STUDIES ON DEFECTIVE STRAINS OF NEWCASTLE DISEASE VIRUS

by

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VOLUME II

RESULTS

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THE CHARACTERISTICS OF VIRUS RELEASED FROM PERSISTENTLY INFECTED CELLS

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THE CHARACTERISTICS OF VIRUS RELEASED FROM PERSISTENTLY INFECTED CELL-LINES COMPARED WITH NDV GROWN IN CELLS OR EMBRYONATED EGGS

A / INTRODUCTION

In this Section, the properties of wild-type NDV grown in cell culture and in fertile hens' eggs are compared with those of the virus released from persistently infected monolayers of BK pi, PK pi and OK pi cells.

A number of workers have compared the properties of wild-type strains of paramyxoviruses with those of virus released from cells persistently infected with paramyxoviruses, and their results have been described in Section I(c) of the Introduction. While differences in immunology and morphology have not been found, there is evidence that infectivity (56), haemolysin activity (114), haemagglutinin temperature stability (135) and the ability to cause cell fusion (139) are all frequently reduced in virus from carrier cell cultures. It has also been shown that the characteristics of a paramyxovirus are not only related to its virulence but may also be influenced by the type and species of cell or tissue in which it has been propagated (49, 56, 172).

In view of these reports, the virus released from

from carrier cultures of BK pi, OK pi and PK pi cells was compared with a number of lentogenic and velogenic strains of NDV grown in embryonated hens' eggs and cell cultures with particular reference to morphology, immunology, infectivity and other biological activities.

B / MORPHOLOGY

Introduction

There is general agreement that virions of NDV are highly pleomorphic when viewed under the electron microscope (12) and that morphological differences cannot be detected between strains of the virus (151).

Compared with laboratory or field strains of the virus there are, unfortunately, very few detailed descriptions in the literature of the ultrastructure of paramyxoviruses released from persistently infected cell cultures. Although abnormal nucleocapsid material occurs in L cells persistently infected with Sendai virus (61), morphological differences have not been shown in virus released from persistently infected cells (118,141).

The morphology of virions released from BK pi, OK pi and PK pi carrier cells.

In this present work, the morphology of virus particles released from monolayers of BK pi, OK pi and PK pi were virtually indistinguishable from those of a number of strains of NDV grown in allantoic fluids of nine-day-old fertile hens' eggs. In negatively-stained preparations, virions released from BK pi cells closely resembled those of the B 1 strain of NDV grown in BHK cells, in that they possessed a fringe of spikes on the outer envelope and an ill-defined inner structure (17, 18). The lack of definition of the herring-bone

herring-bone patterns in the latter electron-micrographs is due to incomplete penetration of stain through the intact envelope. The presence of nucleocapsid material in budding virus particles is clearly shown in ultrathin sections of BHK cells infected with the B1 strain of NDV (Fig. 16) as well as in persistently infected BK pi cells (Fig. 15).

C / IMMUNOLOGY

Because differences occur between strains of NDV in regard to virulence, haemolysin and haemagglutinin activities,it is possible that serological differences will also exist. It is generally agreed, however, that antibody to one strain of NDV is equally effective against other strains of NDV in its ability to inhibit haemagglutination or neuraminidase activity, to fix complement or to neutralise the cytopathic effect of infectious virus (109). On the other hand, recent work by Apostolov and Watereon (172) has shown that, in addition to the haemolytic effect which occurs when red blood cells are incubated in the presence of lentogenic or virulent strains of NDV, lysis is enhanced in the presence of complement and antibody. However this effect is only seen when lentogenic strains are employed and occurs irrespective of whether the antiserum has been prepared against:

i) the strain of NDV that is utilised,

ii) the species of erythrocyte to be haemolysed, or

iii) the specific host tissue in which the virus was grown.

Enhanced haemolysis is believed to be associated with a host-specific hapten, which is incorporated into the membrane of a lentogenic strain but not of a virulent strain of NDV. Thus, this effect cannot be said to represent a true serological distinction between strains.

In the present work, antisera prepared against two

two strains of NDV and the virus produced from PK pi monolayers, have been used in an attempt to differentiate between wild-type virus and the virus released from persistently infected monolayers.

I) HAEMAGGLUTINATION INHIBITION WITH ANTISERUM TO NDV (HI)

The results in Table XIII show that antisera to two virulent strains of NDV (Herts and Lurgan) showed the same degree of haemagglutinin inhibition (HI) when tested against either velogenic or lentogenic strains of NDV. In contrast, the HI titres obtained when these antisera were tested against virus released from carrier cultures (BK pi, OK pi and PK pi) were considerably less, although the differences were less marked with the Herts antiserum than with the Lurgan antiserum.

II) HAEMAGGLUTINATION INHIBITION WITH ANTISERUM TO VIRUS RELEASED FROM PK pi CARRIER CELL CULTURES.

When antiserum to the PK pi strain of virus was employed, there was no appreciable difference in its HI effect on five of the six wild-type strains of NDV nor on two of the three strains of persistent virus. However, the remaining strains (Lurgan and OK pi) both show a reduced sensitivity to PK pi antiserum (Table XIII). It is emphasised that this latter result does not mean that the Lurgan and OK pi strains are closely related to each other but rather suggests that they show less affinity with PK pi virus than do other strains of NDV.

Discussion

TABLE XIII

A COMPARISON OF SEVERAL STRAINS OF NDV, IN REGARD TO THE INHIBITION OF THEIR HAEMAGGLUTININ BY ANTISERA TO THREE STRAINS OF NDV.

STRAIN	PATHOGENICITY OF STRAIN	HIGHEST TITRE TO WHICH THERE WAS INHIBITION EMPLOYING:		
		ANTISERUM PREPARED IN RABBITS AGAINST		ANTISERUM PREPARED IN CHICKENS AGAINST VIRUS RELEASED FROM PK pi CELLS
		LURGAN STRAIN	HERTS STRAIN	
Italien	Virulent	256	512	128
Herts	Virulent	512	512	256
Lurgan	Virulent	512	512	64
B 1	Avirulent	512	512	512
Ulster	Avirulent	128	256	128
Asplin F	Avirulent	128	256	512
PK pi*	N.A.	16	128	256
BK pi*	N.A.	16	128	256
OK pi*	N.A.	32	64	32

*The virus released into the supernatant fluids of cultures of the respective carrier cell-lines.

Discussion

The evidence obtained from this experiment suggests that the virus produced by persistently infected cell-lines BK pi, OK pi and PK pi, has a more distant relationship with the wild-type strains than is known to exist between the wild-type strains themselves. Indeed, it appears that the virus released from the OK pi cells does not bear a close resemblance to that released from the other two carrier cell-lines.

Since the haemagglutinin of the persistent viruses is apparently defective (Table XXIII), it is possible the abnormality of this protein may cause the decreased HI titres observed with these strains. On the other hand, because the haemagglutinin activity per µg protein is reduced (Table XXIII), four HAU of the persistent virus will contain more antigen and thus might neutralise the antiserum at a lower titre. However, this second hypothesis is probably untenable in view of the high HI titres recorded against BK pi and PK pi viruses with the antiserum prepared against PK pi virus. It seems that the persistently infected cells release viruses that are antigenically distinct from wild-type strains of NDV and an attempt was made to clarify this situation by means of the complement fixation test (CFT). Unfortunately the results of CFTs proved inconclusive due partly to the low titres produced (1:32, employing Herts antiserum against the optimum dilution of homologous antigen) and partly to the paucity of viral material available from the persistently infected cells.

III ANTISERUM TO THE ENVELOPE PROTEINS OF NDV

Antibody that had been prepared in rabbits to the envelope proteins of the Herts strain of NDV, inhibited the haemagglutinin of this virus to a titre of 1 : 128. In immunofluorescent studies, areas of brilliant fluorescence were obtained in HeLa cells infected with Herts virus, both with antiserum prepared against whole Herts virus, and with the 'envelope' antiserum, although the pattern of fluorescence was different in the case of the latter antiserum. However, when BK pi cells were stained with the 'envelope antiserum, the fluorescence was much fainter (Fig. 10) than when the antiserum to whole NDV was employed (Fig. 5).

Discussion

These observations confirm the results of other workers (29) and imply that immunofluorescence due to envelope proteins is distinct from that due to other viral components. The decreased sensitivity of the 'envelope' proteins synthesised in BK pi cells to specific antiserum supports the results of the HI tests (vide supra) and suggests that either the formation or the antigenicity of one or more of the envelope proteins of NDV is defective in BK pi carrier cell cultures.

D / INFECTIVITY

Introduction.

The infectivity of the virus in its natural host is of major importance in any consideration of the pathogenesis of a virus infection. In the case of NDV, the virulence of an infection in chickens corresponds to the infectivity and cytopathogenic effect (CPE) of the same strain in chicken cell culture (106). However, other characteristics of NDV can vary from strain to strain but do not necessarily affect the pathogenicity of the virus in chicken tissue (111). It is clear, therefore, that infectivity and virulence either depend on a single viral characteristic, the absence of which causes the strain to be avirulent, or require a combination of properties that is cytocidal.

In the following Section, an attempt has been made to assess the virulence of the virus released from the persistently infected cell-lines, by comparing their infectivity in the natural host with that of other strains of NDV, and in succeeding Sections the relationship of virulence to other properties of NDV will be considered. In addition, two further problems are investigated in this present Section:

i) the susceptibility of three mammalian cell-lines to NDV,

ii) the effect on the infectivity of a strain of NDV of passage through mammalian cells.

I) VIRULENCE OF STRAINS OF NDV IN VARIOUS CELL-LINES

Introduction

It has been recognised for many years that velogenic field strains of NDV are also the most pathogenic for chicken embryos and have the highest intracerebral pathogenicity index in one-day-old chicks. Moreover, an aliquot of virulent virus possesses a greater degree of infectivity for chick embryo fibroblasts than that of an avirulent strain of NDV (106).

However, other workers have found that there is little relationship between the virulence of a strain of NDV for chicken tissues and either its CPE or infectivity in cell cultures derived from mammalian sources (91).

a) The infectivity of wild-type strains of NDV in mammalian cell lines and chick fibroblasts.

This aspect of virulence has been examined in the present work and the results are shown in Table XIV. It is evident from Column 1, that the infectivity per HAU in CF of the lentogenic strains (B1, Asplin F and Ulster) is less than that of the virulent strains (Lurgan, Herts and Italien). These results were confirmed employing purified virus with one lentogenic (B1) and one velogenic (Herts) strain of NDV. However, due in part to the greater sensitivity of the MTCP method and in part to the increased purity of the virus, the absolute infectivity per HAU of the two strains was markedly increased (Table XVI).

T A B L E XIV

THE INFECTIVITY OF SIX STRAINS OF NDV IN CHICK EMBRYO FIBROBLASTS AND THREE MAMMALIAN CELL-LINES

STRAIN OF NDV	INFECTIVITY ($\log_{10}TCID_{50}$/HAU) IN CELL-LINES			
	CF	MDBK	PK(W)K6	HeLa
B 1	2.96	$\bar{1}$.96	$\bar{1}$.96	1.96
F	3.89	$\bar{3}$.89	1.89	3.89
Ulster	4.0	1.14	1.0	3.0
Lurgan	5.89	3.89	3.89	5.77
Herts	7.42	$\bar{1}$.53	2.42	3.42
Italien	7.18	8.18	5.18	5.30

All six strains of NDV were grown in the allantoic cavity of ten-day-old embryonated chicken eggs. Haemadsorption was used as an indication of infection. Infectivity was assayed in one-day-old monolayers of the cell-lines grown in 6" x $\frac{5}{8}$" tubes.

(Table XVI).

It should be noted that, although the presence or absence of haemadsorption was taken as indicating infection of a monolayer by a particular dilution of the virus, the strains would have been placed in the same order of virulence had the denominator chosen been that of CPE or the release of HA from infected monolayers. Haemadsorption was chosen as the method of assay because of its greater sensitivity and accuracy.

It is also of interest that strains that were virulent in chicken cells were generally of higher infectivity for mammalian cell-lines (Table XIV, Columns 2,3 & 4). However the virulent Herts strain was exceptional and had a lower infectivity index in MDBK cells than that of the avirulent Ulster strain. Thus, a strain that is highly pathogenic in chick cells is not necessarily so in mammalian monolayers; while the reverse may be true of an avirulent strain.

An additional feature, not shown in Table XIV, was the large number of red blood cells that were adsorbed to each fibroblast in CF cultures infected with a virulent strain of NDV. In contrast to this, when monolayers were infected with a large amount of a lentogenic strain, so that every cell haemadsorbed, the number of erythrocytes per fibroblast was appreciably less.

In general, chicken fibroblasts were the cell culture most susceptible to any of the strains of NDV

NDV examined, followed by the cell-lines, HeLa, PK(W)K6 and MDBK, in that order (Table XIV). Moreover, the number of erythrocytes adsorbed to each culture cell and the fusion index of an infected monolayer were greatest in CF cultures and least in MDBK monolayers, even when the majority of cells were shown to be infected.

Discussion

The results in this Section confirm that the strains of NDV which cause velogenic infections in chickens, produce higher infectivity titres and greater CPE in chicken fibroblast cultures than lentogenic strains. It may also be concluded that, with certain exceptions, the infectivity of a strain of NDV in mammalian cell-lines is determined both by its virulence in chicken tissue and by the nature of the mammalian cell-line to be infected. Thus these results support the hypothesis that the characteristics of the cell membrane influence the type of CPE that is produced by an infecting virus (74).

b) The infectivity of virus released from mammalian cells.

Since Apostolov and Waterson (172) have recently shown that cellular antigens are incorporated into the membranes of virions released from cells infected with lentogenic strains of NDV, it might be expected that the properties of mature virions would vary with the species of cell in which they were produced. For this reason, the infectivity for CF of NDV released from

from several types of cell was studied in the present work (Tables XIV, XV, XVI, XVII, XVIII). A comparison of the results in Column 1 of Table XIV with those in Table XV, shows that progeny virus released from mammalian cells, infected with persistent or wild-type strains of NDV, was always less infectious for CF cultures than NDV grown in fertile hens' eggs. Thus, the infectivity of the B1 strain of NDV, released from HeLa cells, was much lower (1.17 $\log_{10}$ times) than that of the same strain of virus grown in embryonated hens' eggs and the infectivity of virus, released from persistently infected BK pi cells, was very much less than that of the BK pi virus which had been passed through chicken tissue (Table XVI). Moreover, the amount of progeny virus and haemagglutinin released from MDBK and PK(W)K6 cells infected with the Herts strain of NDV was less than that obtained from HeLa or CF cells (Table XXIV).

On the other hand, the type of cell or tissue in which the infecting virus had been propagated, apparently did not alter the capacity of the virus to grow better in one cell-line rather than another. Thus, the Herts strain of NDV was more infectious to CF than to HeLa, PK(W)K6 or MDBK cultures. Nor were the respective amounts of haemagglutinin released from these cell-lines affected by the type of cell or tissue in which the infecting virus had been propagated.

Summary

Although growth in certain cell-lines may limit the

T A B L E XV

THE INFECTIVITY IN CHICK EMBRYO FIBROBLASTS OF VIRUS RELEASED FROM CELL CULTURES

STRAIN OF NDV	GROWN IN (CELL-LINE)	INFECTIVITY ($\log_{10}TCID^{50}$/HAU) IN CF
B 1	HeLa (sixth pass)	1.79
PK pi	PK pi	0.90
BK pi	BK pi	$\bar{1}$.79
OK pi	OK pi	$\bar{1}$.08
PK pi	HeLa (third pass)	1.32
BK pi	HeLa (third pass)	1.15

Virus grown in the cell-lines indicated, was harvested from the supernatant of such monolayers. Generally, the virus was concentrated before assaying its infectivity in one-day-old monolayers of CF, grown in 6" x $\frac{5}{8}$" tubes. Haemadsorption was employed as an indication of infection.

T A B L E XVI

INFECTIVITY OF COMPLETELY PURIFIED STRAINS OF NDV

STRAIN OF NDV	INFECTIVITY $Log_{10}TCID_{50}$ /µg PROTEIN	$Log_{10}TCID$ /HAU
B 1**	12.15	10.97
Herts**	17.13	15.86
BK pi** (2nd pass)	16.58*	15.76
BK pi ***	4.45	5.18

*When the $TCID_{50}$ is measured in MDBK cells 12 logs diminuition in infectivity is found.

**Virus purified from material grown in the allantoic cavity of ten-day-old embryonated chicken eggs.

***Virus purified from material found in the supernatant fluids of persistently infected cells.

The $TCID_{50}$ was measured, using the haemadsorption endpoint in one-day-old monolayers of chick embryo fibroblasts grown in MTCPs.

T A B L E XVII

INFECTIVITY OF VIRUS RELEASED FROM PERSISTENTLY INFECTED CELLS.

