1971). Glutaraldehyde fixation of the monolayers and the direct method for specific staining of the viral antigen was used throughout the experiments. A control assay was set up in order to confirm the specificity of the peroxidase labelling reaction. Basically, this employs treatment of the monolayers prior to the PLA assay with an unlabelled NDV antiserum. Such blocking effect on the antigenic sites prevents the enzyme-labelled antibodies from attaching to their specific counterparts.

The PLA staining of viral material was clearly seen in the surface antigen present at the cell membrane. In PLA treated cells, the surface label was either continuous or discontinuous, and revealed granular characteristics with considerable thickening of the cell membrane, even without uranyl acetate-lead citrate EM staining (Plate 36). However, Plate 37 illustrates the normal picture of membrane thickening associated with the presence of viral material, in a blocking control assay, which is similar to that seen in untreated MDBK_{pi} cells (Plate 28).

Under higher magnification, micrographs of viral nucleocapsids aligned at the cell membrane, virus in completion or virus already released, confirmed both the specificity of the PLA method and the effectiveness of the blocking process.

The intensity of the labelling of cytoplasmic viral material varied from cell to cell and probably depended upon the degree of penetration due either to the state of preservation of the membrane or to the amount

Plate 36 : Electron micrograph of peroxidase-labelled MDK_{pi} cells (not stained with uranyl acetate-lead citrate), showing cell surface label. (arrows)
x10000

Plate 37 : Electron micrograph of peroxidase-labelled MDK_{pi} cell treated with NDV antiserum prior to labelling (preparation stained as usual with uranyl-acetate-lead citrate.) Note the 'normal' thickening of some areas of the cell membrane associated with viral budding (arrows). x10000

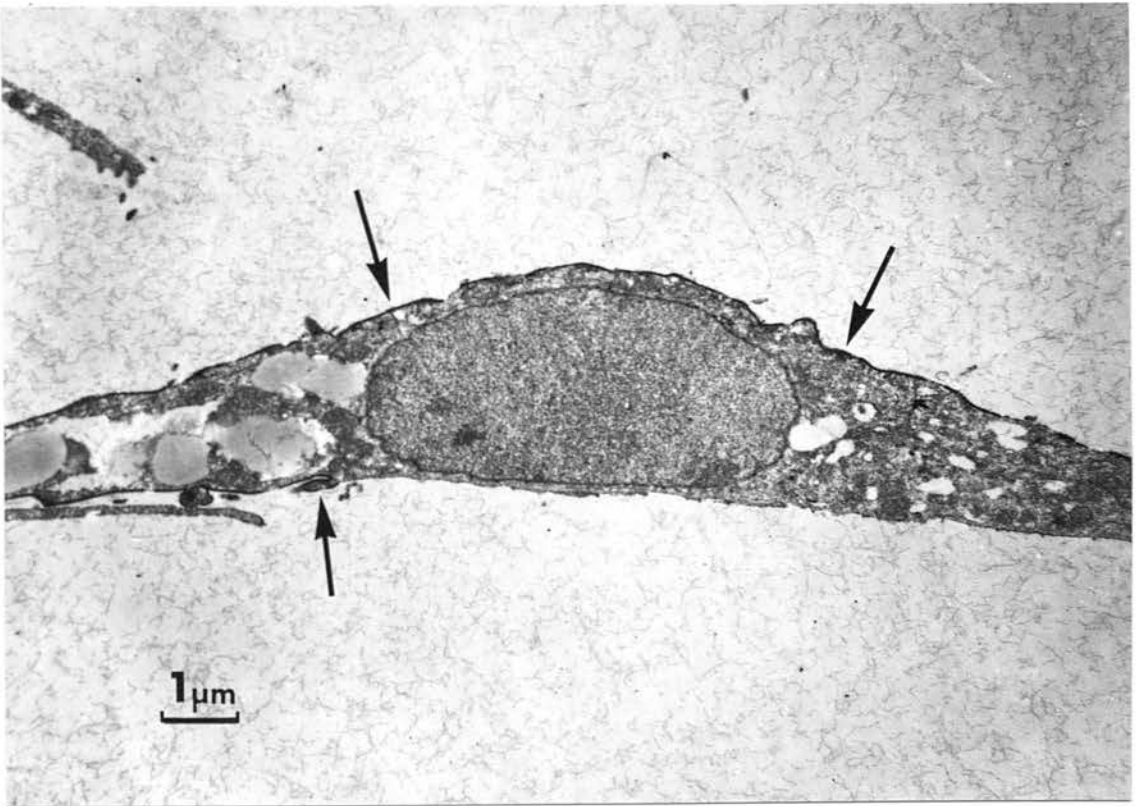
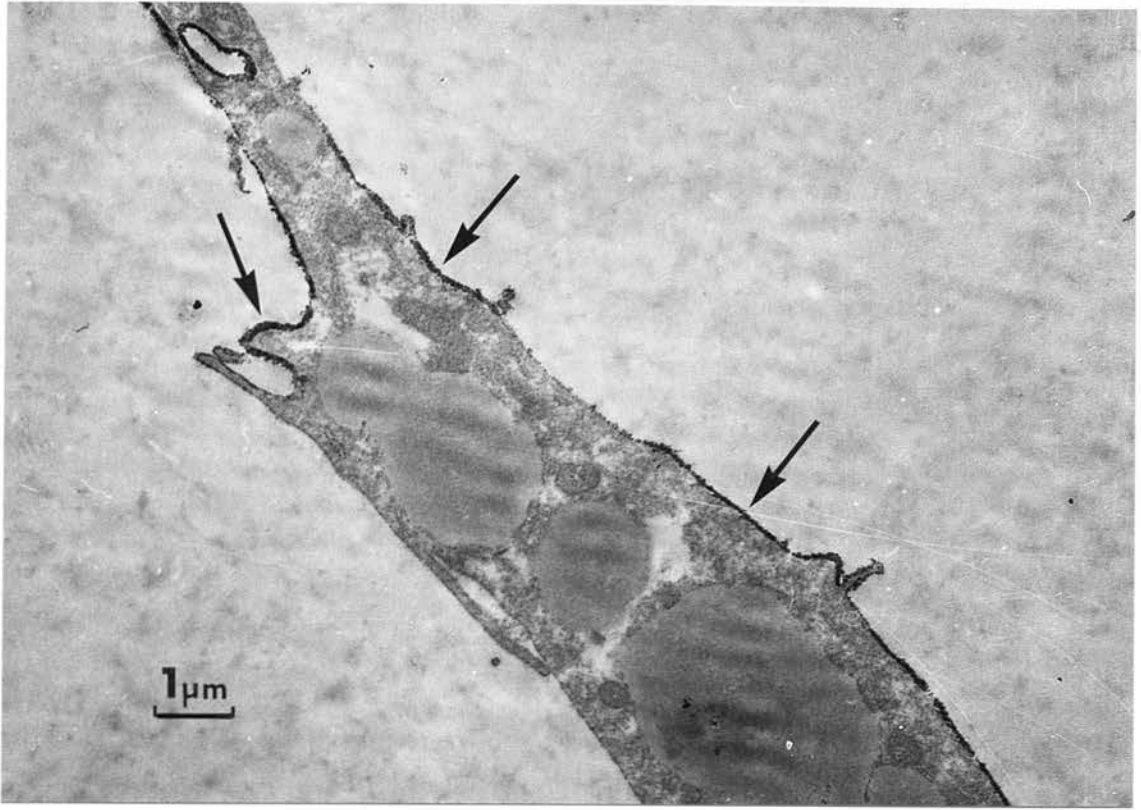


Plate 38 : Electron micrograph of peroxidase-labelled MDBK_{pi} preparation showing granular nucleocapsids scattered in the cytoplasm. Note accumulations of fine grains of label material around RNP structures (arrows)
x80000

Plate 39 : Electron micrograph of peroxidase-labelled preparation of MDBK_{pi} cells showing granular nucleocapsids aligned under the membrane with small dots of label material (arrows). x120000