TIME AFTER SEEDING (DAYS)	CELL LINE	INFECTIVITY $Log_{10}TCID_{50}$/ CELL	INFECTIVITY $Log_{10}TCID_{50}$/ HAU
1*	PK pi	$\bar{4}$.47	N.D.
	BK pi	$\bar{5}$.55	N.D.
	OK pi	$\bar{4}$.04	N.D.
3*	PK pi	$\bar{4}$.45	1.20
	BK pi	$\bar{4}$.0	0.52
	OK pi	$\bar{5}$.70	$\bar{1}$.90
7*	PK pi	$\bar{4}$.47	0.90
	BK pi	$\bar{5}$.32	0.00
	OK pi	$\bar{5}$.70	$\bar{1}$.90

*The cells were refed with MEM plus 5% calf serum, 24 hours before assay.

N.D. not done.

T A B L E XVIII

INFECTIVITY OF VIRUS RELEASED FROM PERSISTENTLY INFECTED CELLS

TIME AFTER SEEDING (DAYS)	CELL LINE	INFECTIVITY $Log_{10}TCID_{50}$/CELL
1*	PK pi	$\overline{4}$.36
	BK pi	$\overline{5}$.82
	OK pi	$\overline{5}$.67
3*	PK pi	$\overline{4}$.46
	BK pi	$\overline{4}$.26
	OK pi	N.D.
7*	PK pi	$\overline{4}$.38
	BK pi	$\overline{5}$.90
	OK pi	$\overline{5}$.48

*The cells were not refed after seeding.

N.D. not done.

Note: Insufficient haemagglutinin was released to allow calculation of $log_{10}TCID_{50}$/HAU.

the amount and the degree of infectivity of the progeny virus produced, infectivity is restored readily by passing the virus through a permissive cell system. Nevertheless, the capacity of the virus to grow better in one cell-line than others is apparently not affected by passage.

II) STUDIES ON THE INFECTIVITY OF THE VIRUS RELEASED FROM THE CARRIER CELL-LINES.

Introduction

In addition to the moderating influence of the cell species in persistent NDV infections, it is possible that some form of adaptation of the virus to the cell plays a role in the initiation and maintenance of the carrier state. Thus it is reasonable to suppose that mutants may be selected which are capable of replicating in the abnormal environment of the persistently infected cells. It has been reported that mutation to a temperature-sensitive form is apparently essential for the establishment of persistence in L cells infected with NDV (124) and there is recent evidence (45) that the acquisition of an RNA-dependent-DNA-polymerase which is not present in normal 'wild-type' NDV, is another contributory factor. Unlike the largely abortive infection of L cells with wild-type NDV (49), a productive cycle of infection occurs in persistently infected cells and although the mutant virus is more infectious to L cells (135), it is less infectious to chicken tissue (120) than the wild-type virus. Therefore,

Therefore, it is probable that the abortive cycle of infection with the wild-type virus is due to the influence of the cell, whereas the persistent cycle of infection is due to mutation of the virus.

Thus, when examining the three carrier cell-lines described in the present work, consideration must be given as to whether a particular characteristic of the released virus is due to the nature of the host cell or is caused by adaptation of the virus to the process of persistence.

a) The effect of the metabolism of the carrier cell on the release of infectious virus.

The results are given in Tables XVII and XVIII of experiments carried out to ascertain the influence of cellular metabolic rate on the release of infectious virus from the carrier cell-lines, BK pi, OK pi and PK pi.

The amount of infectious virus released from all three cell-lines was almost constant, whether measured on the first, third or seventh days after seeding. Because cellular multiplication and hence the metabolic rate slows as the monolayer ages (Table I), the above result implies that the release of virus is largely independent of cellular synthetic processes. A similar conclusion is drawn from the experiments described in Section 3 (I) and (II), although the latter results make it clear that virus released may increase even with the lowering of the metabolic rate of the cell.

b) The temperature-stability of the virus released from the carrier cell-lines.

There is little difference between the amount of infectious virus from cells fed the day before assay and the yield of virus from cultures which had not been refed since seeding of the monolayers (Tables XVII, XVIII). Hence it may be deduced that the stability of virus infectivity is less than 24 hours, at 37°C.

c) Comparison of the infectivity of the virus released from the carrier cell-lines, OK pi, BK pi and PK pi.

The results in Tables XV, XVII and XVIII, show that PK pi virus is between three and ten times more infectious than that liberated from OK pi and BK pi monolayers. Generally a larger amount of infectious virus is present in the supernatant fluids of BK pi cells than in those of OK pi cells. The infectivity titres of all three strains of virus were generally too low to allow them to be compared in any cell-line other than CF.

It is notable that three different values were obtained for the infectivity in chick fibroblasts per HAU of virus released from BK pi cells. When infectivity was assayed in 6"x $\frac{5}{8}$" tubes, the $\log_{10}TCID_{50}$/HAU was $\bar{1}.79$ (Table XV) and infectivity was very rarely detected without concentrating the supernatant fluids. However, when microtitre plates (MTCP) were used, infectious virus could usually be detected without concentrating the released virus, and the $\log_{10}TCID_{50}$/

HAU fluctuated between 0.52 and 0.00 (Table XVII).

Therefore, it is probable that virus overlaid on monolayers grown in MTCPs is more liable to infect a cell, and the greater sensitivity of this method may be due to the increased proximity of virus to cell in the smaller volumes used.

On the other hand, following purification of the virus released from BK pi cells, the $\log_{10}TCID_{50}$/HAU increased to 5.18 when assayed in MTCPs.(Table XVI). In this case the rise in titre is probably due to the removal during purification, of non-infectious virus fragments, containing haemagglutinin. It is emphasised that these two effects are not confined to virus released from the carrier cells, since an apparent increase in infectivity occurs with the Bl and Herts strains of NDV. For example, the $\log_{10}TCID_{50}$/HAU of the unpurified Herts strain when tested in chick fibroblasts, grown in tubes, is 7.42 (Table XIV). However, when assayed in MTCPs the purified virus gave a titre of 15.86 (Table XVI).

In addition, it is notable that infectivity measured as plaque forming units (PFU) per ml varied with the procedure used:-

i) the method was more sensitive when methyl cellulose was employed in the overlay medium in place of agar (Table XX).

ii) a greater number of PFU per $TCID_{50}$ were obtained with Italien virus grown in HeLa cells than with Herts virus grown in chicken embryo fibroblasts (Table XX). This

This is probably related to the cell-type rather than to the strain of virus employed.

In view of the significant differences in the results obtained, from these preliminary experiments, it was decided that in the present work, infectivity was generally to be measured in terms of $\log_{10}TCID_{50}$.

d) <u>The infectivity in five-week-old chicks of the virus released from the carrier cell-lines</u>.

It has been suggested (vide supra) that the virus produced from the persistently infected cultures is a mutant with reduced virulence for chicken tissue. If this were so, it is reasonable to suppose that the virus would be capable of stimulating antibody production without causing pathological changes in the host.

In an experiment to test the above hypothesis, the following three strains of NDV were used:

i) Bl strain, passed six times in HeLa cells (Bl/HeLa virus)

ii) virus released from the carrier cells, BK pi (BK pi virus)

iii) virus released from the carrier cells, PK pi (PK pi virus)

The viruses were concentrated to an equivalent titre as measured by their phosphodiesterase activity.

~~Although commercial vaccines generally contain not more than $10^2 EID_{50}$ of the Bl strain of NDV grown in fertile hens' eggs, pathological effects were produced with inocula containing less than 10^1 EID_{50} of the~~

The majority of birds inoculated with the Bl/HeLa and BK pi viruses died, but the PK pi strain appeared to be less virulent. Birds kept in contact with the inoculated chickens also became infected and produced antibody. These results suggest that the virulence of the virus released from the persistently infected cells had been restored during passage and that in the case of BK pi virus, the mutation to an avirulent form during twelve years of persistence, is unstable. However, as indicated in Table XIX, the reversion to virulence is slower in the case of PK pi virus, and lower doses of this strain might be suitable for use as a vaccine. This possibility is supported by the greater pathogenicity of the Bl vaccine strain of NDV, at the dose level employed. Indeed the fact that Bl is a lentogenic strain of NDV and normally does not kill chickens, suggests that either the material became contaminated with a velogenic strain of NDV before inoculation or else that passage in HeLa cells alters the character of the Bl strain of NDV. The virulence of BK pi virus following passage through embryonated chicken eggs (Table XVI) supports the supposition that this strain would not be a suitable source of vaccine.

Discussion

These conclusions are surprising in view of the greater infectivity titres recorded for virus released

T A B L E XIX

INFECTIVITY AND PATHOGENICITY FOR SIX-WEEK-OLD CHICKENS OF B1 STRAIN OF NDV (PASSAGED IN HeLa CELLS) AND VIRUS RELEASED FROM PERSISTENTLY INFECTED MONOLAYERS.

TIME AFTER INOCULATION (DAYS)	STRAIN OF VIRUS	ROUTE OF INOCULATION	PATHOLOGICAL EFFECT	HIT	STATE OF CONTROL BIRDS
2	B1/HeLa	IM	none	N.D.	Healthy
		ICa	four out of six dead/ proventricular haemorrhagic lesions	N.D.	
	BK pi	IM	nasal discharge	N.D.	
		ICa	five out of six dead/ proventricular haemorrhagic lesions	N.D.	
	PK pi	IM	healthy	N.D.	Healthy
		ICa	healthy	N.D.	
4	B1/HeLa	IM	all birds dead	none	Apparently healthy
		ICa	all birds dead	none	
	BK pi	IM	five out of six dead/ typical P.M. signs of NDV infection	none	
		ICa	all birds dead		
	PK pi	IM	healthy	none	Healthy
		ICa	healthy	none	
7	BK pi	IM	survivor healthy	4	
	B1/HeLa	'In contact'	healthy	4	Healthy
	*PK pi	IM	one out of six with nasal discharge	128 (ave)	Healthy HIT none
		ICa	all healthy	128 (ave)	
13	BK pi	IM	survivor healthy	64	
	B1/HeLa	'In contact'	dead/signs on P.M. typical of NDV		Dead
	*PK pi	IM	four out of six alive and healthy	64 (ave)	Died PM signs of NDV
		ICa	all alive and healthy	32 (ave)	HIT 16

IM - Intramuscular ICa - Intracardiac N.D. - not done
ave - average HIT - haemagglutination inhibition test

*the dead birds were those with the lowest (none) and highest (1028) HITs.

T A B L E XX

PLAQUE FORMATION AND ITS RELATIONSHIP TO $TCID_{50}$

STRAIN of NDV	NATURE OF OVERLAY MEDIUM	CELL-LINE EMPLOYED	PFU (log_{10}) per ml	PFU (log_{10}) per HAU*	PFU (log_{10}) per $TCID_{50}$** (log_{10})
Italien	1% agar	HeLa	10.30	7.99	2.69
Italien	1.5% methyl cellulose	HeLa	13.00	9.68	4.38
Herts	1.5% methyl cellulose	CF	12.08	8.98	1.56

PFU Plaque forming unit.

Both the Italien and the Herts virus were grown in 10-day-old embryonated hens' eggs. Plaque formation was assessed by the presence of haemadsorption. The $TCID_{50}$ with which PFU is compared is that obtained by infection of monolayers grown in 6" x $\frac{5}{8}$" tubes (TABLE XIV).

*PFU log_{10} per HAU of the infecting virus.

**PFU log_{10} per log_{10} $TCID_{50}$ of the infecting virus.

released from PK pi monolayers before passage (Tables XV, XVII and XVIII). However, it may be surmised, that in the case of PK pi, the host cell is susceptible and the reduced virulence of this virus is due to a mutation, while the major factor, in the low infectivity titre of virus released from BK pi monolayers, is the non-permissive nature of the host cell in which it is grown.

This hypothesis is supported by a comparison of the control cell-lines for BK pi and PK pi (MDBK and PK(W)K6 respectively), which shows that healthy PK(W)K6 cells are generally more susceptible to NDV (Table XIV) and release higher amounts of haemagglutinin (Table XXIV).

E / BIOLOGICAL ACTIVITIES OF WILD-TYPE NDV AND VIRUS RELEASED FROM PERSISTENTLY INFECTED CELLS

I) INTRODUCTION

The biological activities of NDV have long been used to assay for the presence of this virus. However, it is only recently that these phenomena have been related to their function in the replication of NDV *in vivo*.

As related in the Introduction, the majority of these biological activities vary from strain to strain and some of them are closely associated with virulence. In addition, it has been found that, in certain persistent infections, some of these phenomenamay be defective and these deficiencies may be related to the induction of the carrier state (123, 11, 42). In other cases, an additional activity (RNA-dependent-DNA-polymerase) has been detected (45).

In the present work, particular attention has been paid to the biological activities associated with the large envelope glycoprotein (haemagglutinin and neuraminidase) which were suspected of being defective in the three carrier lines under study. In addition, the haemolysin and fusion activities were examined briefly because these have been found to be deficient in other persistent infections (11, 42). Unfortunately, within the confines of the present research programme it was not possible to study RNA polymerase, which has

has been shown to be abnormal in L cells persistently infected with NDV.

On the other hand, a preliminary study was made of phosphodiesterase, an enzyme not previously examined in NDV.

It was hoped by examination of these properties that a link could be established, between low infectivity and persistence on the one hand and defective biological activity on the other.

II) HAEMAGGLUTINATION

A detailed description of the function and properties of haemagglutinin is given in the Introduction. In summary:

Haemagglutinin (HA) is the larger of the two envelope glycoproteins of NDV, with a molecular weight of 75,000 daltons (21) and is contained within the spike-like structures seen on the surface of the virions. By means of its agglutinating reaction with neuraminic acid, haemagglutinin attaches the virus particle to the surface of host cells. When erythrocytes are caused to clump by this process, haemagglutination is said to have occurred. The degree of haemagglutinating activity varies from strain to strain but has not been related to virulence (87). However, when the temperature-sensitivity of HA is considered together with that of infectivity, it is found that virulent strains are always temperature-stable in both respects, but avirulent strains maybe thermolabile (35).

a) Temperature-sensitivity of haemagglutinins.

In the present work, the temperature-sensitivity of HA at 56°C was studied employing virus released from BK pi and PK pi monolayers together with egg-grown BK pi, Herts and B1 strains of NDV (Table XXI). The results show that:

i) BK pi and Herts virus have temperature-stable haemagglutinins, while those of PK pi and B1 are thermolabile,

ii) the stability of BK pi virus was unaltered by passage in chicken embryos, although the HA titre per µg protein increased dramatically (Table XXIII).

HA produced by OK pi monolayers is more stable than that associated with BK pi virus at 56°C (Table XXII). However at 37°C a four-fold diminuition of HA titre is obtained with BK pi virus after four days compared with only three days with OK pi virus.

Thus, the three persistent viruses differ in the stability of their HA. On this basis, only PK pi virus can be positively stated to be avirulent in the form in which it is released from the carrier cells. However, possession of a thermostable HA by OK pi and BK pi virus does not imply that they are virulent. Nevertheless, it is interesting in this context that the reversion of BK pi virus to a virulent form, following inoculation into five-week-old chickens Table XIX), is both more rapid and more complete than that of PK pi virus.

T A B L E XXI a

TEMPERATURE STABILITY OF THE HAEMAGGLUTININ OF PURIFIED VIRUS

TIME AT 56°C (MINUTES)	EGG-GROWN BK pi* VIRUS (HAU)	VIRUS RELEASED FROM MONOLAYERS OF BK pi (HAU)	PK pi (HAU)	HERTS STRAIN* OF NDV (HAU)	B1 STRAIN* OF NDV (HAU)
0	2048	32	1024	512	512
2.5	2048	32	512	512	16
5.0	1024	16	4	256	1
7.5	512	4	1	64	-
10.0	128	2	-	32	-
12.5	16	1	-	16	-
15.0	4	-	-	8	-
20.0	1	-	-	2	-

*Grown in 10-day-old fertile hens' eggs.

T A B L E XXI b

THE TIME TAKEN AT 56°C FOR DIMINUITION OF HAEMAGGLUTININ TITRE BY 16 TIMES

STRAIN OF VIRUS	TIME (MINUTES)
Egg-grown BK pi*	10
Cell-grown BK pi**	10
Cell-grown PK pi**	2.5 - 5
Egg-grown Herts*	10
Egg-grown Bl*	<2.5

*Grown in 10-day-old fertile hens' eggs.

**Released from monolayers of BK pi and PK pi carrier cell-lines respectively.

T A B L E XXII

TEMPERATURE STABILITY OF HAEMAGGLUTININ RELEASED FROM BK pi and OK pi MONOLAYERS

TEMPERATURE C^{o}	LENGTH OF TIME (hours) required for a four-fold diminuition in haemagglutinin titre of supernatant fluids from	
	OK pi cells	BK pi cells
56	1	<1
41	72	96
37	72	96
31	72	96
20	72	120
4	>144	>144

T A B L E XXIII

HAEMAGGLUTINATING UNITS PER µg PROTEIN OF VIRUS

STRAIN OF NDV	HAU per µg OF PURIFIED VIRUS	HAU per µg OF PELLETED VIRUS
B1*	15.0	0.72
Herts*	27.0	0.85
BK pi*	47.0	6.15
Ulster*	20.0	N.D.
PK pi*	N.D.	1.16
B1/HeLa**	1.15	0.11
BK pi****	0.18	0.015
BK pi/HeLa**	0.7	N.D.
PK pi****	1.12	0.10
PK pi/HeLa**	0.93	N.D.
Herts/CF***	8.2	N.D.

N.D. Not done

* Grown in 10-day-old fertile hens' eggs.

** Harvested from the supernatant fluids of infected HeLa cells.

*** Harvested from the supernatant fluids of infected chick fibroblasts.

**** Harvested from the supernatant fluids of cultures of the respective carrier cell-lines.

b) Haemagglutinin activity per µg protein of strains of NDV.

Just as infectivity of B1 strain of virus is decreased following passage in mammalian cells, so also is the HA per µg protein of egg-grown virus, higher than that of cell-grown virus (Table XXIII).

Further observations on the variations in HA per µg protein are as follows:

i) The activity of purified virus is approximately 20 times higher than that of pelleted virus (Table XXIII) whether the virus was obtained from fertile hens' eggs or from cell culture.

ii) The HA titre is higher for the virulent Herts strain than for the lentogenic Ulster and B1 strains (Table XXIII).

iii) Although an increase in activity of the order of 300 times follows passage of BK pi virus through embryonated chicken eggs, the increase in the case of PK pi virus is only ten-fold. However, the HA titres of PK pi virus released from the carrier cells is approximately six times higher than that of BK pi virus and, moreover, it is of the same order as that of B1 and PK pi virus grown in HeLa cells. The activity of BK pi virus is increased to that of the latter strains by growth in HeLa monolayers.

Summary.

The following conclusions may be drawn from these data:

data:

1) the three strains of NDV (B1, BK pi and PK pi), grown in HeLa cells have one-tenth of the HA activity of the same strains grown in chicken embryos.

2) the HA activity of virus released from PK pi cells is similar to that of virus grown in HeLa cells but the HA activity of BK pi virus is very much lower. Nevertheless, passage through chicken tissue restores the HA activity of both these viruses to that of wild-type NDV grown in fertile hens' eggs.

3) the HA activity of BK pi virus is increased to that of other cell-grown viruses following passage through HeLa cells.

c) Haemagglutinin activity per cell of cultures infected with strains of NDV.

The results described above for HA activity per µg protein for both the wild-type strains of NDV and the virus obtained from persistently infected cells, are confirmed by HA activity per cell of these viruses (Table XXIV).

The results in Table XXIV, show that the yield of haemagglutinin per cell is similar whether the infecting strain is B1 or Herts. However there is variation between cell types. Thus the HA titre produced per chick fibroblast is 2.5 times greater than that obtained from HeLa cells, and the latter is approximately twice the titre of the haemagglutinin released from MDBK cells. It is notable that the quantity of released haemagglutinin

T A B L E XXIV

TITRE OF HAEMAGGLUTININ RELEASED PER CELL INFECTED WITH VARIOUS STRAINS OF NDV

CELL-LINE	INFECTING STRAIN OF VIRUS	RELEASED HAU PER CELL ($x10^4$)
Persistent infection BK pi	N.A.	0.18*
Persistent infection PK pi	N.A.	0.17*
Persistent infection OK pi	N.A.	0.25*
PK(W)K6 cells	Herts NDV	2.4
MDBK cells	Herts NDV	1.2
MDBK cells	B1 NDV	2.1
HeLa cells	BK pi virus	2.0
HeLa cells	PK pi virus	2.5
HeLa cells	Herts NDV	5.0**
HeLa cells	B1 NDV	3.6
Chicken fibroblasts	Herts NDV	13.0**
Chicken fibroblasts	B1 NDV	13.0**

*The release of haemagglutinin from persistently infected cells was extremely variable. The results shown here are optimum.

**Results are approximate, due to the large number of cells destroyed in these instances.

N.A. - not applicable.

Measured after two days incubation of monolayers in which at least 90% of the cells were infected.

haemagglutinin per HeLa cell infected with BK pi or PK pi virus is similar to that obtained from HeLa cells infected with B1 or Herts strains of NDV. However, a discrepancy occurs in the case of haemagglutinin released from the persistently infected monolayers. It might have been expected that, as is the case for HA per µg protein, the yield per PK pi carrier cell would be ten times greater than that of BK pi cultures, and of a similar value to that produced by B1 virus in HeLa cells. This is not so, since the yields of haemagglutinin from OK pi, BK pi and PK pi monolayers are all one tenth of that released from infected HeLa cells.

Discussion.

This anomaly may be explained if it is assumed that the quantity of virus produced by PK pi cells is one-tenth of that released from BK pi monolayers. Thus, although PK pi haemagglutinin is ten times more active that that of BK pi (Table XXIII), the total amount of haemagglutinin released per cell will be the same. This supposition cannot be proven, but the results in Table XVII show that the yield of infectious virus per HAU of PK pi is about ten times that of BK pi, suggesting that, in the latter carrier cell-line, more non-infectious virus is produced from each cell and that the non-infectious virus bears defective HA.

III) NEURAMINIDASE

Introduction.

Neuraminidase (NA), like haemagglutinin, is associated with the large glycoprotein. It is concerned with the process of virus release, and is believed to function by removing the neuraminic acid of the cell receptors, thus preventing re-attachment of the budding virus particles to the membrane of the infected cell (67). Artificial substrates can be used to measure the activity of this enzyme in a quantitative spectrophotometric assay (34).

Concerned as NA is with the destruction of cellular components, it has long been considered that it may be associated with virulence, if not the direct cause. However, although McNulty *et al.* (108) and Alexander *et al.* (112) have shown that high activity is associated with virulence in purified virus and infected chorio-allantoic-membrane, respectively, the evidence that NA is a cause of virulence is slight.

The relationship of neuraminidase activity to the virulence of a strain of NDV.

In the present work, the association of virulence and NA has been confirmed for virus grown in fertile hens' eggs. Strains of NDV with high infectivity (Herts and BK pi viruses) have neuraminidase with a high specific activity. In contrast, avirulent strains (Ulster and B1 viruses) have specific activities three times lower (Table XXV).

T A B L E XXV

NEURAMINIDASE ACTIVITY OF STRAINS OF NDV

STRAIN OF NDV	SPECIFIC ACTIVITY PER µg PROTEIN* ($\times 10^3$)	
	PURIFIED VIRUS	PELLETED VIRUS
B1**	160.0	16.5
Herts**	600.0	65.0
Ulster**	230.0	N.D.
BK pi**	880.0	110.0
PK pi**	N.D.	40.0
B1/HeLa***	190.0	25.0
BK pi****	2.5	0.6
BK pi/HeLa***	3.3	N.D.
PK pi****	5.8	1.5
PK pi/HeLa***	4.0	N.D.

N.D. not done

* Specific activity measured as nmoles N-acetyl neuraminic acid released per minute.

** Grown in 10-day-old fertile hens' eggs.

*** Harvested from the supernatant fluids of infected HeLa cells.

**** Harvested from the supernatant fluids of cultures of the respective carrier cell-lines.

The situation was found to be more complex in regard to the neuraminidase activity of cell-grown virus. Thus, since B1 virus with normal NA is released from HeLa cells (Table XXV), it is surprising that, although the infectivity of BK pi and PK pi virus is increased by growth in HeLa cells (Table XV), their neuraminidase activity is unaffected and remains extremely low. On the other hand, when BK pi virus is grown in chickens or chicken embryos, there is an increase in NA activity and infectivity to the levels found in virulent strains of NDV (Tables XXV and XV).

Summary and Discussion

The low infectivity of BK pi virus is associated with low HA and NA activities and there is a small increase in infectivity and haemagglutinating ability following passage through HeLa cells. Although growth in HeLa cultures is unaccompanied by a rise in NA activity, when the virus replicates in chicken tissue, all activities are restored to those of a virulent strain of NDV.

A similar process is observed in PK pi virus, but in this case, the increase in infectivity appears to be closely related to restoration of neuraminidase activity, since the haemagglutinin released from this carrier cell-line is less defective than that of BK pi virus.

Therefore, the effect of passage through chicken cells may be either to select for mutants with a normal NA activity or to supply the components necessary for virulence and normal NA activity which are not found

found in mammalian cells.

A fuller discussion of this problem and its functional significance is deferred until the other biological activities of virus produced from the persistent infections have been described.

IV) CELLULAR FUSION

Introduction.

The ability of paramyxoviruses to induce cell fusion can be demonstrated in two ways, namely "fusion from without" and "fusion from within". In the former case, the physiology of the cell membrane is altered following the attachment of the virus particle, while in the second, the effect is due to the presence of viral envelope proteins in the plasmalemma. (The process of cell fusion is described in greater detail in the Introduction). The species and tissue of origin of the cell is believed to affect the degree of fusion possible (74), and some authors consider that fusion is related to the virulence of the infecting strain of NDV (47) but others disagree (111).

a) Fusion from without.

In order to demonstrate fusion from without, large quantities of virus are required (111). Unfortunately, it was difficult to obtain sufficient concentrations of virus released from persistent infections and conclusive results could not be achieved for the cell-lines described in the present experiments.

b) Fusion from within.

However, it was possible to study fusion from within. The results in Table IV show that a low percentage of synkaryons exists in the three persistently infected cell-lines, but a greater amount of fusion occurs when the carrier cells are co-cultivated with chick fibroblasts.

The implication that fusion may depend on the species of cell involved is borne out by the following experiments on homokaryon formation induced by 'wild-type' NDV infections. The quantity of virus employed was well below that required for "fusion from without" and the results (summarised below), were similar whether the B1 or Herts strain of NDV was employed.

i) In infected chick fibroblast monolayers, up to 50% of the cells were polykaryons, each containing as many as 30 nuclei.

ii) In infected HeLa cells, synkaryons made up 25% of the total and each contained between five and ten nuclei.

iii) Following infection of PK(W)K6 cells (the control line for PK pi cells) up to 15% of the cells were polykaryons, with as many as five nuclei per cell.

iv) In no circumstances did synkaryons account for more than 0.1% of an infected monolayer of MDBK cells (the control line for BK pi cells).

Discussion.

In the process of superinfection experiments with

with the carrier cells, it was shown the strain of virus also influenced the degree of fusion. Thus, a ten-fold increase in the number of syncytia was found in OK pi monolayers following superinfection with Herts strain NDV compared with lesser increases obtained with superinfected cultures of PK pi and BK pi cells. This effect cannot be attributed simply to an increase in the number of cells infected because the results in Tables VIII and III show that no fewer that 90% of the cells in the persistently infected monolayers haemadsorbed or contained specific viral antigen when stained with fluorescent antibody.

However, MDBK monolayers infected with NDV contain only 0.1% syncytia, whereas polykaryons make up over 1% of BK pi cells. This would suggest that the BK pi virus induces a higher degree of cell fusion than other strains of NDV. On the other hand, it is possible that during persistent infection a change is induced in the plasmalemma of MDBK cells which renders them more susceptible to cell fusion. The latter possibility is borne out by a comparison with PK pi cells. Both BK pi and PK pi monolayers form similar numbers of heterokaryons following co-cultivation with chick embryo fibroblasts (Table IV), although, in the case of PK pi cells, fewer homokaryons (1.2%) are formed than are produced in control PK(W)K6 monolayers, infected with Herts strain of NDV (15%). This suggests that the fusion ability of both strains of persistent virus is less than that of Herts strain of NDV.

NDV.

It is possible, therefore, that fusion in the persistently infected lines is as much due to an increased ability of the cells to fuse, as it is to a direct consequence of the activity of the glycoproteins of the virus.

It appears from the above results that fusion of cells 'from within' may be dependent on:

i) the strain of virus employed (although no relationship with virulence was established),

ii) the character of the type of cell that is infected,

iii) alterations in the physiology of the plasmalemma of persistently infected cells which render them more liable to cell fusion.

It should be noted that these features may also be relevant to "fusion from without".

V) HAEMOLYSIN

Introduction.

Haemolysin and fusion activities are closely related and both require the presence of the large haemagglutinin-neuraminidase-glycoprotein as well as phospholipid and the small envelope glycoprotein (171). However, these properties vary independently from strain to strain and neither is associated with virulence (111), although enhanced haemolysis in the presence of complement and antibody occurs in avirulent strains due to the incorporation of a specific cellular hapten (172).

hapten (172).

The function of haemolysis is not fully understood. However, since syncytial formation is induced by physiological changes in cell membranes in the presence of the viral envelope glycoproteins (74), and haemolysis is preceded by the fusion of the erythrocyte membrane with that of the virus particle (175), it is probable that haemolysis is a by-product of the cell fusion process. On the other hand, the lysis of culture cells infected with NDV is not related to cell fusion.

Loss of infectivity, fusion ability and haemolysin activity have been demonstrated in various mammalian cell-lines, persistently infected with Sendai virus and these defects have been explained by an abnormality in the small glycoprotein (56, 11).

Haemolysin activity of strains of NDV grown in cell culture or in fertile hens' eggs.

For the reasons given in the section on cell fusion (vide supra), it was not possible to determine whether fusion "from within" or "from without" was genuinely defective in the three carrier cells under examination. However, the results of a comparison between the haemolysin activities of B1 virus grown in chicken embryos or in HeLa cells with virus released from BK pi monolayers is given in Table XXVI. These show that both cell-grown B1 and BK pi viruses possess haemolysin activity eight-fold lower than that of egg-grown B1. The ratio of haemolysin to haemagglutinin is similar whether the B1 strain of NDV is grown in

T A B L E XXVI

HAEMOLYSIN ACTIVITY OF STRAINS OF NDV

STRAIN OF PURIFIED VIRUS	HAEMOLYSIN ACTIVITY* PER µg PROTEIN	HAEMOLYSIN ACTIVITY* PER HAU
BK pi (released from carrier cells)	1,600	18,000
B 1 NDV (grown in HeLa cells)	1,700	2,300
B 1 NDV (grown in fertile hens' eggs)	13,300	1,700

*measured as the number of fowl red blood cells haemolysed per ml.

in chicken or mammalian tissue, because in the latter case both activities are reduced approximately ten-fold. On the other hand, due to the extremely defective HA found in BK pi virus, the ratio in this instance is much higher.

Discussion.

Since haemolysin and cell fusion activities are closely related (171), it is probable that the low haemolysin activity of BK pi virus, is associated with the possible reduction in the ability of the persistent virus to bring about cell fusion.

VI) DISCUSSION OF THE RELATIONSHIP TO PERSISTENCE OF DEFECTIVE HAEMAGGLUTININ, NEURAMINIDASE, CELL FUSION FACTOR, HAEMOLYSIN AND OTHER BIOLOGICAL ACTIVITIES.

The experiments described above on the various biological activities of NDV indicate that full expression of haemolysin, fusion and haemagglutinin activities only occurs when the strains (whatever their derivation) are grown in chicken cells. Furthermore, since the B1 strain of NDV grown in HeLa cells is similarly defective, none of these properties is related to persistence, with the possible exception of the extremely abnormal haemagglutinin of BK pi virus. On the other hand, neuraminidase is fully active in B1 virus released from HeLa cells and is closely related to virulence in all strains of NDV grown in chicken embryos.

In the case of virus released from persistently

persistently infected cells, there is a diminished neuraminidase activity associated with decreased virulence, but these both return to normal levels on passage through chicken cells. Because growth of B1 virus in mammalian cells decreases its infectivity, but not its neuraminidase activity, it is probable that a reduction in neuraminidase activity is related to the maintenance of the persistent infection in BK pi and PK pi monolayers. The reduction in haemagglutinin activity of virus from BK pi cells may also be related to persistence.

The temperature-stability of the haemagglutinins of BK pi and PK pi virus is apparently not related to their persistence. However, it is possible that since the temperature-sensitivity of BK pi virus is unaltered on passage through chicken embryos, the different temperature-sensitivities of the two persistent strains of virus may be related to the properties of the haemagglutinins of the unknown strains of NDV with which the persistent infection was originally induced. On the other hand, as has been suggested in the previous section on the infectivity of the persistent viruses, it is possible that greater mutation has occurred in the case of PK pi virus than BK pi virus and this may be the cause of the temperature-lability of PK pi virus.

Biological activities of NDV, other than haemagglutinin, neuraminidase, cellular fusion and haemolysin include the following:

a) RNA polymerase (Rp), which is concerned with viral

viral RNA synthesis (39).

b) RNA-dependent DNA transcriptase (RdDt) which, so far as NDV is concerned, has been found only in persistent infections of L cells; and may predicate the incorporation of the NDV genome into that of the host cell (45).

c) ATPase (69) and protein kinase (PKA)(68) have been reported in other paramyxoviruses and both of these enzymes are believed to be associated with the synthesis of viral RNA and the assembly of viral proteins.

d) Exo- and endonucleases have been described in Sendai virus (71) and may be concerned both with RNA transcription and with destruction of cellular RNA so that nucleotides are available for viral RNA synthesis. In particular, an exonuclease known as phosphodiesterase (PDA), which can be assayed by means of a simple spectrophotometric method, has been isolated in semi-purified preparations of Sendai virus (70).

The presence of RdDt or deficiencies in any of the other enzymes, might account for the reduced yields of virus from the three persistently infected cell-lines, especially since a temperature-sensitive Rp has been described in cell cultures persistently infected with NDV. Although McNulty, Gowans and Norval (personal communication) have obtained some evidence of RdDt activity in BK pi virus, it was not possible in the context of this present work to investigate any of these activities except for a preliminary examination

examination of PDA.