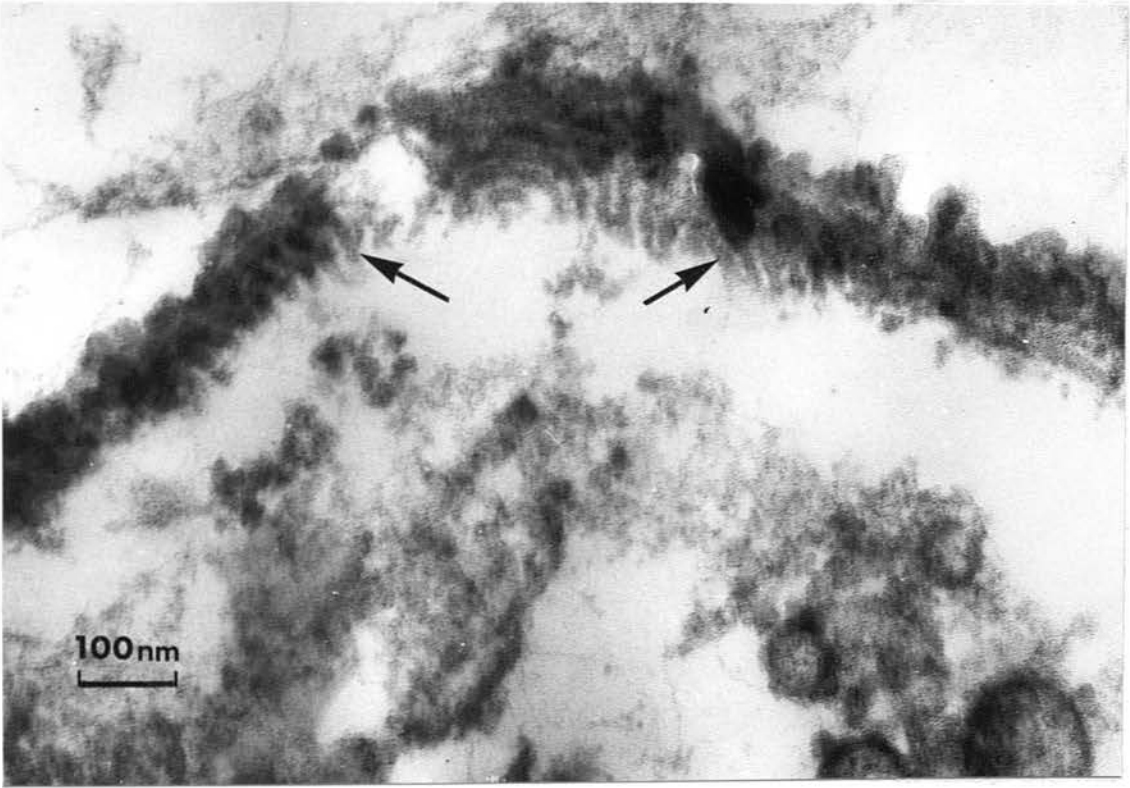
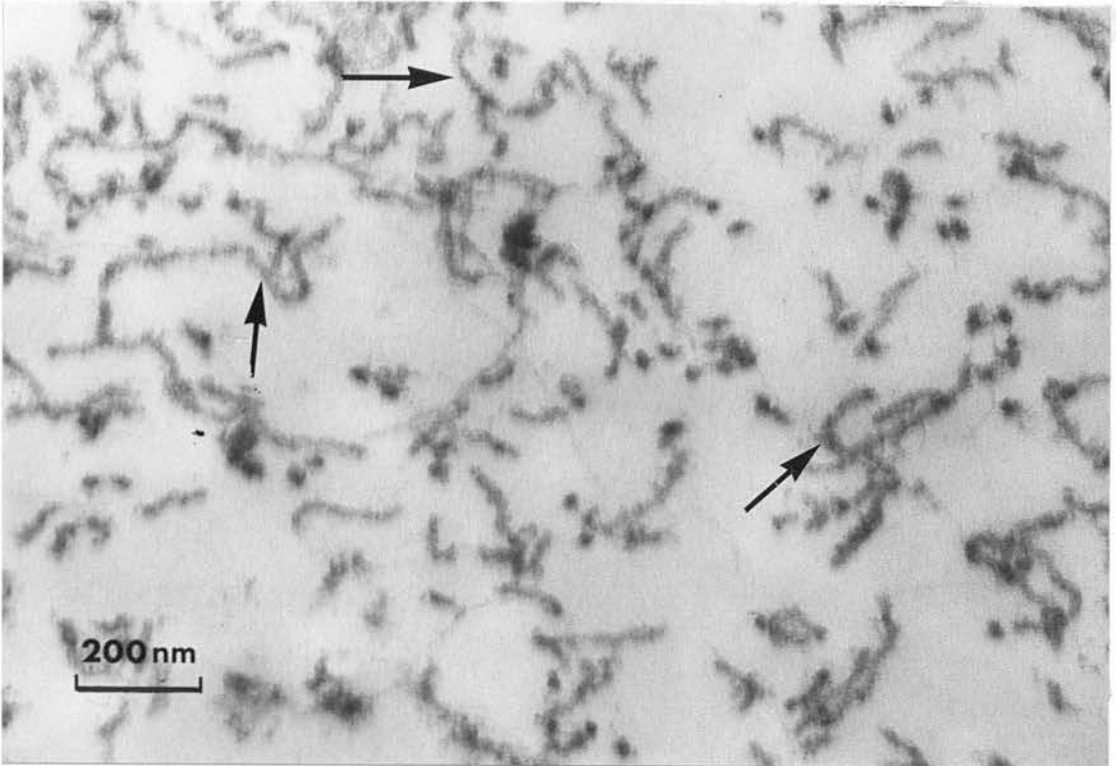
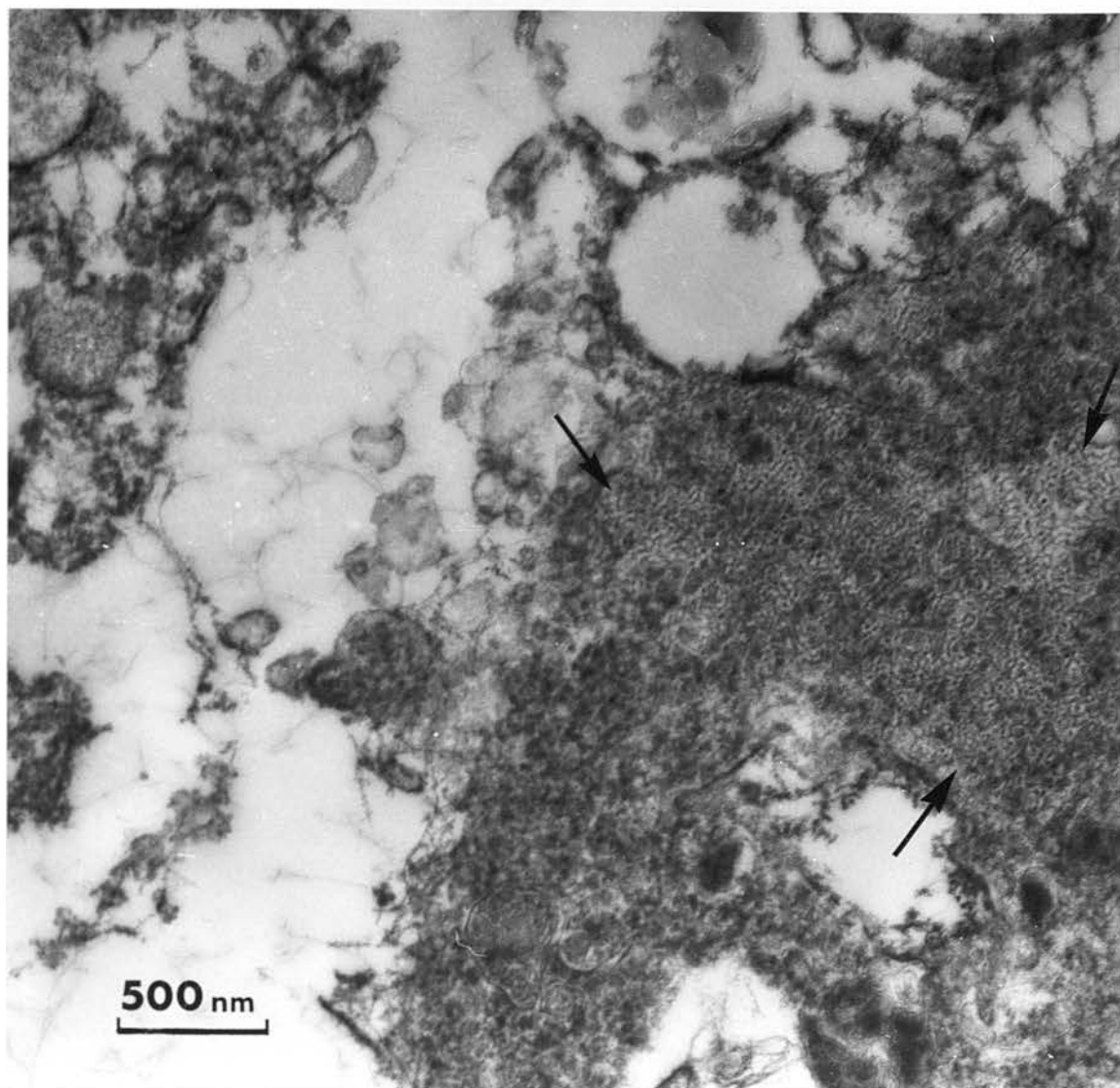


Plate 40 : Electron micrograph of peroxidase-
labelled preparation of MDBK_{pi}
cells showing an intracytoplasmic area
of smooth nucleocapsids which does
not appear to have taken up the label
(arrows). x40000



of PLA taken up by the first antigenic sites encountered on the periphery of the cell.

Granular nucleocapsids could be seen scattered throughout the cytoplasm in PLA treated cells, or aligned under the cell membrane, surrounded by an aureole of discontinuous stained material (Plate 38,39). In blocking controls this type of nucleocapsid revealed characteristics similar to those observed in MDBK_{pi} cells stained by conventional methods.

However, the smooth nucleocapsids present in some of the PLA stained cells did not show any particular deposit of electron-dense material. This might imply, either that the smooth nucleocapsids are not NDV specific or that the stain failed to penetrate sufficiently the cell cytoplasm of PLA treated cells (Plate 40).

5 - Interfering factors in culture fluids of persistent infections

a) Preparation of cell culture fluids

Supernatant fluids from both MDBK_{cs} and MDBK_{pi} cultures were examined for the presence of interfering factors including interferon, which could explain the absence of obvious cytopathic changes associated with NDV infection in MDBK persistently infected cultures.

Confluent cell monolayers cultured in large glass bottles with capacities ranging from 1 to 2.5 litres, were covered with volumes of 50 to 100ml of maintenance medium, containing 3% of heat-treated calf serum.

Culture fluids were collected between 2 and 27 days after the last change of culture medium, and all were subjected to the same treatment. This consisted of the centrifugation of cell culture fluids at 75000xg for 2 to 3 hours in order to pellet any virus present. The supernatants were harvested from the centrifuge tubes, with great care being taken to avoid resuspension of the pelleted material. Infectivity

tests performed on the harvested fluids invariably gave negative results. After being sterilized by filtration, the supernatants fluids were used to treat one-day-old monolayers of the indicator cell system employed, (generally MDBK cells), for at least two hours. Volumes ranging from 0.2 to 1ml of centrifuged fluid were then used to overlay monolayers in culture tubes with an average of 2×10^5 cells/ml.