VII) PHOSPHODIESTERASE

Introduction.

Phosphodiesterases are alkaline exonucleases, operating from the 3-hydroxy terminus of nucleic acids and releasing 5-mononucleotides. Another type of phosphodiesterase which operates at acidic pH, is found in splenic tissue (177, 178) and produces 3-mononucleotides, Alkaline phosphodiesterase was originally investigated in snake venom, and related enzymes have been found in mammalian tissues; the greatest activity being in kidney cell microsomes (179). Phosphodiesterase activity has been reported in concentrated unpurified preparations of Sendai virus (69), but there are no reports of a similar enzyme in NDV.

The function of phosphodiesterase (PDA) in the replicative cycle of paramyxoviruses is unknown. It may be related to the uncoating of viral RNA following entry of virus or, on the other hand, it may be concerned in the replication of viral RNA.

Attempts were made to isolate phosphodiesterase from purified preparations of NDV with a view to confirming its status as a virus-mediated activity of paramyxoviruses. It was also hoped that this enzyme might provide a useful parameter for comparing virulent, avirulent and persistent strains of NDV.

Four characteristics were used to demonstrate that PDA was coded for by viral RNA and was incorporated into

into the mature virion:

a) The increase in PDA activity with increasing purity of the virus (Table XXVII).

b) The isolation of PDA activity in association with the small glycoprotein of NDV (see following section on purification).

c) The inhibition of viral but not cellular PDA by antibody prepared against whole NDV.

d) The demonstration of a pH optimum for the PDA of all strains of NDV different from that of cellular PDA.

a) <u>The increase in phosphodiesterase activity following purification of virus</u>.

The 2-4-fold increase in PDA activity shown in Table XXVII is slight in comparison to the ten-fold difference in HA titre between pelleted and purified virus (Table XXIII). This is due to the relatively high level of cellular PDA activity (Table XXVII) which produces a raised value in the impure viral pellet. However, as shown in Figure 22, the pH optimum is lower than that used for assay of viral PDA.

b) <u>The association of phosphodiesterase with the small glycoprotein of NDV</u>.

Both cellular and viral PDA was inhibited, in the presence of the detergents - Triton X-100 and Nonidet P 40 (NP40) - which separate the viral proteins and expose the core of the virion. This is in contrast to the effect that these compounds have on Rp activity

T A B L E XXVII a

SPECIFIC ACTIVITY* OF PHOSPHODIESTERASE OF NDV AT VARIOUS STAGES OF PURIFICATION

STRAIN OF VIRUS	STAGE OF PURIFICATION			
	VIRUS IN ALLANTOIC FLUID*	PELLETED VIRUS*	VIRUS AFTER FLUOROCARBON TREATMENT*	VIRUS AFTER PURIFICATION IN TARTRATE DENSITY GRADIENTS*
B1 NDV Egg-grown	42	310	350	415
Herts NDV Egg-grown	47	258	540	812
BK pi NDV Egg-grown	N.D.	134	N.D.	330
BK pi NDV Released from persistently infected cells	N.A.	41	N.D.	85
PK pi NDV Released from persistently infected cells	N.A.	126	140	200
B1 NDV Released from infected HeLa cells	N.A.	100	170	280

*Specific activity (nmoles bis-p-nitrophenyl phosphate hydrolysed per hour) per µg protein x 10^3.

T A B L E XXVII b

SPECIFIC ACTIVITY OF PHOSPHODIESTERASE OF CELLULAR PREPARATIONS

CELL-TYPE**	SPECIFIC ACTIVITY*
MDBK	110
BK pi	140
CF	41

*Specific activity (nmoles bis-p-nitrophenyl phosphate hydrolysed per hour) per µg protein x 10^3.

**Cells were disrupted by sonication.

T A B L E XXVIII

THE INHIBITION OF HAEMAGGLUTININ, NEURAMINIDASE AND PHOSPHODIESTERASE BY ANTIBODY AGAINST NDV

STRAIN OF NDV	CONCENTRATION OF ANTIGEN IN HAU	BIOLOGICAL ACTIVITY ASSAYED	pH AT WHICH INHIBITION TITRE WAS ASSAYED	INHIBITION TITRE DUE TO ANTIBODY
Herts	32	Neuraminidase	5.0	256
Herts	4	Haemagglutinin*	7.2	256
Herts	4	Haemagglutinin**	9.0	32
B 1	4	Haemagglutinin	7.2	256
Herts (purified envelope glycoproteins)	512	Phosphodiesterase***	9.0	32
B 1	64	Phosphodiesterase	9.0	4
Herts treated with NP 40	128	Phosphodiesterase	9.0	nil

*HA titre 32

**HA titre 16

***There was no inhibition of the phosphodiesterase activity of disrupted MDBK cells or chick fibroblasts by NDV antiserum when assayed at pH 9.0.

T A B L E XXIX

RELATION OF HAEMAGGLUTININ TO PHOSPHODIESTERASE ACTIVITY OF THE HERTS STRAIN OF NDV

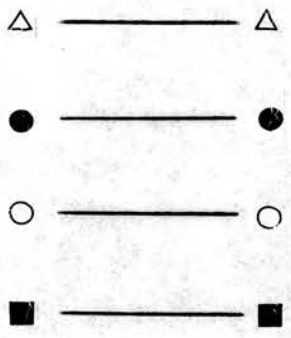
HA TITRE*	PHOSPHODIESTERASE**
512	50
1024	70
2048	105
4096	125

*HAU/ml

**SPECIFIC ACTIVITY expressed as nmoles of bis-p-nitrophenyl phosphate hydrolysed per hour.

Figure 22. Variation of phosphodiesterase activity in relation to pH (read against controls of the appropriate pH).

a) Cells disrupted by sonication



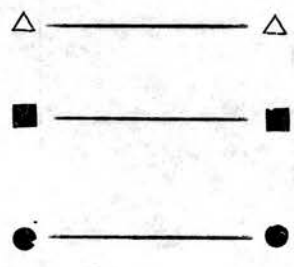
△ ——— △ BK pi cells

● ——— ● chick embryo fibroblast cells

○ ——— ○ MDBK cells

■ ——— ■ HeLa cells infected with B1 strain of NDV

b) Purified virus



△ ——— △ released from BK pi cells

■ ——— ■ B1 strain grown in fertile hens' eggs

● ——— ● Herts strain grown in fertile hens' eggs.

Fig. 22

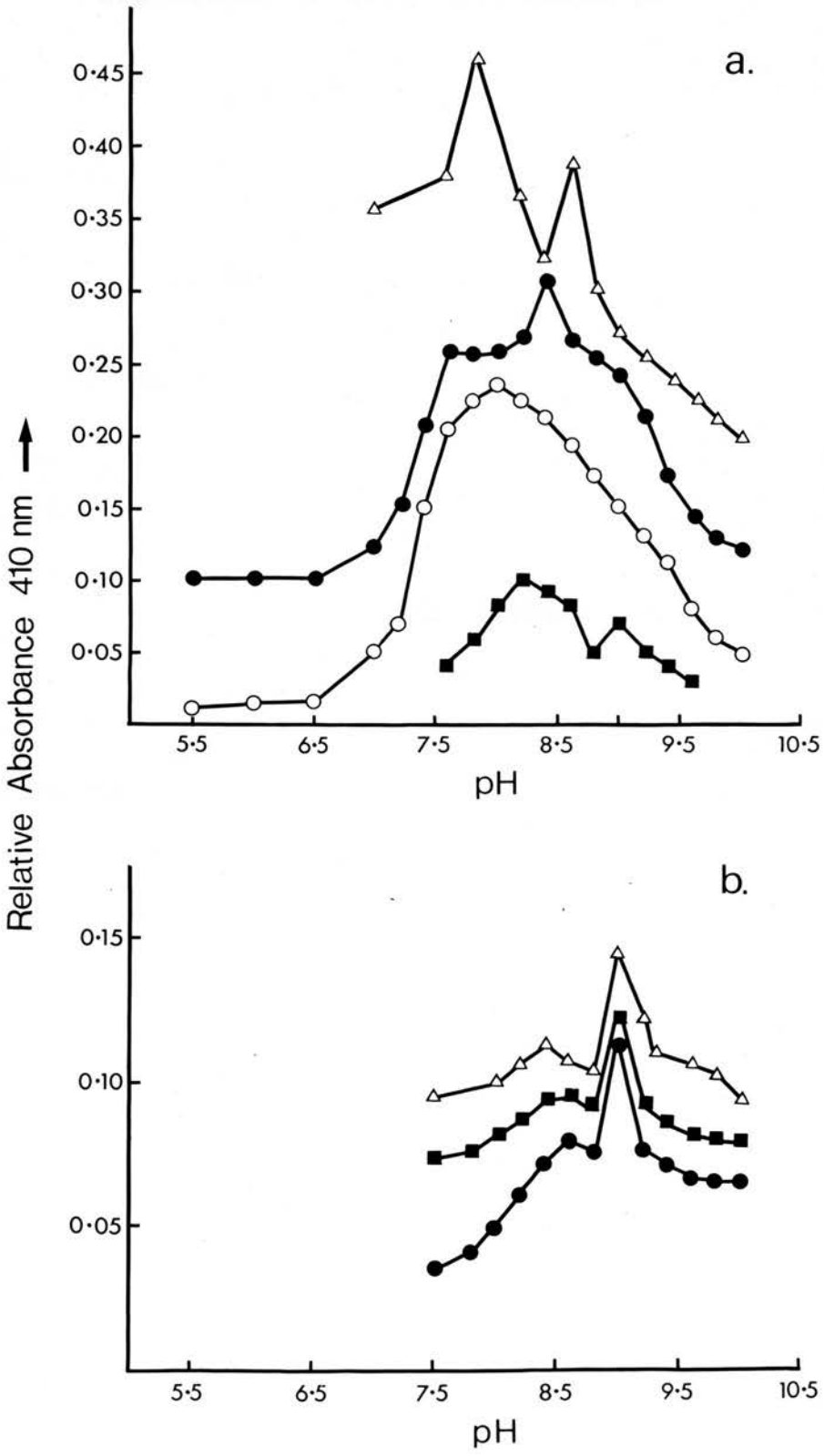
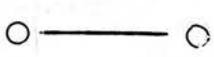

a.
0·45
0·40
0·35
0·30
0·25
0·20
0·15
0·10
0·05
5·5
6·5
7·5
8·5
9·5
10·5
pH
Relative Absorbance 410 nm
b.
0·15
0·10
0·05
5·5
6·5
7·5
8·5
9·5
10·5
pH

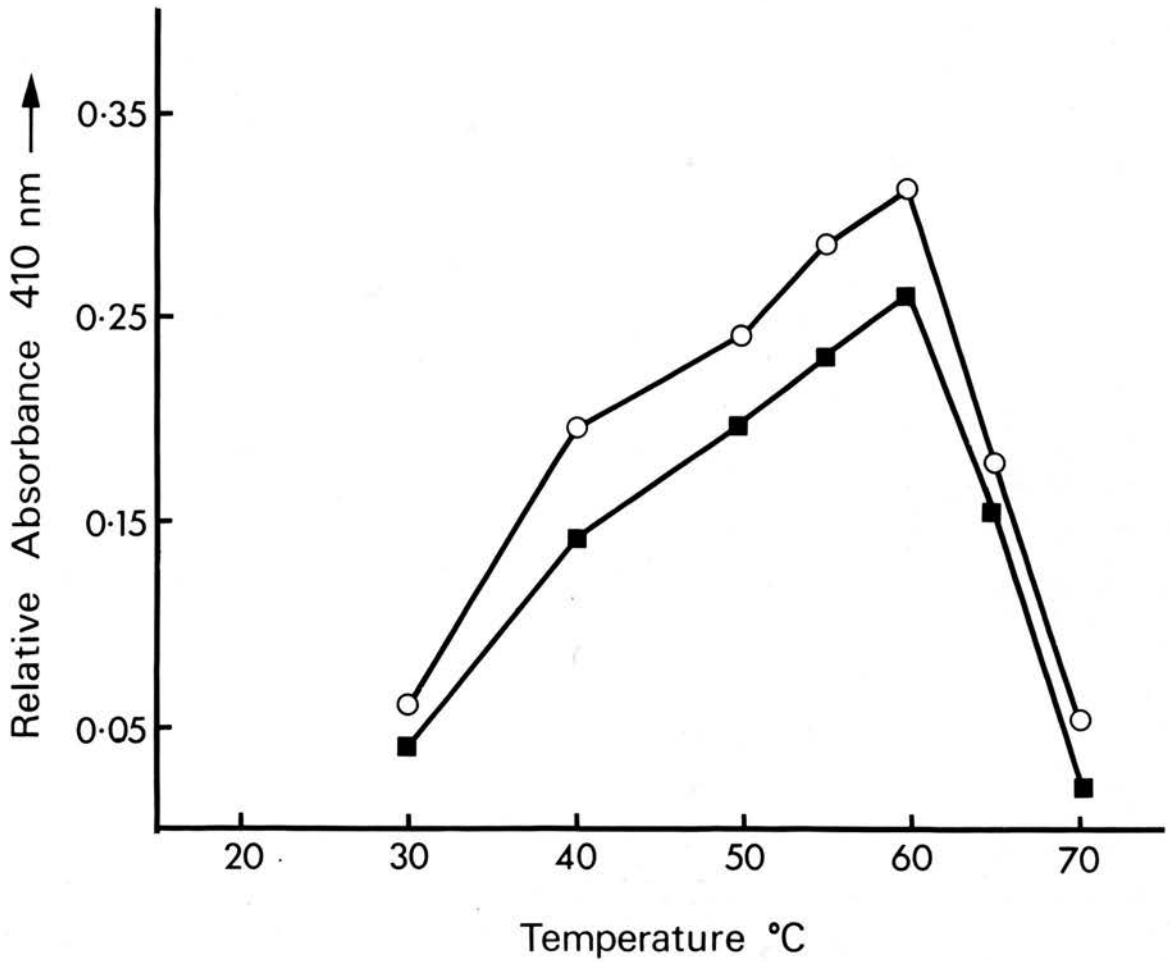
Figure 23. Variation in phosphodiesterase activity in relation to temperature.



Herts virus grown in fertile hens' eggs.

Bl virus grown in fertile hens' eggs.

Fig. 23


Relative Absorbance 410 nm
0·35
0·25
0·15
0·05
20
30
40
50
60
70
Temperature °C

activity and suggests that PDA is not associated with an internal viral component. Indeed, following Triton X-100 treatment of purified virus (Section 2 (G)III), PDA activity is associated with that fraction containing the small envelope glycoprotein (VGP 55). It may be concluded, therefore, that these detergents do not destroy PDA but prevent it from functioning if present during assay.

c) Inhibition of phosphodiesterase activity by antibody against NDV.

The results in Table XXVIII show that the inhibition of activity by antibody was far greater in relation to the envelope fraction than to the complete virion. These findings suggest that the PDA activity is borne on the internal surface of the viral envelope. In these circumstances, it might have been expected that the effect of antibody would be enhanced in the presence of NP40 but owing to the inhibitory action of the detergent, this could not be confirmed. However, the same antiserum had apparently a very much greater inhibitory effect on haemagglutination or neuraminidase activity than on phosphodiesterase (Table XXVIII), but this was probably due to two abnormal conditions. First, since detection of PDA requires a minimum concentration of virus thirty times that needed for HA, the increased amounts of antigen may neutralise the antibody. Second, although HA is not itself inhibited at pH 9.0, antiserum is prevented from functioning under these conditions and,

therefore, a reduced haemagglutination inhibition is obtained at this pH (Table XXVIII). Since 9.0 is the optimum pH for detection of phosphodiesterase activity, it is not surprising that assays for inhibition of the enzyme by antiserum, give similar low titres at this pH. It may be noted that neuraminidase inhibition assays can be carried out at the optimum pH for this activity (5.0) because antiserum is functional at this acidity (Table XXVIII). Because PDA was found to be present in all sera tested (including calf serum used to supplement cell culture medium), the antibody to NDV was partially purified by ammonium sulphate precipitation (as described for fluorescent antibody stains), prior to use in these assays. In spite of the fact that the antiserum was prepared against unpurified NDV grown in the allantoic cavity of embryonated hens' eggs, no inhibition of chicken fibroblast phosphodiesterase was observed (Table XXVIII). Therefore, it may be concluded that phosphodiesterase activity associated with NDV, is specifically inhibited by antibody, prepared against this virus.

Although PDA activity varies between strains (Table XXVII), it is not possible from the available data to ascertain whether or not this enzyme is associated with virulence.

However, it can be stated that the virulent Herts strain of NDV has the greatest PDA activity, while virus released from BK pi monolayers, which had the lowest infectivity of all the strains examined, is also the

the least effective in causing hydrolysis of phosphate diesters. Also of possible significance are the low values for PDA associated with cell-grown virus, compared with that of egg-grown virus. It is emphasised that just as the other biological activities of BK pi virus are restored, after passage through chicken embryos, so also is the PDA activity.

d) The optimum pH of phosphodiesterase activity associated with NDV.

There is no discernible difference between the pH optima for the phosphodiesterases of the Herts, B1 and BK pi strains of NDV; all of which are identical at 9.0 (Fig. 22b). On the other hand, the optimum pH for uninfected cells varies from 8.0 in MDBK to 8.4 in chick fibroblasts, while BK pi cells and HeLa cells infected with the B1 strain of NDV have a double-peaked pH curve. In each case, the major peaks are at 7.8 and 8.2 respectively and the minor peaks, which are probably due to viral PDA, are at 8.6 and 9.0 (Fig. 22a). Since, not only is the optimum temperature for PDA activity very high, but there is also correspondence between the optima recorded for both the B1 and Herts strains of NDV, viral PDA is unlikely to vary in its temperature-stability (Fig. 23). Thus PDA is a virus-specific enzyme, although the presence in cells of a similar enzyme (but with a lower pH optimum) prevents its full exploitation as a means of assaying viral activity.

activity.

PDA is defective in BK pi virus (and to a lesser extent PK pi) but its relationship to persistence and virulence is not clear. However, the location of PDA on the envelope proteins of NDV, in addition to haemolysin, cellular fusion, haemagglutinin and neuraminidase, all of which are defective in the persistent strains of NDV, suggests that malformation of these membrane proteins is related to the reduced infectivity of virus in mammalian cells and the reduced virulence of virus released from them.

F / PURIFICATION

I) INTRODUCTION

Although the properties of a virus may be studied in unpurified preparations, it is only by the use of purified samples that characteristic differences between strains can be elucidated. Purity would be of less import, if the viral species under study had a single invariable parameter common to every strain, against which all variations in other properties of the virus might be assessed. In the case of NDV, the haemagglutinin has, in the past, been used as such a measure. However, it is now known that not only does HA activity decline on storage of the virus but that there is considerable variation in temperature stability and haemagglutinating ability between strains (35, 111).

Since it has not previously been shown that the numerous strains of NDV are readily distinguishable by their susceptibility to antibody, the use of a standard unit of antigen, as defined by its ability to neutralise antibody, might be envisaged as a means of standardisation. Unfortunately, the advantages of the use of this criterion are reduced first, by the presence of several antigens in NDV which are capable of variation in the promotion of biological activity and second, the difficulty of providing a reference antiserum of constant efficacy.

Thus, measurement of the relevant properties in terms of their activity per μg of purified viral protein, was

was considered to give the best absolute comparison between strains. Moreover, the use of purified virus eliminates the possibility of contamination by cellular components with activities of a similar nature.

Purity of a virus sample may be judged:-

i) by demonstrating that a similarly treated preparation obtained from an uninfected source contains no activities otherwise attributed to virus,

ii) by demonstrating that the virus prepared from a single source is constant in its nature and contains no substance of non-viral origin.

These criteria can be satisfied by examination of the material under the electron microscope, and by analysis of the number, relative proportions and molecular weights of the proteins by polyacrylamide gel electrophoresis (PAGE); and monitored by the increase in activity per μg of protein of specific viral properties.

Several methods have previously been used to purify paramyxoviruses, including:-

a) separation on size, by differential filtration through columns of cellulose or agarose beads

b) separation on the basis of biological activity, by agglutination of viral material to erythrocytes and by employing neuraminidase to bring about elution of these particles

c) separation by means of differential centrifugation, on the basis of relative mass or density of the

the virus compared with that of contaminating material.

The method employed in the present work is based on that of Evans and Kingsbury (19) and may be divided into four stages.

i) contaminants of high mass are first removed by pelleting them at relatively low accelerations (300g),

ii) contaminants of low mass are then removed by pelleting the virus at greater accelerations (40,000g).

iii) some cellular components can be removed because they are more soluble in fluorocarbon than in a buffer prepared in water.

iv) thereafter the virus is placed on top of a linear density gradient of potassium tartrate and subjected to very high acceleration (287,000g). After centrifugation of relatively short duration (1-2 hours), bands of virus form at its isopycnic density. This step is then repeated. Cell fragments of the same density as the virus, but with lower sedimentation rates, do not contaminate the virus samples due to the short periods of centrifugation employed.

II) EFFICACY OF THE PURIFICATION METHOD

NDV, obtained from infected allantoic cavities of embryonated chicken eggs, was purified by the method described and the viral proteins were separated by electrophoresis on polyacrylamide gels (PAGE) following solubilisation with sodium dodecyl sulphate. The stained gels were scanned with a Unicam SP 500

500 spectrophotometer with a Gilford linear transport attachment and the profiles obtained are shown in Fig. 24 a,b,c and Fig. 29 c. Similar treatment of allantoic fluids from uninfected eggs produced no discernible bands in tartrate density gradients, nor did a portion of the gradient of the same density at which virus banded, contain protein in sufficient quantity to be analysed by PAGE.

The efficacy of the purification procedure was further established by comparing the proteins present with the specific activity per μg of protein of various biological properties of NDV at the several stages described above. These results are presented in Table XXX and Fig. 24, 25, 26. The number of proteins present in infected allantoic fluid (Fig. 25b, 26a) is greater than that in virus which has been twice centrifuged through a potassium tartrate gradient (TDG 2)(Fig. 24 a,b,c). Moreover during the purification procedure there is an increase in the relative proportions of the proteins found in the TDG 2 (Fig. 26a and 26c). An increase in the activity per μg protein of all three biological activities examined is also found; the greatest being for haemagglutinin (<1000-fold) followed by neuraminidase activity (>100-fold) while PDA activity is 7-20 times greater (Table XXX b). There is a relatively smaller increase for all these properties in the case of B1 strain compared with Herts strain. This may be due to the presence of a greater number of virus particles

T A B L E XXX a

THE EFFECT OF PURIFICATION ON THE BIOLOGICAL ACTIVITY OF NDV

STRAIN OF NDV	STAGE OF PURIFICATION	PROTEIN CONCENTRATION (µg/ml)	HAEMAGGLUTININ ACTIVITY*	NEURAMINIDASE ACTIVITY**	PHOSPHODIESTERASE ACTIVITY***
B1/E	Infected allantoic fluid	6,600	0.02	0.88	54
	Pelleted virus	1,400	0.72	15.7	120
	Fluorocarbon-treated virus	500	5.1	22.8	350
	After first tartrate density gradient	100	7.9	91.9	375
	After second tartrate density gradient	40	12.8	113.5	400
Herts/E	Infected allantoic fluid	3,400	0.02	2.21	40
	Pelleted virus	1,450	0.35	52.7	240
	Fluorocarbon treated virus	380	1.35	77.7	500
	After first tartrate density gradient	65	5.0	287.4	592
	After second tartrate density gradient	25	20.5	483.1	769

Bl/HeLa	Infected supernatant fluids	3,300	0.005	0.1	N.D.
	Pelleted virus	1,100	0.32	27.0	100
	Fluorocarbon-treated virus	750	0.62	45.0	170
	After first tartrate density gradient	70	0.80	115.0	200
	After second tartrate density gradient	23	1.20	119.0	280
BK pi	Pelleted virus	800	0.015	5.0	41
	Fluorocarbon-treated virus	540	0.12	2.5	72
	After first tartrate density gradient	65	0.18	2.5	85
	After second tartrate density gradient	42	0.22	1.0	140

N.D. Not done

Bl/E: The Bl strain grown in 10-day-old fertile hens' eggs.

Herts/E: The Herts strain grown in 10-day-old fertile hens' eggs.

Bl/HeLa: The Bl strain released from HeLa cells.

BK pi: The virus released from monolayers of BK pi carrier cells (assays were not performed on the supernatant fluids).

* Haemagglutinin activity expressed as HAU/µg protein.

** Neuraminidase activity expressed as nMoles N-acetylneuraminic acid/minute/µg protein $x10^3$.

*** Phosphodiesterase activity expressed as nMoles of bis-p-nitrophenylphosphate hydrolysed/hour/µg protein $x10^3$.

T A B L E XXX b

TOTAL INCREASE IN BIOLOGICAL ACTIVITY DURING PURIFICATION

STRAIN OF NDV	INCREASE IN SPECIFIC		
	HAEMAGGLUTININ ACTIVITY	NEURAMINIDASE ACTIVITY	PHOSPHODIESTERASE ACTIVITY
B1/E	640	130	7
Herts/E	1025	220	20
B1/HeLa	240	1200	2.8*
BK pi	15*	0.2*	3.5*

B1/E the B1 strain grown in 10-day-old fertile hens' eggs.

Herts/E the Herts strain grown in 10-day-old fertile hens' eggs.

B1/HeLa the B1 strain grown in HeLa cells.

BK pi the virus released from cultures of BK pi carrier cells.

The total increase in activity from 'allantoic fluid' to 'second tartrate density gradient' stage was estimated from Table XXX a.

*In these cases the total increase in activity from the 'pellet' stage was estimated from Table XXX a.

Figure 24. Densitometer profiles of polyacrylamide gels of viral proteins electrophoresed under reduced conditions, stained with Coomassie brilliant blue and scanned at 580 nm. The position of the marker dye front is shown by an unlabelled arrow and the positions of the major polypeptides of NDV, designated by their approximate molecular weight (x 10^{-3}), as determined by Moore and Burke (21), are shown in the scale. The origin of the gel is on the left of the profile and at the top of the photograph.

Three different strains of NDV were grown in fertile hens' eggs and completely purified as described. Photographs of the stained gels (b) and (c) are shown for comparison. The viral proteins are labelled in Fig. 24 a.

a) Italien strain

b) Herts strain

c) B1 strain

Fig. 24

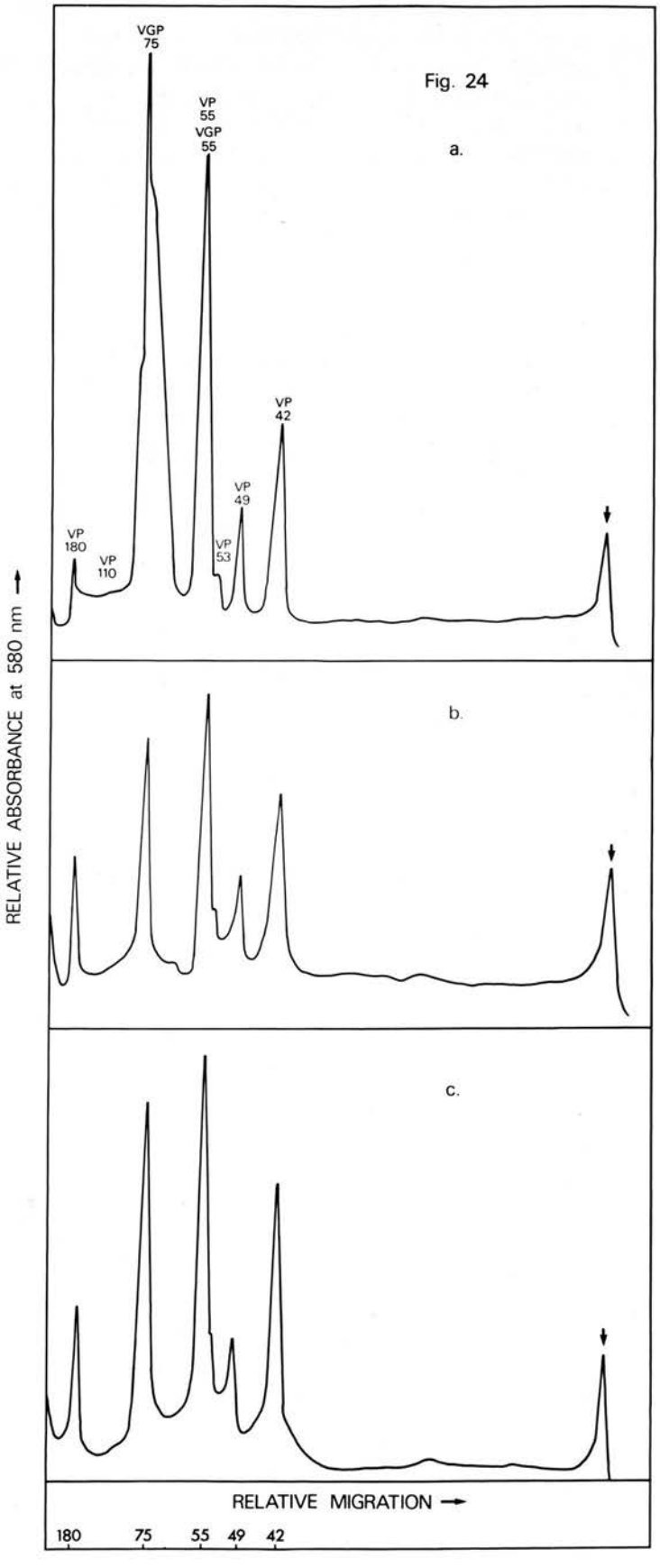

VGP 75
VP 55
VGP 55
VP 42
VP 49
VP 180
VP 110
VP 53
a.
b.
c.
RELATIVE ABSORBANCE at 580 nm
RELATIVE MIGRATION
180
75
55
49
42

Figure 25. Densitometer profiles obtained in a similar fashion to those in Fig. 24.

a) Herts strain of NDV, grown in chick embryo fibroblast cells and completely purified as described, showing marked differences from the profile of the same strain grown in fertile hens' eggs (Fig. 24 b).

b) Herts strain of NDV grown in fertile hens' eggs and completely purified as described (shown in Fig. 24 b), co-electrophoresed with an equal quantity (μg) of calf serum (shown in Fig. 31 c). Note that this profile is different from both Fig. 24 b and Fig. 25 a.

c) Herts strain of NDV, grown in the allantoic fluid of fertile hens' eggs (compare with Fig. 26 a).

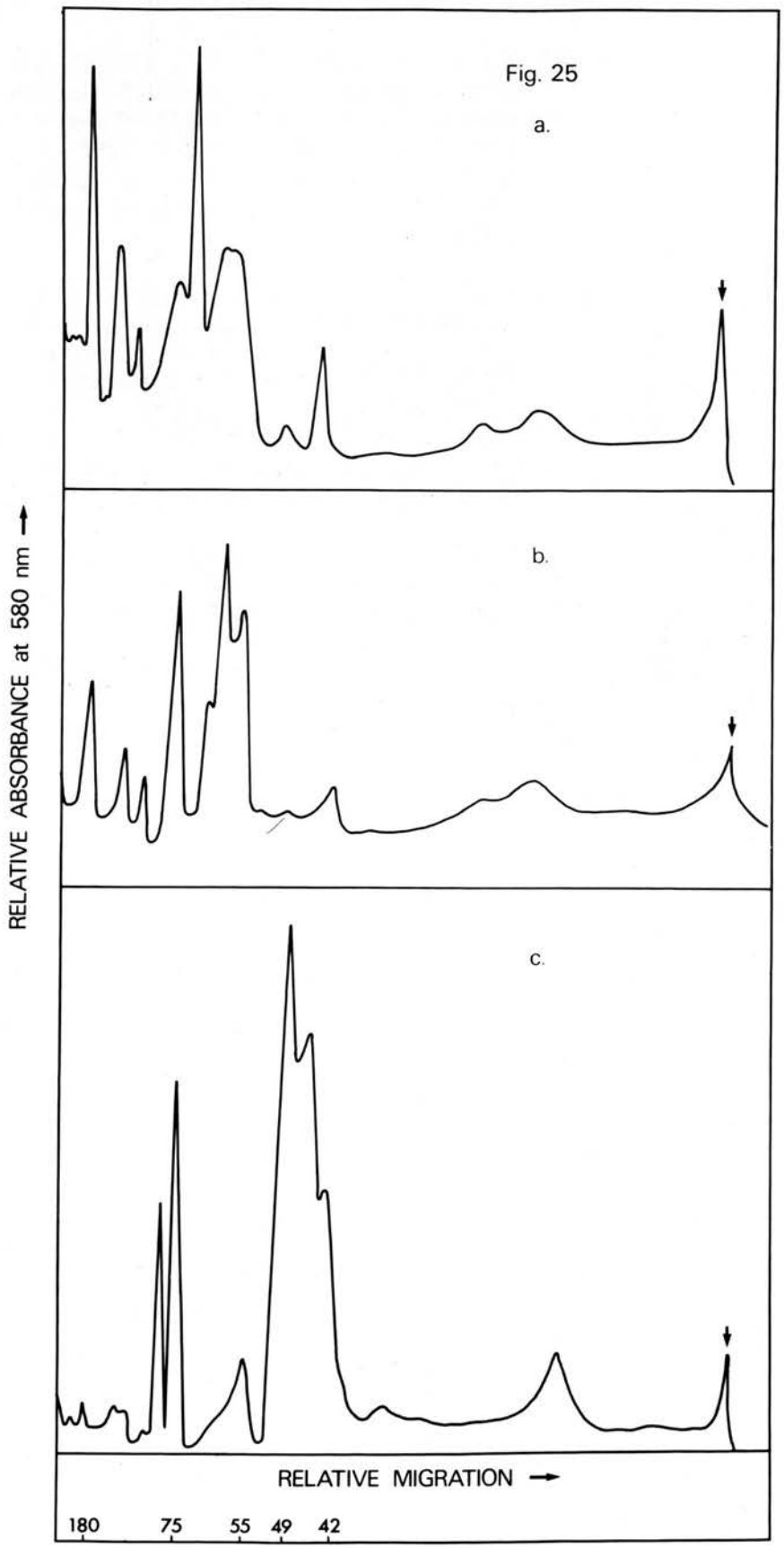


Fig. 25
a.
b.
c.
RELATIVE ABSORBANCE at 580 nm
RELATIVE MIGRATION
180
75
55
49
42

Figure 26. Densitometer profiles obtained in a similar fashion to those in Fig. 24, showing the B1 strain of NDV grown in fertile hens' eggs, at various stages of purification. Photographs of the stained gels are shown for comparison.

a) Virus in allantoic fluid.

b) Virus after pelleting.

c) Virus after treatment with TCTF (fluorocarbon).