For the interferon studies, 10 to 20ml of the centrifuged fluids were incubated for two hours at 56°C , and the indicator cells cultures always treated for a period of at least 12 hours.

The treated and untreated cultures, employed for the assays of inhibitory factor and interferon, were then challenged with several viruses (NDV, Sendai virus, EMV and M6 virus) and their growth cycle studied as described in Materials and Methods.

b) Viral inhibitory factors in persistently infected fluids

Ter Meulen and Martin (1976), observed that a virus-specific factor was produced in cultures of Vero cells persistently infected with canine distemper virus which inhibited the replication of the distemper virus but not that of related or unrelated viruses. They also found that this viral inhibitory factor (VIF) was labile and was neither inactivated by ultraviolet light nor sedimented by ultracentrifugation. Because their persistently infected cell system often suffered a crisis, the authors realised that the phenomenon could only be detected when the proportion of infected cells in the cultures was low.

In view of these interesting observations, a series of experiments were conducted in MDBK_{cs} and MDBK_{pi} in an attempt to ascertain whether a similar interfering effect might be present in cell culture systems persistently infected with other paramyxoviruses, such as NDV.

The data obtained from these experiments show that culture fluids (treated by centrifugation, as described earlier) taken from MDBK_{cs} two

TABLE 8

ASSAY FOR VIRAL INTERFERING FACTORS IN MDBK_{cs} AND MDBK_{pi} CULTURE FLUIDS

Assay	Culture	Age (days)	Days after last refeed	Challenge with NDV ^{a)} Control Test	VIF	Presence of interferon	Challenge virus	
1	MDBK _{cs}	9	6	7.5	4.5	+	ND ^{b)}	
2	MDBK _{cs}	3	2	7.5	7.5	-	ND	
3	MDBK _{cs}	3	2	7.0	6.0	+	-	BMV
3	MDBK	3	2	7.0	7.0	-	ND	
4	MDBK _{pi}	13	6	7.5	7.5	-	-	BMV
5	MDBK _{cs}	14	11	7.5	6.8	±	-	BMV
6	MDBK _{cs}	11	9	6.5	4.5	+	-	BMV
7	MDBK _{cs}	14	11	- ^{c)}	-	-	-	BMV
8	MDBK _{pi}	24	19	6.0	6.3	-	-	BMV
9	MDBK _{cs}	15	10	6.5	5.2	+	-	BMV
10	MDBK _{cs}	15	9	5.8	4.5	+	-	BMV
	<i>idem</i> after 16 hours' centrifugation			5.8	4.8	+		
11	MDBK _{cs}	12	9	5.5	3.5	+	-	BMV
	<i>idem</i> after 24 hours' centrifugation			5.5	3.5	+		
	<i>idem</i> after 24 hours' at 4°C			5.8	3.5	+		
12	MDBK _{cs}	17	14	5.8	3.5	+	-	NDV Sendai virus
	<i>idem</i> after 8 days at 4°C			5.8	3.8	+		
13	MDBK _{cs}	33	18	5.8	4.5	+	-	NDV Sendai
14	MDBK _{cs}	25	13	6.5	4.5	+	-	NDV
15	MDBK _{pi}	21	14	6.5	6.5	-	-	NDV M6
16	MDBK _{cs}	22	18	7.1	5.1	+	+	M6
17	MDBK _{cs}	17	12	7.1	5.8	+	-	M6
18	MDBK _{cs}	51	27	5.8	4.5	+	-	M6
19	MDBK _{pi}	48	27	5.8	5.8	-	-	M6

a) Viral titres expressed as log₁₀/TCD₅₀/0.2ml.

b) Not done

c) No reliable results obtained due to faulty dilution of the virus.

or more days after the last medium change, and allowed to act on indicator cell monolayers, gave them a 10^1 to 10^3 -fold protection against infection with IDV (Table 8). Similarly, fluids treated in the same manner from MDBK control cultures when assayed under the same conditions gave no indication of possessing such properties.

In order to ascertain whether the novel protective effect might interfere with viral replication after its initiation, or if it somehow confers on previously treated cells a refractory state to viral infection, a further experiment was carried out. In Table 8, experiment 2 refers to a carrier culture from which fluid was collected and treated as described. Viral inoculation was accomplished 1.5 hours before the addition of the centrifuged fluid, which was allowed to remain in contact with the indicator cultures for a period of three days. Since identical viral titres were obtained in both test and control assays, this suggested that the timing of the treatment is critical for the disclosure of the interference effect.

In the following experiments, the viral titres obtained in the treated monolayers were similar whether or not the centrifuged fluids were removed or allowed to remain during the adsorption period of the challenging virus.

When it was established that fluids should be allowed to act on indicator cell systems, prior to viral inoculation, attempts were made to ascertain the effects of time lapse following the last change of medium, on the amount of VIF present in MDBK fluids. As the data presented in Table 8 show, the inhibitory factor is already present and can be detected in the supernatant fluids within 2 days of refeeding, but there was no clear evidence that any correlation could be drawn between the build up of VIF and the interval between culture medium changes, even up to 27 days. These findings, together with those

related to the age of the cultures, suggest that VIF production depends largely upon the particular culture involved.

As the period of centrifugation routinely employed was of 2 to 3 hours, small amounts of previously treated fluids from experiments 10 and 11 were recentrifuged at 100000xg, for 16 and 24 hours, respectively. However, since no difference was obtained in the level of protection against NDV infection between the normal samples and those prepared by prolonged centrifugation, this suggests that the inhibitory factor is not readily pelletable.

In order to test the lability of the VIF found in MDEK fluids, samples of centrifuged fluids were held at 4°C for periods ranging from 1 to 8 days (experiments 11 and 12). During the first 24 hours, no differences were obtained and, even after 8 days, only a 0.3 of log₁₀ loss of protection was observed.

Finally, the specificity to NDV of the inhibitory factor in the carrier cultures was studied by diluting successive amounts of rabbit anti-NDV serum in centrifuged fluids, prior to treatment of the indicator cells. At the same time, controls were set up using normal culture medium incorporating identical dilutions of anti-NDV serum. In experiments 13, 14 and 16, final dilutions of 1/25, 1/50, 1/100 and 1/200 of the NDV antiserum were employed. Three to five rinses with maintenance medium were required after the antiserum treatment in order to remove the excess of antibody present. Cultures were then challenged with NDV, as described previously. The results shown in Table 9 indicate that antiserum treatment reduces the viral titres between 5 to 100 fold in control cultures, even after careful and extensive removal of any antiserum remaining. As test indicator cultures did not reveal titres significantly different from those of the controls, it was assumed that the inhibitory effect of the culture fluids had been neutralized by the NDV antiserum.

TABLE 9

NDV SPECIFICITY OF THE VIF PRESENT IN MDEK_{CS} CULTURE FLUIDS $(\log_{10} \text{TCD}_{50}/0.2\text{ml})^{\text{a)}}$

Antiserum dilution	Antiserum treated cultures ^{b)}		Non-treated cultures	
	Control	Test	Control	Test
1/25	5.5	5.1	7.1	5.1
1/50	4.5	4.5	6.5	4.5
1/100	4.5	4.1	6.5	4.5
1/200	5.1	4.3	5.8	4.5

a) Viral titres expressed as $\log_{10} \text{TCD}_{50}/0.2\text{ml}$.

b) Anti-NDV rabbit serum (HI 1/512) was diluted in MDEK_{CS} centrifuged fluids. In the case of controls the antiserum was diluted in maintenance medium.

Culture fluids from MDBK_{pi} were also examined for the presence of VIF. Experiments 4,8,15 and 19 of Table 8, refer to attempts made using identical methods to those with MDBK_{cs}, but they consistently show that there was no evidence of an inhibitory factor in fluids obtained from cultures with the regulated type of infection.

c) Differentiation of viral inhibitory factor and interferon

Study and characterization of interfering agents require that a fundamental distinction should be drawn between viral-specific interfering factors and interferon or interferon-like substances. The basic feature for such distinction is the fact that while the interfering agents are virus-specific, as is shown by their ability to block only infections with the corresponding type of virus, interferon action is a non-specific mechanism of viral interference which, being induced by a given virus, might protect cell cultures from infection with related or even, unrelated viruses.

In the present study, four different viruses, namely NDV, Sendai virus, BMV and M6 virus, were used to ascertain the presence of interferon and to study the blocking effect of VIF on infection with viruses other than NDV.

Centrifuged fluids of MDBK_{cs} and MDBK_{pi} cultures were used either treated or untreated for two hours at 56°C. Fluids from indicator cell cultures, propagated in tubes, were discarded and replaced with 1ml of those treated or untreated testing fluids, for at least 12 hours. In most experiments, the overlay period was carried out overnight. The viral challenge, using either NDV or one of the viruses mentioned above was performed after removal of the test and control fluids.