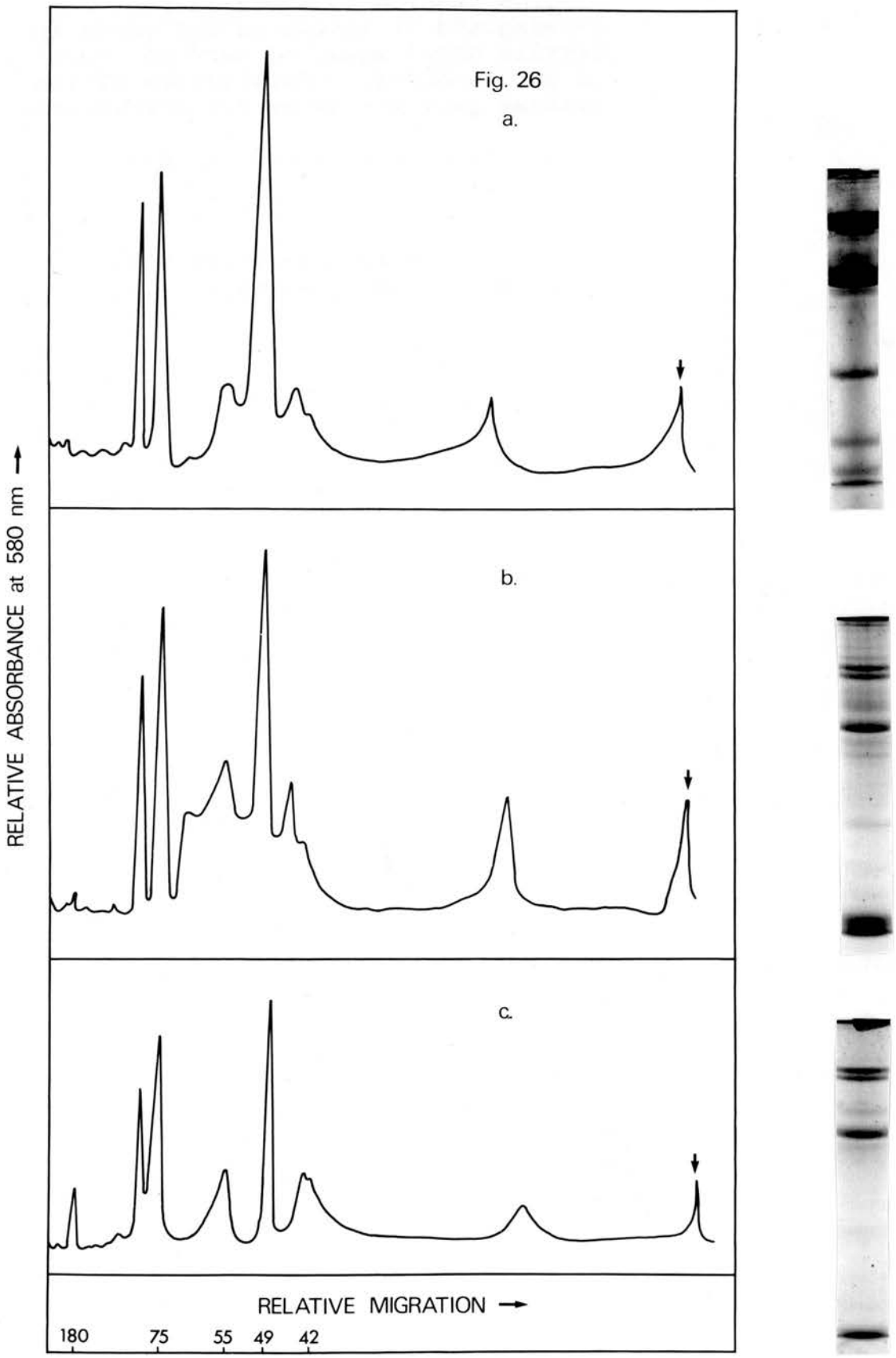


Fig. 26
a.
b.
c.
RELATIVE ABSORBANCE at 580 nm
RELATIVE MIGRATION
180
75
55
49
42

Figure 27. Densitometer profiles obtained in a similar fashion to those in Fig. 24 showing the B1 and PK pi strains of NDV released from cell culture and purified as described.

a) B1 strain released from HeLa cells (compare with Fig. 24 c).

b) PK pi strain released from HeLa cells.

c) PK pi strain released from persistently infected PK pi cells.

Note. The gel profiles of all these viruses as well as that in Fig. 25 a are very similar. In these figures, VGP 75 is a shoulder of a large peak of about 70,000 daltons and VP 55 and VP 42 are generally small in size.

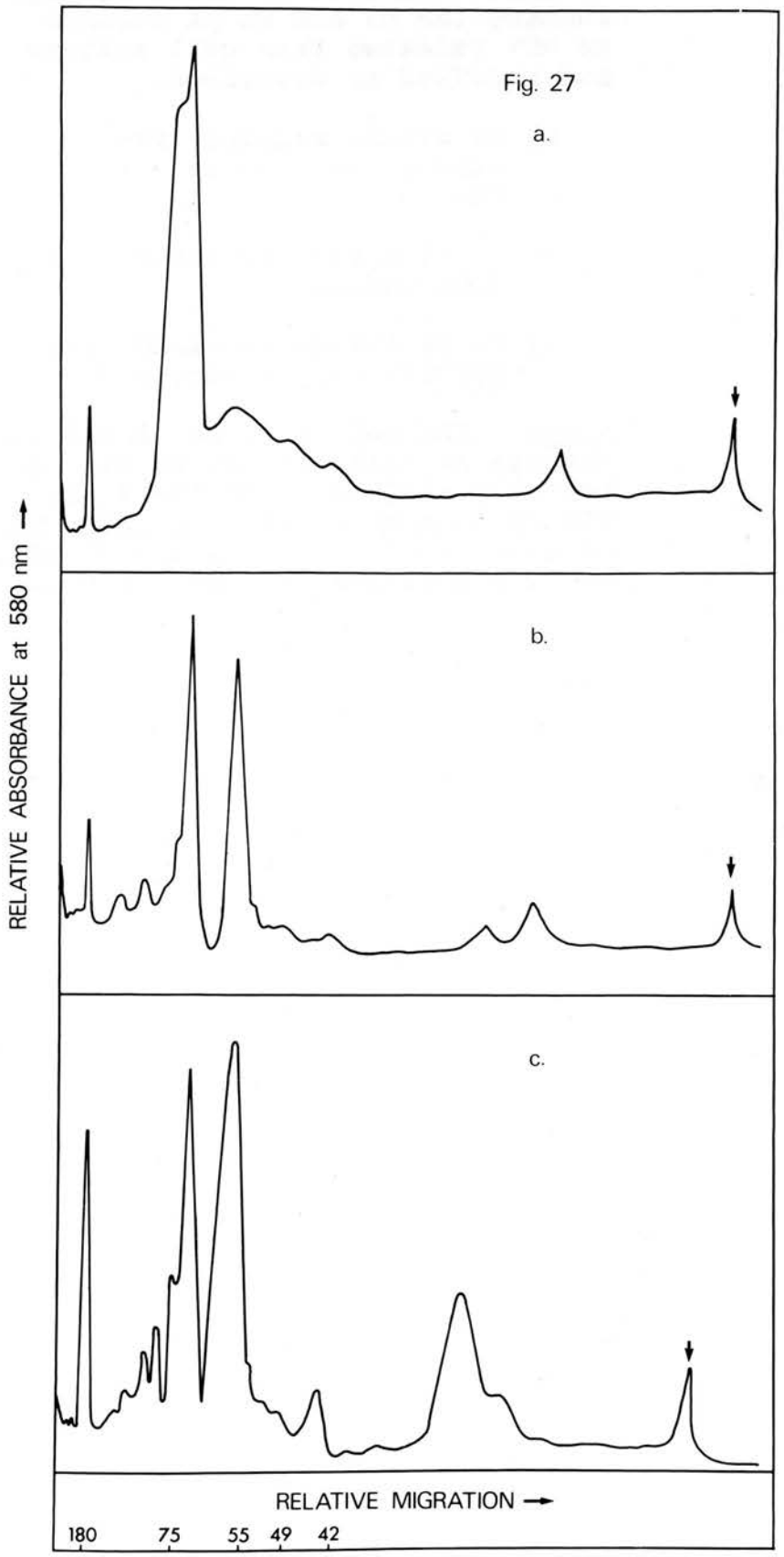


Fig. 27
a.
b.
c.
RELATIVE ABSORBANCE at 580 nm →
RELATIVE MIGRATION →
180
75
55
49
42

Figure 28. Denisometer profiles obtained in a similar manner to those in Fig. 24, showing the BK pi strain of NDV grown in cell cultures and purified as described. The peak designated VP 70 is labelled.

a) Virus released from BK pi cells (a photograph of the stained gel is shown for comparison).

b) BK pi strain grown in HeLa cells.

c) BK pi strain released from persistently infected BK pi cells but stained with PAS in place of Coomassie brilliant blue.

Note the absence of VGP 75 in Fig. 28 a and its presence in Fig. 28 b. VP 55 and VP 42 are both small in size compared with egg-grown strains of NDV (Figs. 24,29).

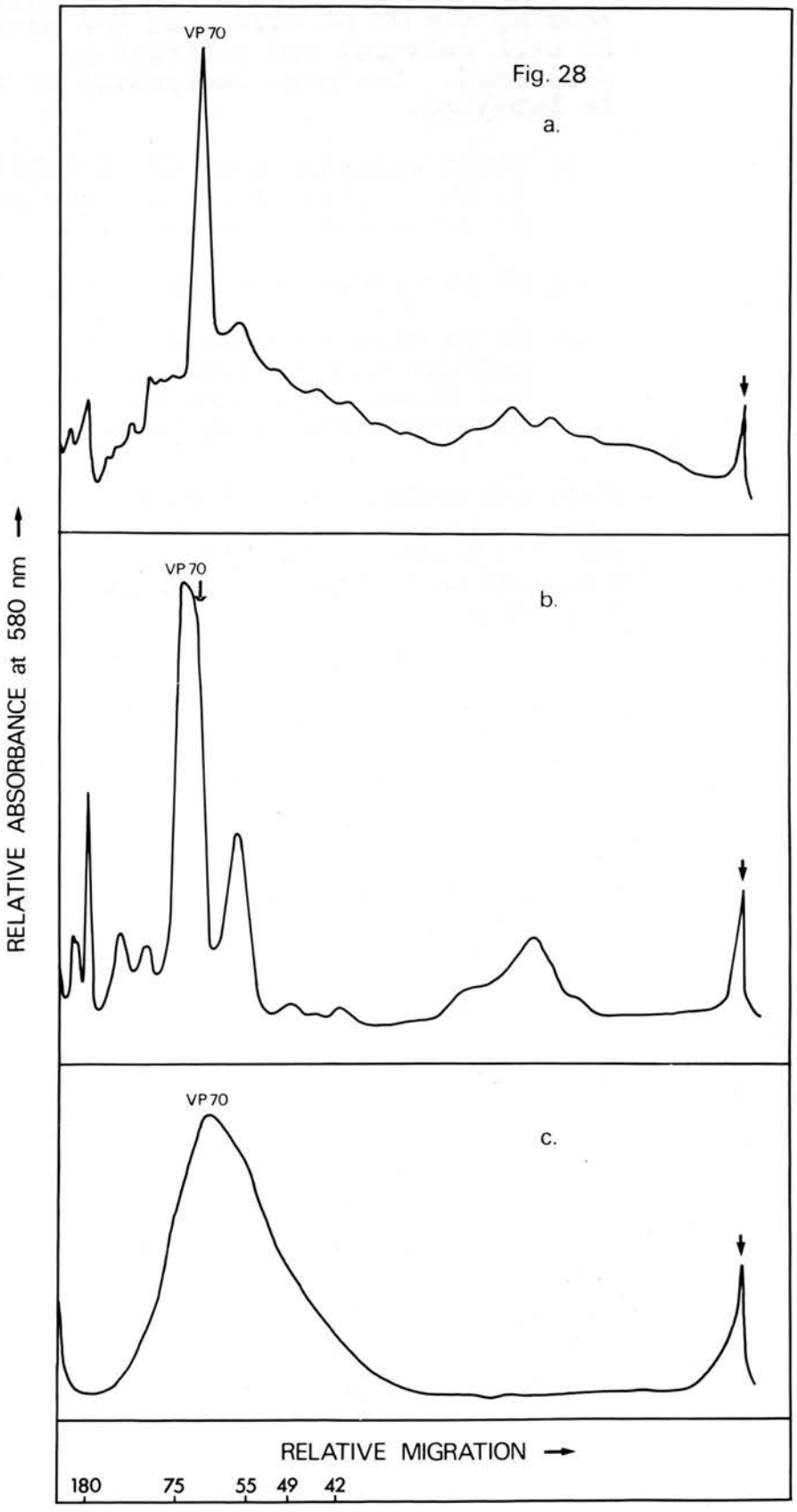


Fig. 28
a.
VP 70
b.
VP 70
c.
VP 70
RELATIVE ABSORBANCE at 580 nm
RELATIVE MIGRATION
180
75
55
49
42

Figure 29. Densitometer profiles obtained in a similar fashion to those in Fig. 24.

a) BK pi strain grown in fertile hens' eggs and completely purified as described.

b) Bl strain of NDV grown in BHK cells and completely purified as described.

c) Ulster strain of NDV, grown in embryonated hens' eggs and completely purified as described.

Note that after passage through chicken eggs, the profile of BK pi virus (Fig. 29 a) resembles that of egg-grown strains of NDV (Figs. 24 a,b,c and Fig. 29 c).

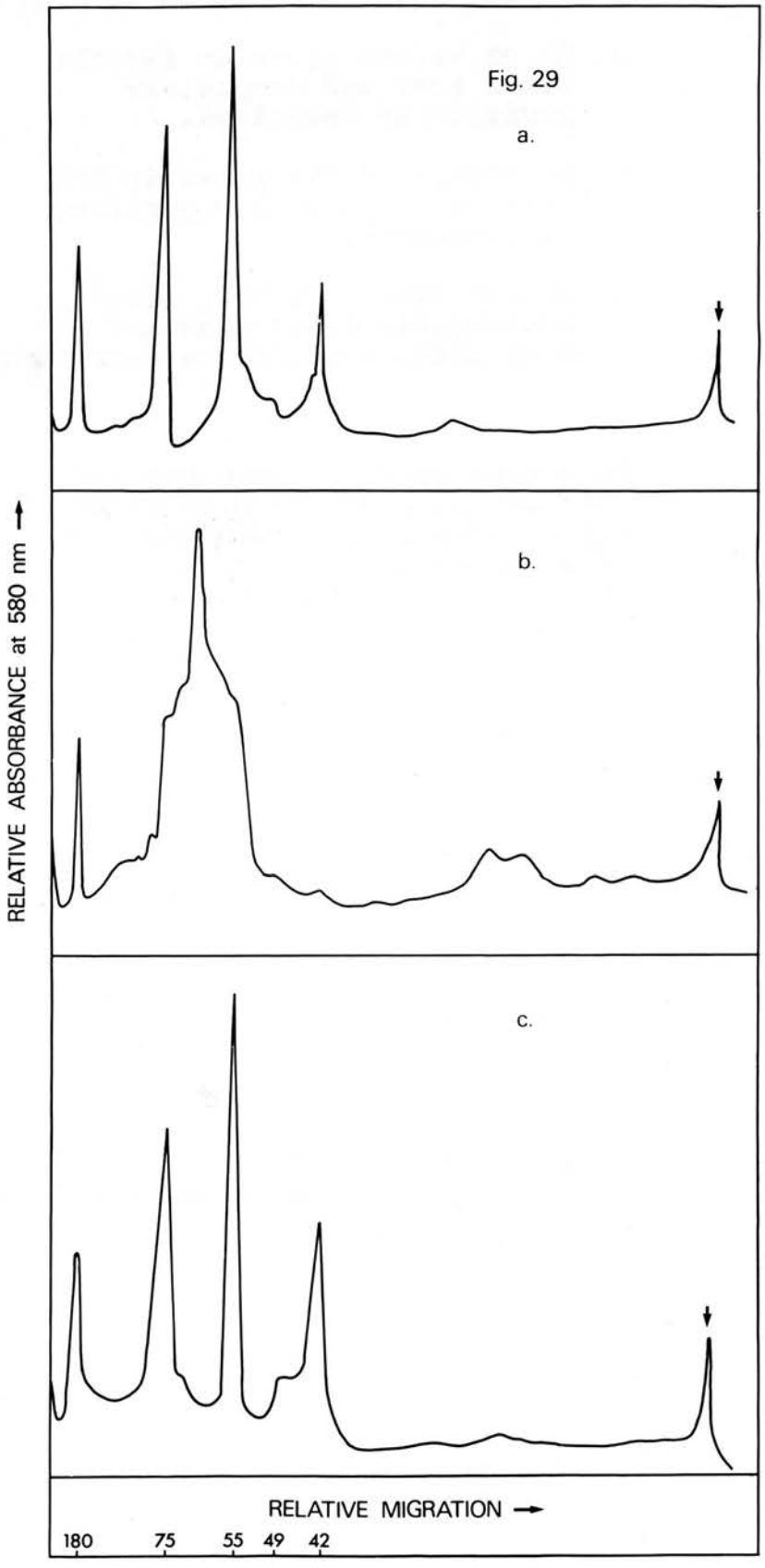


Fig. 29
a.
b.
c.
RELATIVE ABSORBANCE at 580 nm →
RELATIVE MIGRATION →
180
75
55
49
42

Figure 30. Densitometer profiles obtained in a similar fashion to those in Fig. 24, showing the staining of glycoproteins with PAS.

a) fetuin (F) and ovalbumin (A), stained with PAS.

b) fetuin (F) and ovalbumin (A), stained with Coomassie brilliant blue.

c) Italien strain of NDV, grown in fertile hens' eggs and completely purified as described, stained with PAS. Compare this with Fig. 24 a, in which the equivalent gel was stained with Coomassie Brilliant blue. Two peaks of approximately 75,000 and 55,000 daltons are seen.

Ovalbumin (A) is poorly stained with PAS, while fetuin (F) is well stained (Fig 30 a). Both proteins are stained with Coomassie brilliant blue (Fig. 30 b).

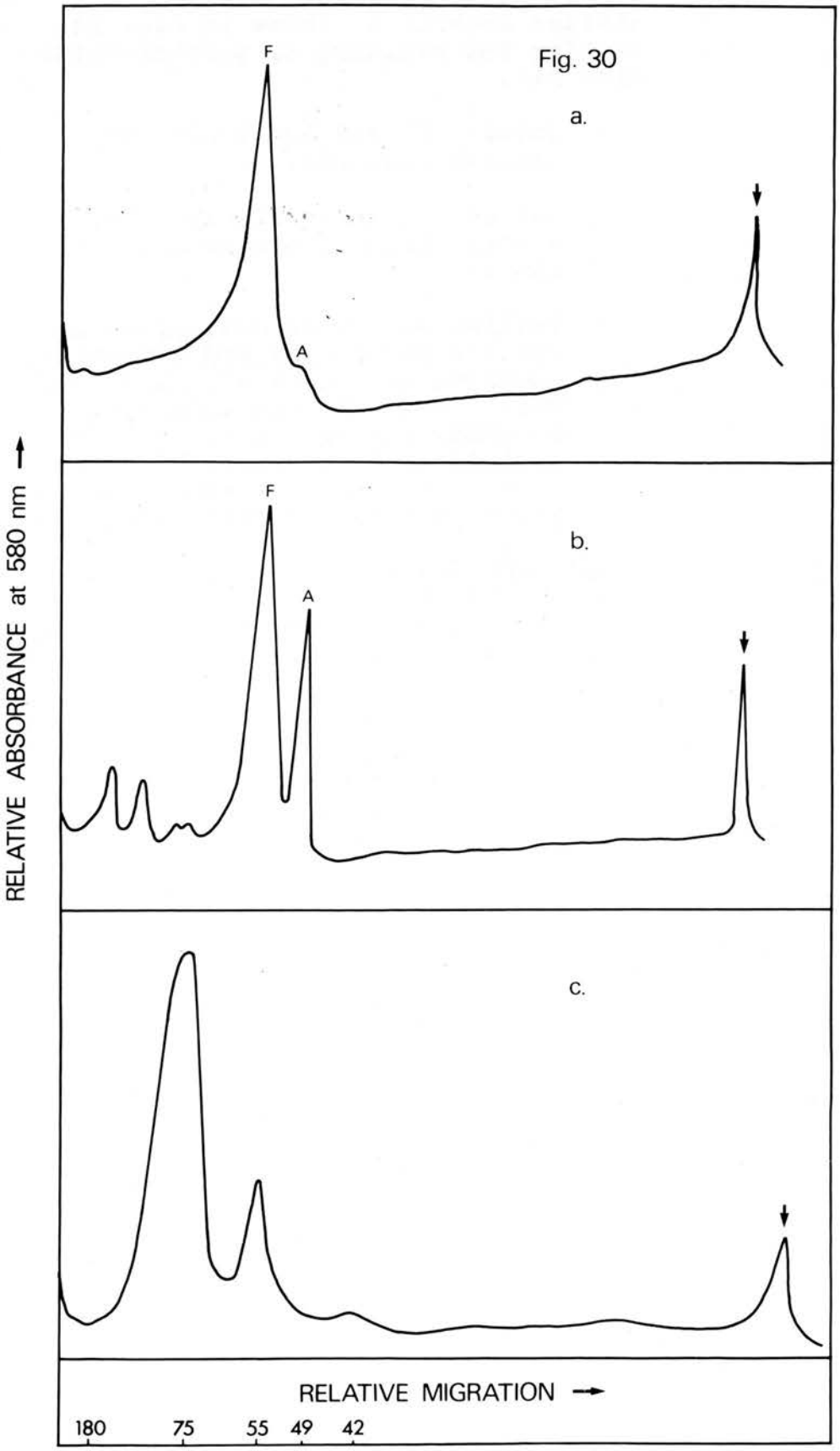


Fig. 30
a.
F
A
b.
F
A
c.
RELATIVE ABSORBANCE at 580 nm
RELATIVE MIGRATION
180
75
55
49
42

particles in allantoic fluid following B1 infection. Thus, because the high protein concentration in allantoic fluid is due mainly to host material, the specific activity will be higher. The relatively small increase in PDA is due to the presence of cellular PDA in the allantoic fluid.

III) COMPARISON OF PROTEINS OF PURIFIED NDV BY PAGE

In Figs. 24, 27, 28, 29, a comparison is made of the gel profiles obtained from purified preparations of B1, Herts, Italien, Ulster and BK pi strains of NDV grown in fertile hens' eggs (Fig. 24 a,b,c and Fig. 29 a, c), the virus released from PK pi and BK pi monolayers (Fig. 27 c and Fig. 28 a), B1, PK pi and BK pi virus grown in HeLa monolayers (Fig. 27 a,b and Fig. 28 b) and B1 virus released from BHK cells (Fig. 29 b).

a) The proteins of strains of NDV, grown in fertile hens' eggs.

It may be seen that three major and up to seven minor peaks are obtained with nearly all strains of NDV grown in chicken eggs (Figs. 24, 29). The approximate molecular weights (m.w.) of these polypeptides were as follows:

DESIGNATION	PROBABLE NATURE	m.w.(daltons)	MAJOR/MINOR COMPONENT
VP 180	protein	180,000	minor
VP 110	protein	110,000	minor
VGP 75	glycoprotein	75,000	major
VP 70	protein	70,000	minor

DESIGNATION	PROBABLE NATURE	m.w.(Daltons)	MAJOR/MINOR COMPONENT
VP 55	protein	55,000	major
VGP 55	glycoprotein	55,000	minor
VP 53	protein	53,000	minor
VP 49	protein	49,000	minor
VP 45	protein	45,000	minor
VP 42	protein	42,000	major

Where possible these proteins have been correlated with those reported in NDV by Moore and Burke (21). It is interesting to note that two of the proteins (VP 70 and VP 45) were not found by Moore and Burke and that two proteins (VP 52 and VP 51) described by these authors were not observed in the present work. The two glycoproteins (VGP 75 and VP 55) were located by examination of duplicate gels of Italien stained either with Coomassie brilliant blue, which stains all proteins (Fig. 24 a), or with periodic acid - Schiff's reagent (PAS), which stains carbohydrate (Fig. 30 c).

The relative sizes of the protein peaks varied but the gel profile was consistent for any particular strain; and the three major proteins were always more prominent than the minor proteins. With the exception of the Italien strain, more of VP 55 was present than of VGP 75. The minor proteins (VP 110 and VP 70) are absent or extremely small in most profiles of virus grown in fertile hens' eggs and may not be truly of viral origin. Since VP 45 is a

a major constituent of allantoic fluid (Fig. 26), it is probable that this protein is a contaminant, although it is consistently present in the Herts strain of NDV (Fig. 24 b).

b) The proteins of NDV released from cell cultures.

The PAGE profiles obtained from proteins of cell-grown virus differed considerably from those of egg-grown strains of NDV, although a similar purification method was employed.

From a comparison of the electrophoretic patterns of B1 virus grown in BHK (Fig. 29 b) and HeLa cells (Fig. 27 a), together with purified preparations of cell-grown BK pi and PK pi virus (Fig. 28 a,b and Fig. 27 b,c), the following proteins may be identified with molecular weights of the approximate value shown:-

DESIGNATION	m.w.(daltons)	MAJOR/MINOR COMPONENT
VP 180	180,000	minor
VP 75	75,000	major
VP 70	70,000	major
VP 55	55,000	major
VP 49	49,000	minor
VP 42	42,000	minor

VP 55 and VP 42 appear to have a lower electrophoretic mobility than is normally found in virus grown in hens' eggs, while VP 110 and VP 53 are absent, or so reduced as to be indistinguishable from

from peaks due to other proteins. PAS staining of BK pi virus (Fig. 28 c) shows the presence of glycoproteins of molecular weights between 50,000 and 80,000 daltons but the sensitivity of this method is such that the precise location of these glycoproteins is impossible. However, the major peak appears to coincide with VP 70 and carbohydrate is present in the region of VGP 75 and VGP 55 although the relative quantity present in each is unknown. Obviously PAS staining suffers from the defect that proteins containing a high percentage of carbohydrate will be more densely stained than glycoproteins with a greater proportion of amino-acids irrespective of the quantity of the two proteins present. Moreover, it was found that the Shandon Disc Electrophoresis system used in this laboratory did not permit more than a total 50 µg protein to be loaded on to a single gel and the PAS method employed (205) did not stain bands containing less than 25-30 µg protein. Thus in most instances viral glycoproteins were not present in sufficient quantities to be stained by this method.

It is probable, therefore, that the following proteins are present in purified preparations of cell-grown NDV:
VP 180, VGP 75, VGP 70, VGP 55, VP 55, VP 49 and VP 42. (The prefix VGP indicates a viral glycoprotein). A protein of 62,000 daltons may be present in the leading edge of VP 55 (Fig. 27 a).

Discussion.

In comparison with virus grown in embryonated hens' eggs, the B1 strain of NDV grown in cells shows the following characteristics:

i) a reduction in the proportion of VP 55 and VGP 55 and VP 42,

ii) the appearance of a protein of 70,000 daltons (VGP 70).

A similar electrophoretic pattern was found for PK pi virus (Fig. 27), whether released from persistently infected cells or infected HeLa monolayers. However, VGP 75 was almost totally absent from virus released from BK pi cells (Fig. 28 a) and was replaced by a major protein of 70,000 daltons, but after passage through HeLa cells, both proteins were seen in the gel profile, which now resembled that of other strains of virus grown in HeLa cells (Fig. 27 a,b). The reappearance of VGP 75 after growth in HeLa cells is associated with an increase in haemagglutinin activity (Table XXIII). Moreover, both B1 and BK pi strains of NDV, grown in fertile hens' eggs have normal gel profiles (Figs. 24 c and 29 b) and higher haemagglutinin activities than those of any cell-grown strain of NDV (Table XXIII).

There are two possible causes of the differences observed between cell-grown and egg-grown NDV namely,

α) contamination of the viral samples by serum or cellular proteins

β) defective production of virus in cell culture.

culture.

The purity of the samples is difficult to assess due to the possibility of the viral proteins and their biological activities being defective. However, neuraminidase activity is the same in both cell-grown and egg-grown B1 virus and the increase in activity is greater in the former during the purification procedure (Table XXX). This suggests that removal of cellular contaminants by the methods employed is at least as effective for material derived from mammalian monolayers as for virus contained in avian allantoic fluid.

However, the possibility of contamination by serum proteins was examined by electrophoresing Herts strain of NDV together with calf serum (Fig. 25) and also re-purifying Herts virus (derived from chicken embryos) after mixing it with serum. A similar experiment was performed with purified BK pi virus that had been released from persistently infected monolayers (Fig. 31). The re-purified Herts virus had a normal PAGE profile. Co-electrophoresis of serum and virus showed that the major serum peak had a molecular weight of approximately 60,000 daltons and that neither this nor a minor protein of 66,000 daltons were related to the increased size of VP 70. However, it is probable that the serum protein of 60,000 daltons is associated with the broad peak of this approximate molecular weight found in profiles of cell-grown virus (Figs. 27, 28) and that serum components are responsible for the two unidentified bands of low

low molecular weight. Thus, although contamination by serum in the nutrient medium may slightly affect the purity of preparations of virus grown in mammalian cell monolayers, the polypeptides (prefixed 'VP' or 'VGP') isolated from these samples are not related to those found in serum. When BK pi virus is co-electrophoresed with BK pi cells, an increase in the size of one peak of approximately 70,000 daltons is found (Fig. 32 b). This protein is not present in uninfected MDBK cells (Fig. 33 a) and thus suggests that the additional polypeptide (VGP 70) is associated with viral infection, and is not a cellular contaminant.

c) The proteins of purified Herts virus released from CF cells.

It was observed that the PAGE profile and other properties of purified Herts strain released from CF cells (Fig. 25 a) was intermediate in many aspects between those of virus produced from mammalian cells and those of NDV grown in embryonated hens' eggs.

Major peaks were seen at the following approximate molecular weights: 180,000, 110,000, 90,000, 70,000, 62,000, 57,000, 49,000 and 44,000. In addition, two minor components with rapid electrophoretic mobility were seeb.

Discussion.

It is possible that, in common with other cell-grown virus, a certain degree of contamination occurs due to

Figure 31. Densitometer profiles obtained in a similar fashion to those in Fig. 24.

a) Co-electrophoresis of equal quantities (µg) of purified B1 virus grown in fertile hens' eggs (shown in Fig. 24 c) and virus released from BK pi cells (shown in Fig. 28 a), indicating that VP 70 does not coincide with VGP 75 and that the VP 55 of egg-grown virus is partially superimposed on the small peak seen in BK pi virus.

b) Co-electrophoresis of equal quantities (µg) of purified BK pi virus (Fig. 28 a) and calf serum (Fig. 31 c). The position of VP 70 is indicated and does not coincide with any of the proteins of calf serum.

c) Calf serum (a photograph of the stained gel is shown for comparison).

Fig. 31

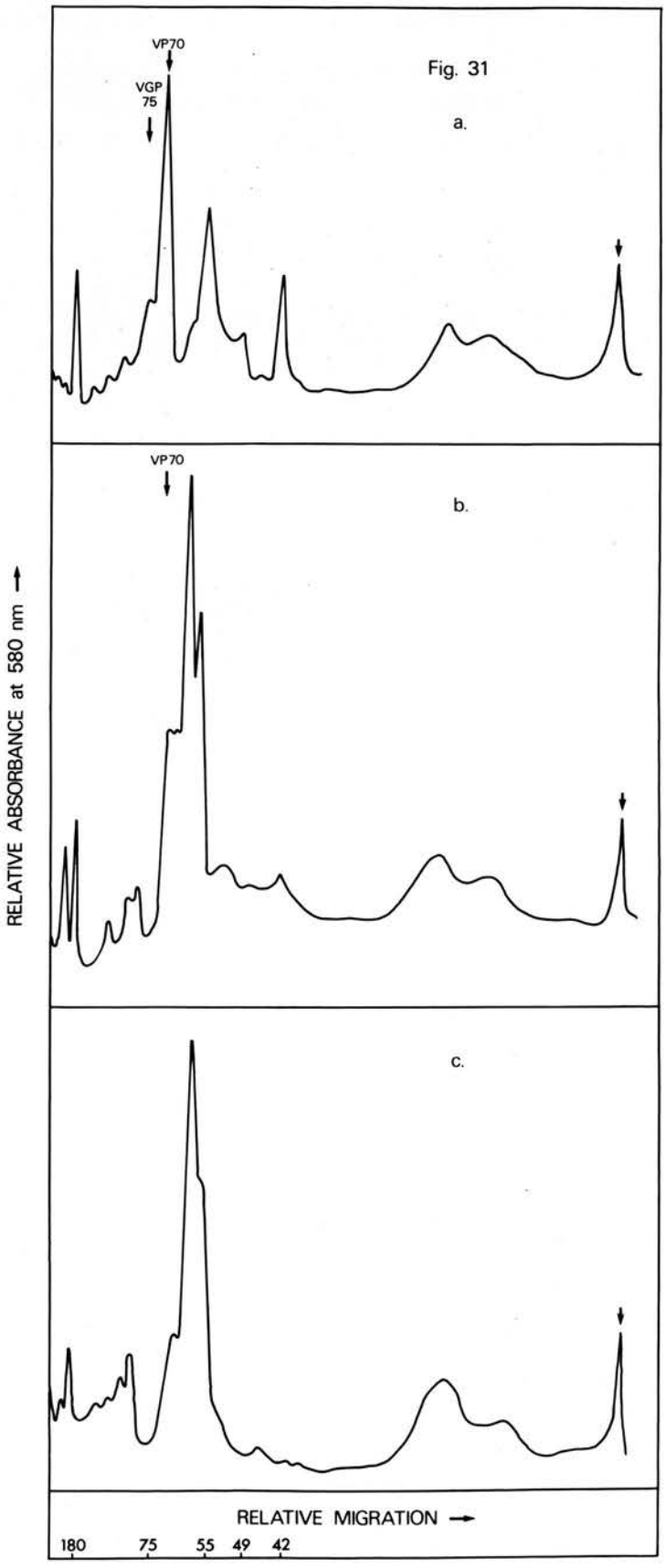

VP70
VGP 75
a.
VP70
b.
c.
RELATIVE ABSORBANCE at 580 nm
RELATIVE MIGRATION
180
75
55
49
42

Figure 32. Densitometer profiles obtained in a similar fashion to those in Fig. 24. The positions of the major cellular polypeptides of uninfected MDBK cells and that of VP 70 are shown by the labelled arrows.

a) Co-electrophoresis of equal quantities (μg) of completely purified virus released from BK pi cells (Fig. 28 a) together with disrupted MDBK cells (Fig. 33a).

b) Co-electrophoresis of equal quantities (μg) of completely purified virus released from BK pi cells (Fig. 28 a) together with disrupted BK pi cells (Fig. 33b).