Cell monolayers from indicator cultures which were treated with fluids heated at 56°C, and challenged with BMV (experiments 3 to 15), NDV (experiments 12,14 and 15), or M6 virus (experiments 15 to 19),

TABLE 10

INFECTIVITY TITRES AFTER TREATMENT WITH HEATED AND UNHEATED
 MDBK CULTURE FLUIDS^{a)}
 ($\log_{10} \text{TCD}_{50} / 0.2 \text{ml.}$)^{cs}

Experiment	NDV		Sendai virus		BMV		M6	
	Control	Test	Control	Test	Control	Test	Control	Test
3	NT				4.5	3.8		
4	NT				4.2	3.5		
5	NT				3.5	3.5		
6	T ^{c)}				3.5	3.7		
	NT				3.5	3.5		
7	T				4.3	4.0		
	NT				3.8	3.8		
8	T				4.0	3.8		
	NT				3.5	3.5		
9	T				3.5	4.0		
	NT				3.8	4.1		
10	NT				2.8	3.1		
11	T				2.5	2.8		
	NT				2.8	2.8		
12	T	5.8	5.8		3.2	3.5		
	NT	5.8	3.5	3.8	3.8	2.8	2.8	
13	T				3.2	2.5		
	NT			3.8	3.8	2.8	2.8	
14	T	6.5	6.2		3.5	3.2		
	NT	6.5	4.5		3.8	3.8		
15	T	6.5	6.2		3.5	3.2	2.5	1.5
	NT	6.5	6.5		3.8	3.5		
16	T						2.5	1.0
17	T						1.7	1.5
	NT						3.5	3.5
18	T						3.5	3.5
	NT						2.8	2.8
19	T						2.8	3.5

a) Results expressed as $\text{TCD}_{50} / 0.2 \text{ml.}$ The parameters used were haemadsorption 3 days after NDV or Sendai virus challenge, or visible cytopathic changes 5-7 days after BMV or M6 virus infection.

b) Centrifuged MDBK_{cs} culture fluids (unheated), used to overlay indicator cell monolayers overnight.

c) Idem, heated at 56°C for 2 hours.

gave results similar to those obtained with untreated fluids, except in the case of NDV challenge (Table 10). These findings show that heat treatment destroyed the VIF present in MDBK_{cs} fluids and also, that related or unrelated viral infections were not affected by the inhibitory factor present.

The data accumulated seem to indicate that no interferon can be detected in MDBK_{cs} and MDBK_{pi} by the methods used, although two results obtained after challenge with M6 virus (experiments 15 and 16) showed a significant drop in the titre of the test assay compared with that of the control. If these results are taken into account, it would be reasonable to suggest that the fact that no VIF was ever detected in MDBK_{pi} cultures seems to indicate that this form of interference bears no relationship to the presence of interferon.

6 - Attempts to detect proviral information

The peculiar characteristics of MDBK_{pi}, in which almost the entire cell monolayers shows viral infection, without detectable alteration of cell metabolism, and a very low amount of infectious virus being released in the surrounding fluid suggests that the prolonged permanence of the virus in the cells is due to integration of viral information into cellular chromosomes.

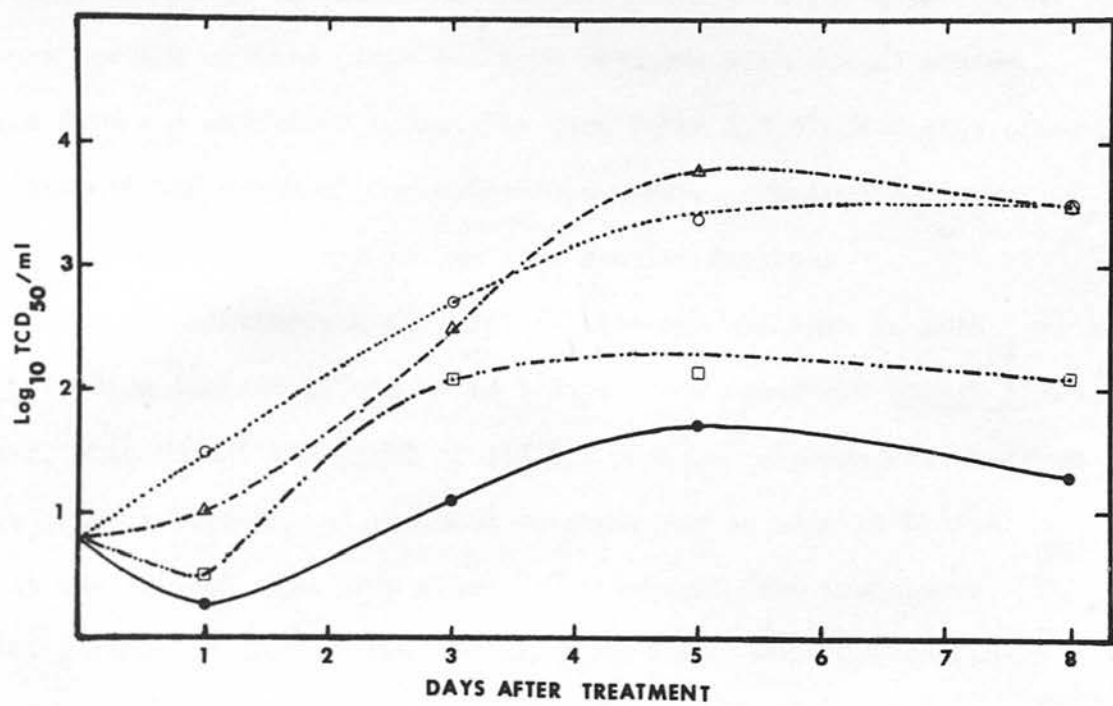
The activation of latent virus in non-producing cell lines has been achieved by several different methods such as co-cultivation with susceptible cells (Benveniste et al., 1974) and induction by inhibitors of cell protein synthesis (Aaronson and Dunn, 1974) or by halogenated pyrimidines (Aaronson et al., 1971; Klement et al., 1971; Lowy et al., 1971; Stewart et al., 1972). Although these experiments were concerned with C-type RNA viruses, they all relate to the induction of naturally integrated viral information.

Graph 12 : Effect of different concentrations of Iododeoxyuridine (IdU) on MDBK_{pi} cells.

Cell cultures of MDBK_{pi} cells propagated in 4oz bottles, and containing approximately 6×10^6 cells, were incubated at 37°C. Culture medium supplemented with 20, 50 and 100 µg/ml of 5-iodo-2'-deoxyuridine (IdU) was allowed to remain on the cell monolayers for a period of 24 hours, when it was replaced with normal medium. Samples of supernatant fluids taken 1, 3, 5 and 8 days after the end of treatment were examined for released virus.

Concentrations of IdU:

□.....□ , 20 µg/ml; ○.....○ , 50 µg/ml;
△.....△, 100 µg/ml; ●——● , MDBK_{pi} control cultures.



In view of this, a number of experiments were carried out in an attempt to detect the presence of integrated viral genomes in our two models of persistent infection.

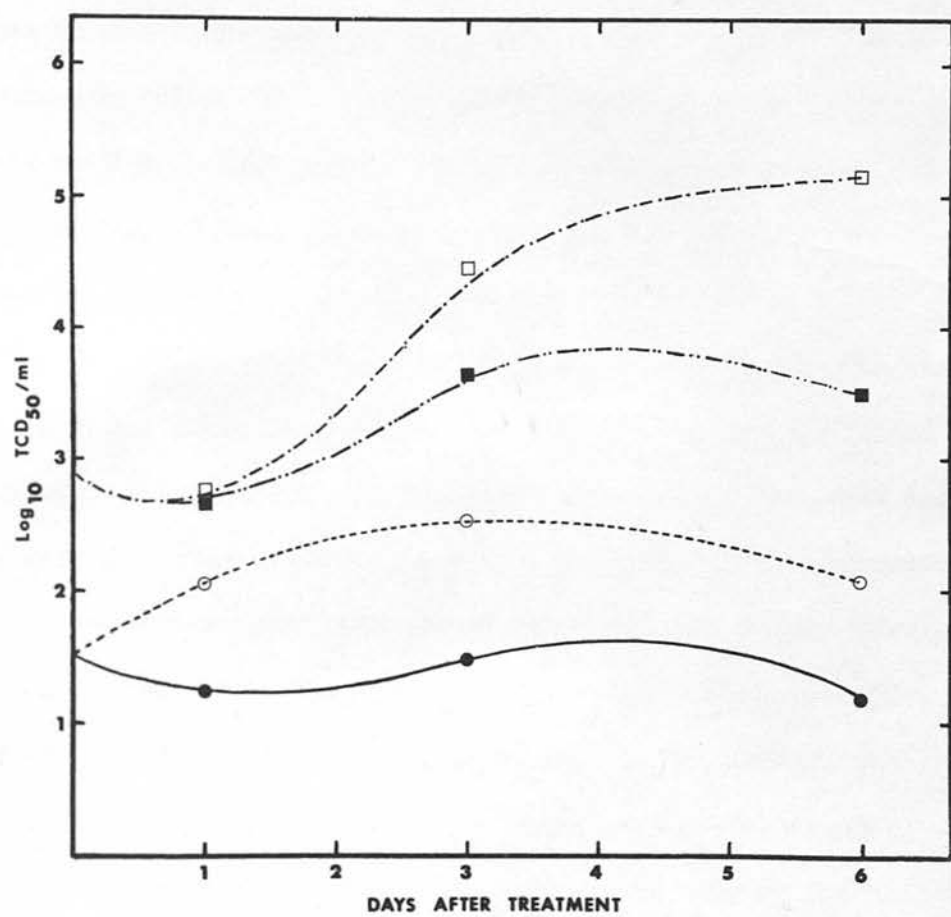
a) Enhancement of the release of infectious virus in persistent infections by DNA analogues

Cell cultures from MDBK_{pi} and MDBK control kept in 4oz bottles and containing approximately 6×10^6 cells, were incubated with culture medium containing 20, 50 and 100 $\mu\text{g}/\text{ml}$ of 5-iodo-2'-deoxyuridine (IdU). The drug was allowed to remain on the cell monolayers for a period of 24 hours and the culture fluid was then replaced with normal medium. Samples from the culture supernatants were taken 1, 3, 5 and 8 days after the treatment and examined for infectious virus. Control cultures were treated with drug-free medium in a similar fashion.

A significant increase in viral release was obtained in MDBK_{pi} with all three drug concentrations up to 3 days after treatment (Graph 12). However, with 50 and 100 $\mu\text{g}/\text{ml}$ of IdU the maximum response was observed 5 days after treatment and remained constant for as long as 8 days.

It was noticed also that after IdU treatment, the monolayers, including those of MDBK control cells, showed some cell destruction denoted by an increased number of cells becoming rounded and detaching from the glass surface. Similarly, the pH of the culture fluid was observed to rise slightly. These symptoms were more marked in cultures treated with the higher concentrations of IdU. With the removal of the drug from the cultures, all recovered their normal characteristics, with the recovery taking longer in the more severely affected.

From these findings it was decided to use a standard dose of 50 $\mu\text{g}/\text{ml}$ of IdU in further experiments. These were set up to confirm the results of the previous experiments and to compare the effect of IdU on MDBK_{pi} with that on the NDV carrier state, MDBK_{cs}.



Graph 13 : Effect of Iododeoxyuridine (IdU) on persistent infections.

Cultures of both MDBK_{pi} and MDBK_{cs} cells were treated with 50 μ g/ml IdU. The treatment fluids were removed after 24 hours' incubation and replaced with normal medium. Supernatant fluids were taken 1,3 and 6 days after the end of treatment and examined for infectious extracellular virus.

- , MDBK_{pi} control cultures;
- ⊖---⊖ , MDBK_{pi} cultures treated with IdU;
- ⊞---⊞ , MDBK_{cs} control cultures;
- ⊞---⊞ , MDBK_{cs} cultures treated with IdU.

The data obtained showed that IdU treatment not only increases NDV production but also the release of the virus from the cells in both systems (Graph 13). This suggested that, in both cases, control of viral expression was dependent on cellular DNA. It should be noted that the degree of activation achieved through IdU treatment, although always present, has varied in all experiments. This implies that the effect of the drug is also dependent upon the particular conditions of the virus-cell relationship at the moment of the treatment. Furthermore, the effect of the drug on the level of virus production was still evident 5 to 8 days after its removal whereas the cells had shown complete recovery by this time.

b) Isolation and transfer of cellular DNA

Hill and Hillova (1971) have shown that when cellular DNA was taken from rat XC cells containing latent Rous sarcoma virus, and added to chicken embryo fibroblasts, these latter cells became actively infected with the virus. This achievement was followed by other successful demonstrations of transfection, all of them with RNA tumour viruses (Cooper and Temin, 1974; Svoboda et al., 1974). More recently, other types of RNA virus were also found to be capable of integration into the host genetic material, as in the case of the successful transfer of the respiratory syncytial virus infection through the DNA of the infected cells (Simpson and Iinuma, 1975). Several hybridization studies also revealed the integration of measles and tick-borne encephalitis viruses into cellular chromosomes (Zhdanov and Parfanovich, 1974; Zhdanov et al., 1974). Furthermore, Furman and Hallum (1973) have found a reverse transcriptase in preparations of a mutant of NDV arising from persistently infected L cells.

In an attempt to demonstrate the presence of viral genetic material in the DNA of the persistently infected cells from both systems under

the present study, cell cultures from the MDBK control line and the two NDV persistent infections were used. These were propagated in Roux bottles for periods of 3 to 7 days until confluent monolayers were obtained, when they were used for the isolation of cellular DNA. Similarly, MDBK cell cultures were inoculated 20 hours before DNA isolation began, with 1 MOI of Herts NDV, and served as a cell-virus infection control. A total of at least 10^8 cells for each culture were lysed and processed for DNA isolation as described in Materials and Methods.

The DNA content of all isolates was determined by the technique described in Material and Methods. The amount of DNA present in the samples varied from 25 $\mu\text{g/ml}$ to 150 $\mu\text{g/ml}$.

To confirm the specificity of the assay, samples of various DNA isolates were treated with deoxyribonuclease at a final enzyme concentration of 10 $\mu\text{g/ml}$. The mixture was incubated for one hour at 37°C and then used for transfection experiments in the same manner as untreated DNA.

The recipient cell cultures used in most of the assays were twenty-hour-old monolayers of BHK-21 cells, although young MDBK cell cultures were also used in some of the experiments.

The inoculum was diluted either in PBS, modified Hanks' solution (BSS), EDTA high salt buffer, NaCl-tris solution, or Hepes buffered saline (HeBs), according to the various methods tried. Similarly, recipient cell monolayers were sometimes pre-treated with DEAE-dextran in a NaCl-Tris buffered solution at pH 7.4. In some experiments, DNA isolates were diluted in HeBs solution and precipitated at room temperature for 25 minutes by the addition of calcium chloride at a final concentration of 125mM, prior to inoculation.

The adsorption period varied with the different method employed, and ranged from 10 minutes up to 5 hours. Sometimes, the inoculum

was removed and monolayers washed with the appropriate buffer. Sometimes, no wash was performed after the removal of the inoculum. However, on some other occasions the inoculum was allowed to remain and monolayers were simply overlaid with the culture medium.

After addition of the DNA, the recipient cell cultures were incubated either at 35°C or 37°C, for periods varying from 2 to 14 days. Some inoculated cultures were passaged four times over a period of two months. Cultures were checked daily for visible cytopathic changes, and coverslips were removed every two days and examined for the presence of viral antigen by immunofluorescence staining.

During the period of the experiment, no trace of transfection was obtained with any of the DNA isolates. Also, the recipient monolayers did not show any signs of morphological or functional alteration.

DISCUSSION

In 1962, a persistent infection of Madin's bovine kidney cell line (MDBK) was induced accidentally in this laboratory and shown subsequently to have the characteristics of a regulated or steady-state infection (Edwards, 1972; Fraser et al., 1976). The origin of this infection is not known but is thought to have been caused by the Herts 33 virus since this was the only strain of Newcastle disease virus held in the laboratory at this time. Early attempts to demonstrate the presence of infectious extracellular virus in cultures of the persistently infected cells (MDBK_{pi}) proved unsuccessful, but more recent studies have shown that cytopathic changes typical of NDV infections occur in several permissive cell cultures inoculated with MDBK_{pi} fluids (McNulty et al., in press). Possible reasons for these discrepant results include the use of improved techniques and the fact that significant changes had occurred in the characteristics of the persistently infected cultures between the two periods of investigation. The latter is believed to be the more likely explanation since the cultures have been maintained for several years by trypsinization at weekly intervals.

In view of these observations an attempt was made in this present work to induce a persistent infection of MDBK cells with the Herts 33 strain of NDV, under carefully controlled conditions. This was done in order to study the mechanisms involved in the establishment of a persistent infection in MDBK cells, and to provide a more practical model for further comparative studies at the biological and molecular levels.

For the induction of this carrier state cell system (hereafter named MDBK_{cs}) cultures of healthy MDBK cells were persistently infected with NDV (Herts 33 strain) by continuous undiluted passage of virus

harvests. Four different multiplicities of infection were used at the start of the experiment because other authors have shown that the initial inoculum plays a decisive role in establishing persistent infections with paramyxoviruses (Henle et al., 1958; Wheelock and Tamm, 1961a).

The approach used to induce the MDBK_{cs} cell system, involved adaptation of egg-grown NDV to MDBK cells rather than successive propagation of the surviving infected cells. The choice of method was deliberate, since it is well known that the latter procedure usually gives rise to persistently infected cell systems of the regulated type where: a) only very small amounts of infectious virus is released spontaneously (Walker and Hinze, 1962a; Fernandes et al., 1964; Norrby 1967; Haspel et al., 1973), or b) where viral release can only be induced by co-cultivation with permissive cell cultures (Knight et al., 1972; Wroblewska et al., 1976), or c) where no infectious virus is released at all (Katz and Koprowski, 1973; Bachmann et al., 1975; Menna et al., 1975a). On the other hand, since there is clear evidence that infectious virus is readily produced and is easily detectable in carrier state cultures it seemed likely that this type of cell system would serve adequately as controls in studies on regulated type infections.

The main characteristics of our MDBK_{cs} system were remarkably similar to those of an NDV carrier state in Earle's L cells described earlier (Henle et al., 1958; Bergs et al., 1958; Deinhardt et al., 1958; Henle et al., 1959; Henle, 1963), in that the number of infected MDBK_{cs} cells was consistently low in most of the 25 subcultures studied. However, it is interesting to note that in five of the subcultures, the percentage of infected cells rose dramatically, and this was accompanied by cytolytic activity.

These features, called crises, have also been observed in cell systems persistently infected with rabies virus (Wiktor and Clark, 1972), measles virus (Ehrnst et al., 1974), influenza virus (Perekrest et al., 1974) and canine distemper virus (ter Meulen and Martin, 1976), and appear to undergo cyclic variation. In the present study, the proportion of infected cells in monolayer cultures was determined by both haemadsorption and fluorescent antibody staining techniques, and it is clear from the results obtained (Table 2) that the latter is by far the more sensitive method for detecting infected cells. These findings are consistent with those of Nagata et al. (1972) who found that the haemagglutinin antigen detected by immunofluorescence within the cytoplasm of BHK-21 cells persistently infected with Sendai virus, acquired the ability to haemadsorb only after it had become incorporated into the cellular membrane. Thus, the present findings might be explained by the inability of viral material to reach the surface of the cell or to modify the plasma membrane (Duc-Nguyen, 1968). This abnormality in the developmental cycle of the virus would still permit detection of viral antigen by fluorescent staining techniques in the absence of haemadsorption.

Several reports concerning NDV (Henle et al., 1958) and other membrane-forming viruses such as measles (Norrby, 1967) and rabies (Wiktor and Clark, 1967) suggest that persistently infected cultures have a slower rate of growth compared with that of uninfected controls. This was also shown to be the case with MDBK_{cs} cultures, although the total yield of cells from infected and control cultures was similar after 8 days of incubation. This is in contrast to the results obtained by Henle (1958) who observed lower yields in carrier L cells infected with NDV. The morphology of MDBK_{cs} cells after the first subcultures was noticeably different from that of the original MDBK cells. In

appearance they closely resembled the morphology of MDBK_{pi} monolayers which were shown to consist of transformed cells (Edwards, 1972).

However, experiments based on the methods designed by MacPherson and Montagnier (1964) gave inconclusive results and it was not established whether the MDBK_{cs} cultures also contain transformed cells.

It is generally agreed that infected cells in carrier-state cultures do not divide or grow into colonies, and that clones obtained from carrier cultures are free from virus (Walker, 1964). In the present work, several attempts were made to obtain clones of either MDBK_{cs} or MDBK_{pi} cells but without success. Direct observations of MDBK_{cs} monolayers showed that at least some infected cells could undergo mitosis and divide, and that progeny cells continued to show surface haemagglutinins. However, these results do not necessarily prove that infected MDBK_{cs} cells divide and grow to form infected colonies, because Wheelock and Tamm (1959) have shown that some infected cells in HeLa monolayers primarily infected with NDV were able to undergo one cellular division.

A number of authors have suggested that virus transmission during subcultivations of many persistently infected cultures of the regulated type seemed to occur at cell division or across intracellular bridges (Walker and Hinze, 1962b; Fernandes et al., 1964; Norrby, 1967; Wroblewska et al., 1976). This type of persistent infection cannot be cured even by prolonged serial propagation of the cultures in media supplemented with specific antiserum, and it has previously been reported that MDBK_{pi} cell cultures could not be cured even after five weeks of treatment with NDV antiserum (Fraser et al., 1976). Nevertheless, in the case of MDBK_{cs} cultures, complete cure was achieved within about a month after the beginning of treatment. This observation suggests that the release of infectious virus plays a decisive role in the

maintenance of the carrier state in MDBK_{cs} cultures, along with some sort of mechanism that controls or balances the output of infectious virus.

The ability of the MDBK_{cs} cells to support the growth of homologous virus and related or unrelated viruses, is remarkably similar to that shown by regulated types of infection. According to Walker (1964) and Wiktor and Clark (1972) the maintenance of some carrier state cultures depends on the production of interferon, which provides protection for the majority of the cells in the monolayers, and in these circumstances the carrier cultures resist superinfection with both homologous and unrelated viruses. This was not the case with MDBK_{cs} cultures which, by contrast, showed no resistance either to another member of the genus Paramyxovirus (Sendai virus) or to an unrelated herpes virus (bovine mammillitis virus), although they were highly resistant to infection with the homologous infecting virus (NDV).

Several studies were conducted in an attempt to ascertain whether resistance to superinfection by homologous virus is caused by lack of adsorption of the superinfecting virus to the persistently infected cells. Rustigian (1966) working with both measles virus and HeLa cells persistently infected with measles virus observed that fewer viral particles became attached to the infected cells than to the controls. However, studies conducted by Staneck et al. (1972) in BHK-21 cells persistently infected with lymphocytic choriomeningitis virus led to the conclusion that the same number of viral particles attached to infected or uninfected cells. More recently, ter Meulen and Martin (1976), using Vero cells persistently infected with canine distemper virus observed that superinfection with the same virus or with measles virus resulted in some cytopathic changes appearing after 24 hours, which disappeared completely 48 hours post-infection. These workers

suggested that defective interfering (DI) particles might be responsible for the blockage of viral cytolytic replication. This is in line with the hypothesis proposed by Huang and Baltimore (1970) which attributes a major role to viral interfering particles in the establishment and maintenance of persistent infections. More recently, Holland and Villarreal (1974) confirmed that DI particles in a BHK-21 cell system persistently infected with vesicular stomatitis virus (VSV) suppress virion transcriptase and interfere with fully infectious VSV particles, thereby preventing replication and expression of normal cytopathogenicity. Similarly, Hall et al., (1974) observed DI particles in many virus preparations which were used to establish persistent infections, as well as in supernatant fluids of cells persistently infected with measles virus.

Other workers have shown that BHK-21 cultures persistently infected with VSV cannot be superinfected with the homologous virus, although they are capable of supporting the growth of heterologous viruses (Holland and Villarreal, 1974). This is also the case in Vero cells persistently infected with canine distemper virus, which were established by continuous passage of undiluted virus harvests (ter Meulen and Martin, 1976). These latter workers also described the production of viral inhibitory factor (VIF) which is released into the medium of the persistently infected cultures and inhibits the replication of canine distemper and measles viruses. A similar effect was also detected in MDBK_{cs} cultures and will be discussed later.

Unlike the MDBK_{pi} cell system described by Fraser et al. (1976) the present MDBK_{cs} virus was found not to be temperature-sensitive, at least in early stages of the persistent infection (passages 5 to 12). However, further work would be necessary to ascertain whether t. s. mutants have been selected during the later passages of this cell system, similar to that described by Holland et al. (1976) in their

BHK-21 cells infected with VSV. These additional experiments might also have shown whether or not the occurrence of t.s. mutants in our present model was of a transient nature, as reported in other cell systems (Haspel et al., 1973; Kawai et al., 1975).

In order to understand the cellular and viral mechanisms involved in the establishment of different types of cell-virus equilibrium, studies at the molecular level were conducted. These consisted of studies on purified virus from both persistent infections (propagated in embryonated eggs or released in the cell culture medium), or radio-isotopic labelling of viral components or virus-induced products.

The release of virus in the cell culture fluids of both MDBK persistent infections was very low. Attempts were made to induce an higher yield by co-cultivating the persistently infected cultures with a suitable indicator cell system (BHK-21 or CEF). Although extensive cell fusion resulted, only a slight increase in the amount of released virus occurred. In fact, high titres of infectious virus were always detected in the carrier cultures throughout the period of the experiments, without proportional amounts of virus being concentrated and purified from those fluids. Further studies are needed to explain whether the method employed for viral purification is appropriate for cell culture fluid concentration, or alternatively if the particle : infectivity ratio is close to unity, then only a small number of infectious viral particles would be sufficient to be detected when a highly susceptible indicator cell line is used in infectivity determinations.

Only virus from MDBK_{cs} cells was able to be grown in embryonated hen's eggs. Various attempts were made to cultivate MDBK_{pi} released virus in eggs but none was successful, and earlier attempts made in this laboratory gave conflicting results (Edwards, 1972; Fraser et al., 1976; McNulty et al., in press; Ruben, J., unpublished observations).

However, when suitable indicator cell cultures (BHK-21 and CEF) were inoculated with virus released from MDEK_{pi} cells, syncytial foci showing haemadsorption were observed 48 hours after infection. Attempts were made to enhance the viral presence by successive passage of the supernatant fluids from those infected indicator cell cultures but without success, and even the evidence of viral activity disappeared. Earlier experiments using cell suspensions or supernatant fluids from mixed cultures of MDEK_{pi} and CEF cells which showed extensive cell fusion, did not reveal infectious virus when inoculated in the allantois of embryonated hen's eggs or intracerebrally into one-day-old chicks (Fraser et al., 1976). These results seem to suggest that both cell fusion and haemadsorption activities indicate the presence of viral components in the cell membrane of the infected cells (Duc-Nguyen, 1968; Bratt and Gallaher, 1969; Gallaher and Bratt, 1974). The results also seem to indicate that the released virus in MDEK_{pi} cells may well be able to modify the plasma membrane of the infected indicator cells and induce fusion or attachment of erythrocytes, but is unable to undergo a complete cycle of replication or be properly assembled at the cell surface.

Egg-grown Herts NDV and MDEK_{cs} viruses were used as controls in comparative studies of the polypeptide composition of the various purified isolates of the two MDEK persistent infections. As shown in Table 6 the present results are in agreement with those of several other workers for the distribution and size of the various polypeptides of NDV. It should be noted that when isolates from the allantoic fluid of embryonated eggs were centrifuged in continuous sucrose or tartrate gradients for equilibrium sedimentation, two distinct areas of viral distribution were detected. Early studies by Granoff (1955) have shown that NDV produced in embryonated eggs consists of a mixture of

two distinct types of particles, which have been designated as infectious haemagglutinins (IH) and non-infectious haemagglutinins (NIH). In contrast to IH, NIH particles are smaller, deficient in viral RNA and in haemolytic activity, and do not produce a von Magnus type of interference (Granoff, 1955; Rott et al., 1962; Rott and Schäfer, 1964; Clavell and Bratt, 1972). The area in the gradient lying at a density of about 1.18 is similar to that obtained by those workers for the sedimentation of NDV from which typical distribution of the virus polypeptides and viral activity were observed. The other area, comprising lighter viral particles sedimenting at a density of 1.12, revealed an unusual polypeptide composition with a significantly reduced NP/F peak. Also, lower haemagglutinin and neuraminidase activities and a markedly lower infectivity titre were obtained.

These results seem to be consistent with those of La Montagne et al. (1975) who in a recent study concerning the biological and chemical characteristics of IH and NIH particles, observed that the distribution of the major proteins in infective and noninfective haemagglutinating particles from egg-grown NDV virus was significantly different. They found that the amount of proteins NP/F and M present in the NIH particles was reduced relative to protein HN. However, the haemagglutinin activity was found to be higher in NIH than on IH particles, whereas the specific neuraminidase activity did not differ in the two particle types.

Confirmation that the peak HN consists of a glycosylated polypeptide and that another glycoprotein co-migrates with the NP/F peak, was obtained in periodate-Schiff' stained gels. These results are in agreement with those of several other workers (Mountcastle et al., 1970; Meager and Burke, 1973; Scheid and Choppin, 1974). On the other hand

it is apparent that the egg-grown viruses assayed have a considerably higher proportion of the lower molecular weight glycoprotein in comparison with the HN glycopolyptide. Such findings are consistent with those of Scheid and Choppin (1973), but contradict those of Moore and Burke (1974). Furthermore, it was often observed that a number of small peaks appear between protein L and the HN peak. Similarly, Nagai et al., (1976b) observed in lentogenic strains of NDV grown in cell cultures (BHK-21F and MDEK), the occurrence of an HN precursor (HN_0) with a mol.wt. of 82000 which is converted by proteolytic cleavage into the haemagglutinin-neuraminidase glycoprotein (74000 mol.wt.). More recently, Samson (1976) studying the heterogeneity of the charge of NDV HN protein observed that it consisted of a population of seven peptides identical in molecular weight but of different charges.

On the other hand, the presence of a viral glycoprotein precursor in several cell culture systems infected with NDV or other paramyxoviruses has been recognized since the works of Samson and Fox (1973) and Scheid and Choppin (1974). This glycopolyptide (F_0) with 65000 mol.wt. is a precursor of the smallest virion glycoprotein F (53000 mol.wt.) and can be cleaved to yield F by treatment in vitro of the virions with trypsin. The cleavage of F_0 is known to be necessary for the expression of cell fusing and haemolytic activities. As the activation of the glycoproteins is not required for virus assembly, virus particles containing the precursor F_0 may be formed showing reduced infectivity (Nagai et al., 1976b). The glycoprotein pattern of the two viruses isolated from MDEK persistently infected cell cultures showed no component associated with the NP/F areas. However, glycosylated stained material was found in near areas of higher molecular weights. To gain evidence that it was related to any

precursor glycoprotein, similar studies should be conducted in trypsin-treated viral preparations.

The synthesis of viral proteins in both MDBK_{pi} and MDBK_{cs} was studied by co-electrophoresis of the two virus samples, labelled with different radioisotopes, in the same gel. In a parallel gel, Herts NDV was run under similar conditions. As shown in Plate 16, the radioactive trace of MDBK_{cs} cell-associated virus components closely corresponds to that of the Herts NDV grown in embryonated eggs, although the relative amount of the polypeptides present seems to differ significantly. This finding suggests that the intracellular viral polypeptides of the carrier state are all synthesized, although possibly in different proportions. Of the three main peaks, the relative proportion of the NP/F peak is reduced but, from the data available, it is not clear which of the two proteins (NP and F) is reduced in concentration. Alternatively, the polypeptide normally observed in the lower molecular weight peak shoulder (U1) may be missing, thereby contributing to the overall reduction of the peak size. On the other hand, a number of small peaks corresponding to high mol.wt. polypeptides appear as shoulders on both sides of the HN peak. The components on the left slope might be related to the occurrence of the precursor polypeptide (HN₀) of the HN protein, and are similar to the standard Herts NDV profile. The shoulder observed on the right slope of the HN peak of MDBK_{cs} intracellular virus might well be F₀, and if so, would explain the reduced proportion of the NP/F peak. However, further experiments using a labelled precursor of the glycosyl moiety are necessary to confirm these findings.

Several minor peaks of lower mol.wt. were seen on the radioactive trace of MDBK_{cs} cell-associated virus, which are absent in egg-grown NDV preparations. Kaplan and Bratt (1973) reported the presence of a

non-glycosylated polypeptide of 33000 mol.wt., which they observed to be primarily associated with the intracellular membrane, in the gel profiles of cell culture grown virus.

However, the composition of MDBK_{pi} intracellular virus is strikingly different. No trace of shoulders were found in the HN peak, and the relative proportions of HN and NP/F peaks are similar to the egg-grown virus. However, the matrix protein (M) is absent, but not the two viral polypeptides of unknown function or the lower mol.wt. peak corresponding to that of MDBK_{cs} cell-associated virus. It should be noted that the M protein, which is associated with the inner surface of the viral envelope, is known to be the recognition site for nucleocapsid during the process of viral assembly (Mountcastle et al., 1971; Choppin et al., 1972). Furthermore, it was shown recently that the nucleocapsid protein does not combine with the viral envelope glycopeptides unless the M protein is present (Yoshida et al., 1976). The finding that a defect in the viral polypeptide production occurs on NDV replication in persistently infected MDBK cells of the regulated type, and not in carrier state, seem to confirm the hypothesis that in these persistent infections viral replication is blocked at one of the last stages of maturation (Rima and Martin, 1976).

On the other hand, defects related to the lack of surface polypeptides were described in temperature-sensitive mutants isolated from persistently infected cell cultures with influenza virus (Palese et al., 1974) and Sendai virus (Portner et al., 1975). In both cases, the non-infectious virus particles were produced only when the mutants were grown at non-permissive temperature. The experiments conducted with Sendai virus clearly established that HN polypeptide was essential for virus-cell binding, either as a required structure, or because

of its neuraminidase activity, or both. Furthermore, Fraser et al., (1976) reported that BK_{pi} (MDBK_{pi} in the present study) virus did not show neuraminidase activity and, as in the present study, the defect is present even at the permissible temperature, 37°C. It also should be pointed out that the absence of neuraminidase activity in the released virus might be associated with a defect in the composition of the HN polypeptide, which could be related with deficient or erroneous assembly of the virus particles at the cell surface. Recent studies conducted in SV5 virus (McSharry et al., 1975) and Sendai virus (Shimizu and Ishida, 1975; Yoshida et al., 1976) have shown that the nucleocapsid of these viruses form complexes with the M protein. These associations are believed to be essential for the incorporation of the nucleocapsid into the modified areas of the plasma membrane where the envelope glycoproteins are arranged.

It is interesting also to note that the present findings bear close similarities with the observations reported in subacute sclerosing panencephalitis (SSPE) cell cultures where the failure to isolate infectious virus was attributed to the defectiveness of the virus rather than to some other factor (Katz and Koprowski, 1973). In fact the present results seem to indicate that incomplete or defective viral particles which are non-infectious might be released by MDBK_{pi} cells, and also that the defect located at viral assembly leads probably to the accumulation of viral components at the cell surface or in the cytoplasm.

These assertions were confirmed by experiments using both RNA and protein synthesis inhibitors. Both drugs failed to induce a significant increase in the release of infectious virus from MDBK_{pi} cells, although the synthesis of viral RNA in actinomycin D treated cells seemed similar to that of a primary MDBK infection with NDV.

These results are similar to those obtained by Knight et al. (1972; 1974) which showed that the release of measles virus from persistently infected HEF cells by co-cultivation with BSC-1 cells was not sensitive to inhibition of protein and RNA synthesis. They concluded that a block at a late step in virus replication, possibly concerning the maturation of infectious virus at the cell membrane, seemed the most likely. Menna et al. (1975a) also found that although the haemadsorption activity of the BGM/MV cells could be enhanced by treatment with actinomycin D, no detectable levels of measles haemagglutinin were observed. More recently, Flanagan and Menna (1976) working with the same model confirmed the results of those authors by inducing a similar effect with the inhibition of DNA transcription (with actinomycin D or enucleation of cells by cytochalasin B) or inhibition of protein synthesis (cycloheximide). These findings suggested that the enhancement of haemadsorption observed is probably due to a derepression effect caused by the drugs or procedures employed, on the cellular mechanism that blocks the synthesis of the viral haemagglutinin.

On the other hand, MDBK_{cs} cells treated with either actinomycin D or cycloheximide showed an increased release of infectious virus. These results were similar to those obtained in experiments using MDBK cells primarily infected with NDV, at low multiplicities of infection. It is known that NDV, as well as other paramyxoviruses, show marked differences in interaction with various types of host cells (Holmes and Choppin, 1966; Alexander et al., 1973; Hecht and Summers, 1974; Scheid and Choppin, 1975; Homma, 1975). The studies conducted in MDBK cells primarily infected with NDV, revealed that RNA synthesis in infected monolayers recovered after the first 9 to 11 hours, while virus production significantly increased after the same period. As the registered increase in the overall RNA production is not due to a corresponding enhancement in the viral RNA production,

it is probable that MDEK cells have a repressor mechanism which is able to control NDV replication.

The present results suggest that in the carrier state viral expression is controlled by mechanisms similar to those in primary infections, but unlike those operating in the MDEK_{pi} system.

It seems likely, therefore, that the defect in viral replication of MDEK_{pi} cells at a late stage of the virus assembly, might account for the intracellular accumulation of viral components. In cell cultures subcultured at weekly intervals this accumulation is normally confined to the cytoplasm, although fluorescing antigen is often seen in the nucleus (Plate 10). These findings confirmed earlier reports of primary NDV infection of different cell lines (HEp-2, bovine kidney and bovine thymus cells) in which viral antigen appeared in the nucleus of some cells at late periods of infection (Johnson and Scott, 1964; Omar, 1965; Zhdanov et al., 1966). The latter workers observed that the appearance of viral material is confined to the nucleolus and might be due to its transportation from the cytoplasm. However, when MDEK_{pi} cell cultures were subjected to prolonged cultivation, accumulation of viral material different from that associated with the nucleoli were seen both by fluorescent antibody staining techniques and in electron microscopy of thin sections. Normal nucleoli were generally found in those cells showing intranuclear inclusions, which indicate a different mechanism of accumulation. From the extensive studies conducted to compare SSPE and measles viruses, it was observed that nucleocapsid structures are the viral components usually accumulated, both in the cytoplasm and nucleus (Oyanagi et al., 1971; Norrby, 1972; Dubois-Dalcq and Barnosa, 1973; Dubois-Dalcq et al., 1974). Some of these authors also reported that two types of nucleocapsid structures were seen in both SSPE and measles infected

cells namely, smooth and granular filaments. They have different diameters, 15 to 17nm and 22 to 25nm respectively, and differ also in their distribution, with the smooth filaments appearing both in nuclear and cytoplasmic aggregates, and the granular filaments being confined to the cytoplasm.

The present results concerning MDBK_{pi} cell cultures closely resemble those reported in SSPE and measles infections, with the aged cultures showing large intranuclear aggregates always consisting of smooth nucleocapsids. These findings are in agreement with those of Raine et al. (1971), where aggregation of viral nucleocapsids of smooth characteristics only occurred after 14 days, during a long-term measles infection of a nervous cell culture system obtained from hamsters.

Dubois-Dalcq and Barnosa (1973) using peroxidase-labelled antibody (PLA) in Vero cells infected with SSPE virus, observed that only granular nucleocapsid filaments were labelled and not the filaments of the smooth type. They suggested that the antigenic nature of the granular nucleocapsids in the cytoplasm may be different from that of the smooth nucleocapsids. These findings were later confirmed by Dubois-Dalcq et al. (1974) in a comparative study of productive and latent SSPE viral infections. These workers suggested that the abnormal maturation observed is probably related to a deficiency in synthesis or interaction of one viral protein associated with the sheath of material covering the granular nucleocapsids. In consequence, the smooth type of nucleocapsid is incapable of alignment under the plasma membrane and interacting with it to induce budding.

The PLA experiments both by direct methods and in blocking assays, conducted in the present study, confirmed that the granular nucleocapsids appearing in the cytoplasm or aligned in the plasma

membrane were specific for the peroxidase-labelled anti-NDV globulin prepared in rabbits. However, the labelled preparations showing smooth nucleocapsids seem to indicate that they were not antigenically identical. These findings, if confirmed, seem to contradict the results obtained with immunofluorescence techniques in the same cells, where intranuclear material is specifically labelled by the fluorescing antibody. It should be pointed out, however, that the results refer to PLA experiments using glutaraldehyde fixation, as attempts to fix MDBK_{pi} cells with formaldehyde were unsuccessful. The use of the latter fixative is regarded as a better method of labelling intracellular components (Dubois-Dalcq and Barbosa, 1973).

The mechanisms responsible for the maintenance of the persistency of NDV in MDBK cells, in both MDBK_{pi} and MDBK_{cs}, were shown to be different. In MDBK_{pi} cell cultures the defectiveness of the virus mutants produced, being non-infectious, probably accounts for the perpetuation of the infection. It should be noted that according to the hypothesis put forward by Preble and Youngner (1975), in the establishment and maintenance of persistent infections of the regulated type, one of the first alterations occurring on the carried virus is the acquisition of the "temperature-sensitive" phenotype. The agent infecting MDBK_{pi} cells was confirmed to be temperature-sensitive at 41°C, and that more virus was released from cells at 31°C than at 37°C (Fraser et al., 1976). However, further investigations should be carried out at temperatures lower than 37°C, in order to find out where the permissible temperature is and if, at that temperature, the synthesis of viral polypeptides becomes normal.

On the other hand, MDBK_{cs} cell cultures yield small amounts of highly infectious viral progeny which is capable of undergoing productive infections either in the same or in other cell systems.

The regulation of this type of persistent infections is ascribed to the presence and function of DI particles (Huang and Baltimore, 1970). Many reports suggest that the synthesis of DI particles has been implicated in the maintenance of persistent infections namely, measles/HeLa cells (Rustigian, 1966) measles/Lu 106 cells (Norrby, 1967), Western equine encephalitis virus persistency in cultures of L cells (Chambers, 1957), BHK-21/13S cells persistently infected with Parana virus (Staneck and Pfau, 1974), and also in L cells persistently infected with NDV (Rodriguez et al., 1967). It has also been demonstrated that DI particles are essential for maintaining a BHK-21 cell culture persistently infected with vesicular stomatitis virus (Holland and Villarreal, 1974). However, more recently ter Meulen and Martin (1976) observed that Vero cells persistently infected with canine distemper virus produced and released into the medium a labile component which had strong inhibitory activity against the replication of CDV and measles virus. These workers proposed that the regulation of the persistence in their system may be due to this virus-induced regulatory protein, rather than related to DI particles (which are pelletable and ultraviolet-sensitive) or to interferon (which protect the cells also against unrelated viruses).

As shown in the Results (Section 2-5), we have detected in MDBK_{cs} cell cultures a viral inhibitory factor (VIF) which, as in the case of the CDV/Vero cells system, was not sedimented by prolonged ultracentrifugation of the culture fluids, protected indicator cell monolayers against infection by NDV but not related or unrelated viruses, and was blocked by NDV antiserum. However, the inhibitory effect present in MDBK_{cs} which was studied throughout the experiments 2-3 days after viral challenge, could be demonstrated only when indicator cell cultures were treated before infection, and was stable

for at least 8 days at 4°C, but destroyed by heating for 2 hours at 56°C. These findings were apparently in disagreement with those found by ter Meulen and Martin. They reported that the inhibitory effect was demonstrable even when the indicator cultures were treated within one hour after challenge. It should be noted, however, that the results described by those workers were obtained within a period of 24 hours after infection.

The production of the inhibitory factor in MDBK_{cs} cell cultures was already detectable in the fluids within 2 days of refeeding, but no correlation was found between the interval of culture medium changes and the amount of VIF observed. Similarly, the age of the culture did not reveal any effect on VIF production. These results suggested that the inhibitory factor was either continuously produced independently of age or physiological state of the culture, or the indicator system used was not sensitive enough to detect variations in the VIF production. Furthermore, it was noticed that frequent changes of the maintenance medium increased the proportion of infected cells in the carrier cultures, although no accurate determinations were obtained.

Generally, no interferon was detected in MDBK_{cs} cell cultures, although in one experiment an effect attributable to interferon was observed. However, MDBK_{pi} cell cultures, which never revealed any VIF effect, showed similar activity. This seems to indicate that there is no relation between VIF and interferon.

The fact that the viral inhibitory factor was only found in MDBK_{cs}, and never detected in MDBK_{pi} cell cultures, confirmed the suggestion that different mechanisms are involved in the maintenance of the two persistent infections. In the case of MDBK_{cs}, which bears close relation with the CDV/Vero cell system, it seemed likely

that it is a virus-induced product which is responsible for the stability of the infection. This protein could be the result of the participation of subgenomic material derived from DI particles in protein synthesis (ter Meulen and Martin, 1976).

Recently it has been suggested that integration of RNA virus genomes into the DNA of persistently infected cells plays a role in persistent infection (Zhdanov, 1975). Attempts to explain the mechanisms of DNA provirus formation in cells chronically infected with RNA viruses comprise the interaction between infectious and oncogenic RNA viruses (Zhdanov, 1975), the mediation of cellular RNA-dependent DNA polymerases (Simpson and Iinuma, 1975), and the demonstration of reverse transcriptase in persistently infected cells (Furman and Hallum, 1973).

The recovery of infectious proviral DNA from mammalian cells persistently infected with respiratory syncytial virus (Simpson and Iinuma, 1975), and the observation of an RNA-dependent DNA polymerase in L cells persistently infected with NDV (Furman and Hallum, 1973), seem to indicate that integration of RNA viral genomes into cellular chromosomes of MDBK persistent infection is likely to occur.

Due to lack of facilities necessary to investigate the complementarity of the viral RNA with cellular DNA from the persistently infected cells, using hybridization techniques, our studies were concerned mainly with attempts to enhance the release of infectious virus by treatment of the cell cultures with DNA analogues (Aaronson et al., 1971; Klement et al., 1971; Lowy et al., 1971; Stewart et al., 1972), and also attempts to transfect isolated DNA provirus information into susceptible recipient cell cultures (Hill and Hillova, 1971; Cooper and Temin, 1974; Svoboda et al., 1974; Simpson and Iinuma, 1975; Haase et al., 1976).

In the present work both MDBK_{cs} and MDBK_{pi} cell cultures showed an increased yield of released virus when treated with iododeoxyuridine (IdU), noticeable up to 8 days after treatment. The fact that both systems react in a similar manner seems to confirm that some kind of common cellular control is present in MDBK cells infected with NDV. However, these findings per se did not explain whether viral expression was dependent on cellular DNA or if the excess of released virus was due to the expression of integrated viral information. The fact that integration of viral genomes in cellular DNA is not exclusive to persistent infections was demonstrated in primary infections of RSV in BEK and Hep-w cells by Simpson and Iinuma (1975). In this study no investigations were conducted on infectivity of virus particles present in the enhanced yields from MDBK_{pi} cells. It is interesting to note that in a BGM cell culture persistently infected with measles virus, which is believed to be caused by a block at an early stage of the virus replication cycle, it was demonstrated that no enhancement of the haemadsorbing activity or release of the infectious virus was obtained after treatment with bromodeoxyuridine (Menna et al., 1975a).

The failure to transfect the presumably integrated viral information from MDBK cells persistently infected with NDV, during the period of the experiment, may be due to the techniques used rather than confirmation of the absence of infectious proviral DNA in MDBK persistently infected cells. It should be pointed out, however, that Holland et al. (1976) studying two carrier states of BHK-21 cells persistently infected with vesicular stomatitis or rabies viruses, observed no integration of the viral genomes into the cellular chromosome. On the other hand, the persistence of NDV mutants in MDBK_{pi} cells is difficult to understand without the integration of viral information into the cellular chromosome. This is the case with aged MDBK_{pi} cell cultures which under certain

circumstances showed a large majority of the cells with no viral antigen (Plate 25), but regained the usual characteristics 3 to 5 days after propagation. As most, if not all, of the virus released by the cells is non-infectious, it is unlikely that they became infected by released particles. However, hybridization studies using NDV RNA and the DNA of the persistently infected cells would be necessary to confirm this hypothesis. Also, the search for a RNA-dependent DNA polymerase associated with the persisting mutant would be a useful approach for further understanding of the mechanisms involved in maintaining viral persistence in MDBK cells.

The present results also showed the potential capacity of NDV to adapt and form an endosymbiotic relationship with mammalian cells. Besides the artificial conditions of the in vitro model, similar situations might well occur in vivo, thereby explaining sudden outbreaks of Newcastle disease.

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