Note the similarity of the profiles in Figs. 32 a and 32 b as well as the increase in the number of peaks of an approximate molecular weight of 70,000, seen in Fig. 32 a compared with those in Fig. 33 a.

c) Uninfected HeLa cells, disrupted by sonication.

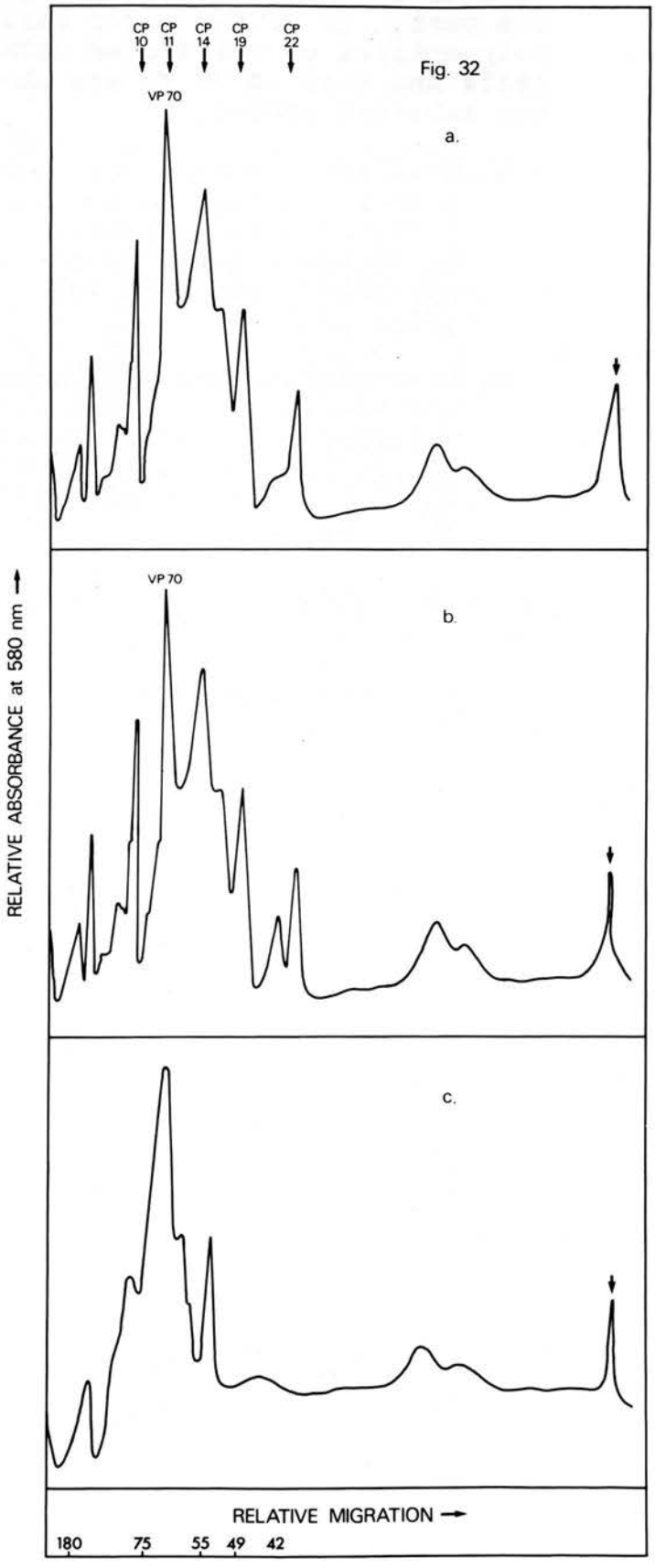

CP 10
CP 11
CP 14
CP 19
CP 22
Fig. 32
VP 70
a.
VP 70
b.
c.
RELATIVE ABSORBANCE at 580 nm →
RELATIVE MIGRATION →
180
75
55
49
42

Figure 33. Densitometer profiles obtained in a similar fashion to those in Fig. 24. The labelled arrows indicate the positions of VP 70 and the major proteins of uninfected MDBK cells.

a) Uninfected MDBK cells, disrupted by sonication (a photograph of the stained gel is shown for comparison).

b) BK pi cells, disrupted by sonication (a photograph of the stained gel is shown for comparison).

c) Uninfected MDBK cells, disrupted by sonication (Fig. 33a) co-electrophoresed with equal quantities (μg) of completely purified B1 virus grown in fertile hens' eggs (Fig. 24 c).

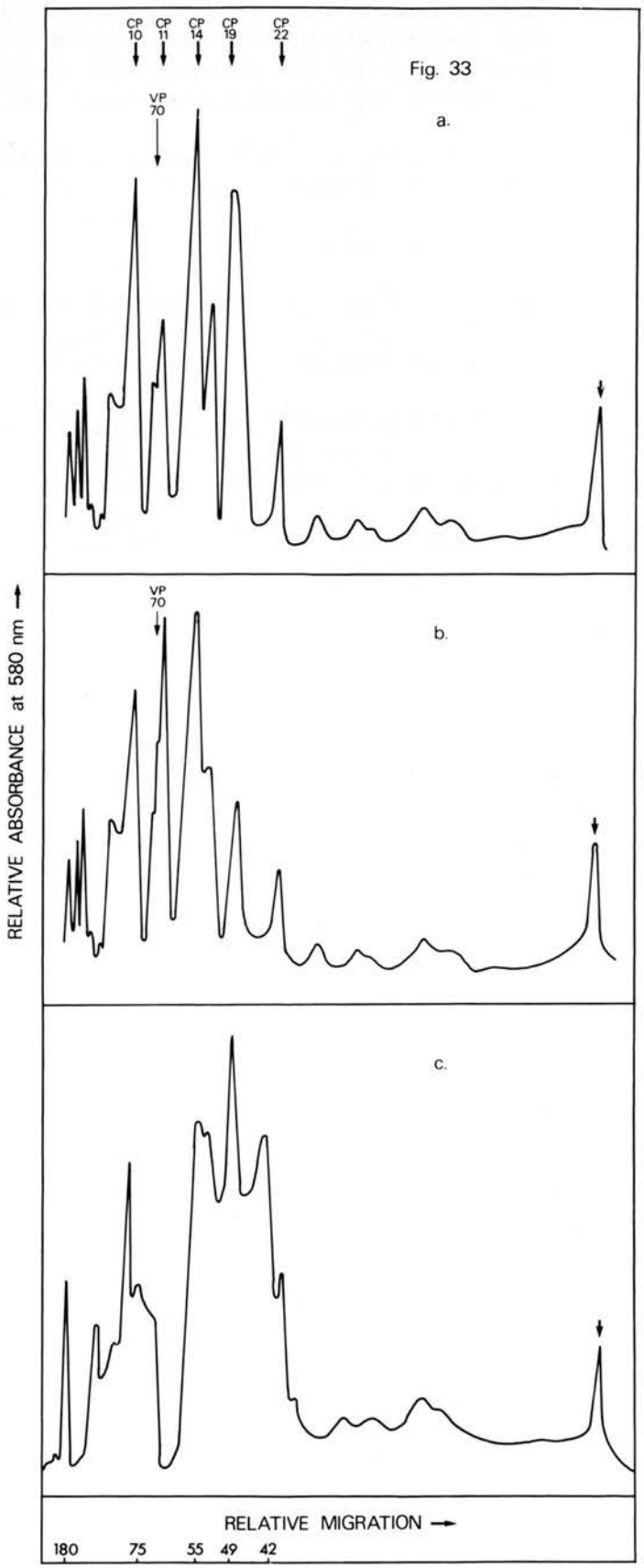
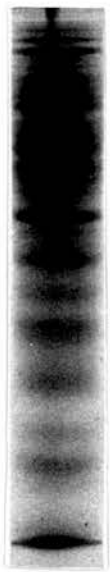

CP 10
CP 11
CP 14
CP 19
CP 22
VP 70
a.
VP 70
b.
c.
RELATIVE ABSORBANCE at 580 nm →
RELATIVE MIGRATION →
180
75
55
49
42

Fig. 33



to the serum in the supernatant fluids. This may account for the peaks of 110,000, 90,000 daltons and those of low molecular weight which are common to all virus particles released into tissue culture fluids. On the other hand, the 110,000 protein may correspond to VP 110 seen in the profiles of virus grown in fertile hens' eggs. It is probable that the polypeptides of 57,000 and 44,000 daltons represent the VP 55 and VP 42 observed in virus purified from chick embryo allantoic fluid. While the 62,000 dalton protein may relate to the largest of the serum polypeptides (Fig. 31 c), it may be viral in origin, since proteins of this approximate molecular weight have been shown to be synthesised by NDV in infected CF cells (50). Thus, although other cell-grown viruses may contain small amounts of VGP 75, VP 55, VP 42 and VP 49, (Figs. 27 a,b,c and 28 a,b), Herts strain grown in CF cells (Herts/CF virus) contains more of these normal viral structural proteins. However, the extraneous peak, VP 70, found in the profiles of the former viruses is also seen in the latter.

Examination of Herts/CF virus under the electron microscope revealed that the virus particles were fragile and contained degenerate nucleocapsid. However, the specific haemagglutinin activity of this virus was nearly eight times higher than that of B1 virus grown in HeLa cells (Table XXIII) and this may be related to the more normal densitometer profile of the former virus (Fig. 25 a) compared with the latter (Fig. 27 a).

(Fig. 27 a). Thus, some of the extra peaks seen in electrophoretic patterns of cell-grown virus may be due to contamination with serum polypeptides and certain of the viral proteins may be destroyed by the action of certain components of the nutrient medium. On the other hand, the 70,000 and 62,000 dalton polypeptides appear to be of viral origin and evidence will be presented in Section H to show that these proteins may be synthesised in cells infected with NDV.

d) PAGE of NDV under non-reduced conditions.

When purified virus released from mammalian cells is electrophoresed following solubilisation with SDS but under non-reduced conditions, the gel profile produced closely resembles that obtained when PAGE is performed under similar conditions on NDV, grown in fertile hens' eggs (Figs. 34, 35). In these circumstances, only six bands of protein are seen on stained gels, five of which correspond to VP 180, VGP 75, VP 55, VP 49, VP 42 while the sixth is a protein of about 62,000 daltons (VP 62). In the case of virus released from BK pi cells and B1 strain of NDV grown in embryonated chicken eggs (Fig. 34, 35), the major proteins are VGP 75 and VP 55. Of the other polypeptides, VP 180 and VP 42 are small and VP 49 and VP 62 are not always present. On the other hand, B1 virus grown in HeLa cells, the Herts strain of NDV produced in fertile hens' eggs, or CF cells and PK pi virus contain large proteins of 180,000 and 55,000

Figure 34. Densitometer profiles of polyacrylamide gels, run under non-reduced conditions, stained with Coomassie brilliant blue and scanned at 580 nm; but otherwise produced in a similar manner to those in Fig. 24 (photographs of the stained gels are shown for comparison).

a) completely purified virus released from BK pi cells (seen under reduced conditions in Fig. 28 a). The position of VP 70 is indicated by the labelled arrow.

b) completely purified Herts strain of NDV, grown in fertile hens' eggs (seen under reduced conditions in Fig. 24 b).

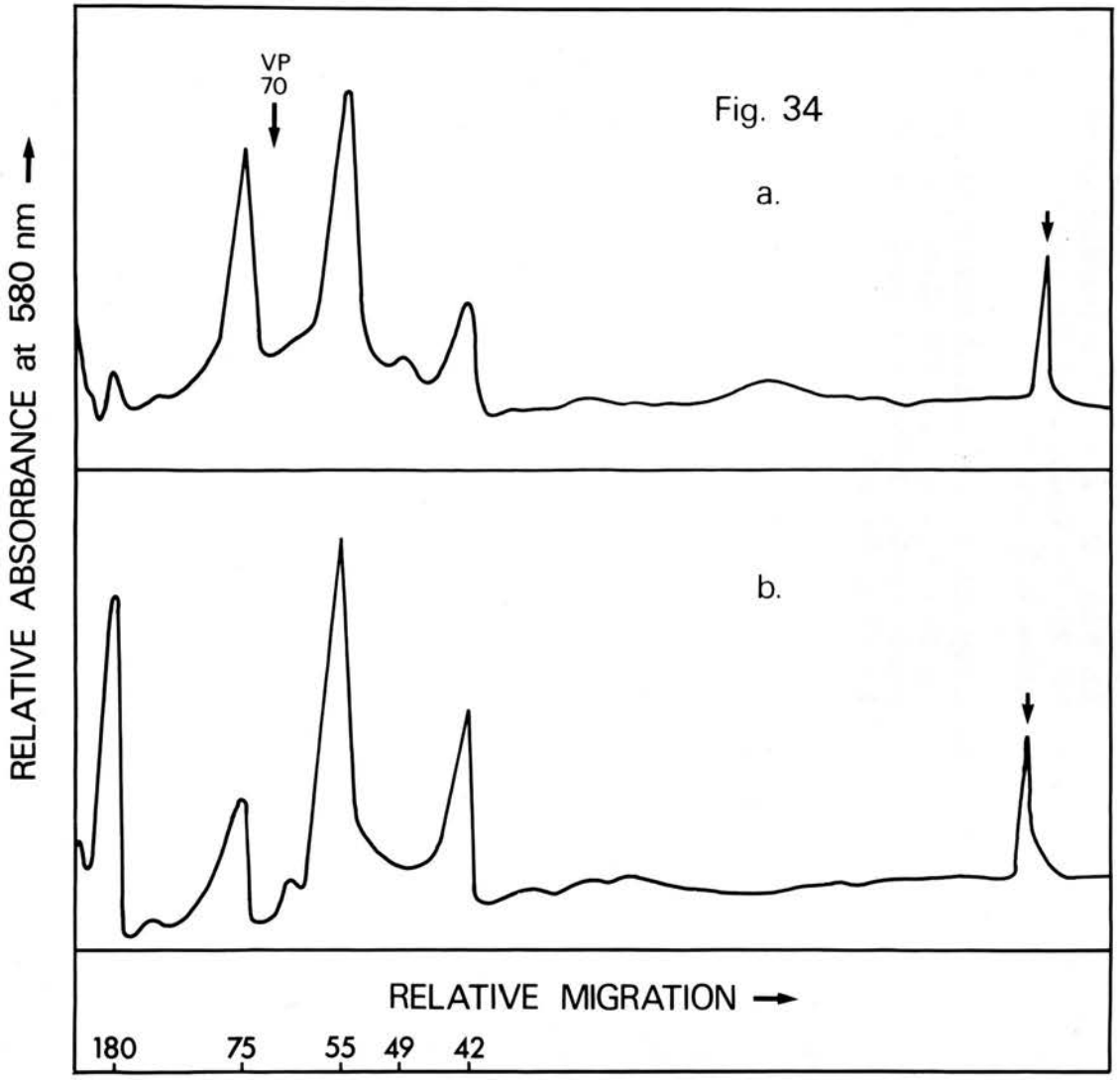

VP
70
a.
b.
RELATIVE ABSORBANCE at 580 nm
RELATIVE MIGRATION
180
75
55
49
42

Fig. 34

Figure 35. Densitometer profiles obtained in a similar fashion to those in Fig. 34, showing the B1 strain of NDV.

a) completely purified virus grown in fertile hens' eggs (seen under reduced conditions in Fig. 24 c).

b) completely purified virus released from infected monolayers of HeLa cells (seen under reduced conditions in Fig. 27 a).

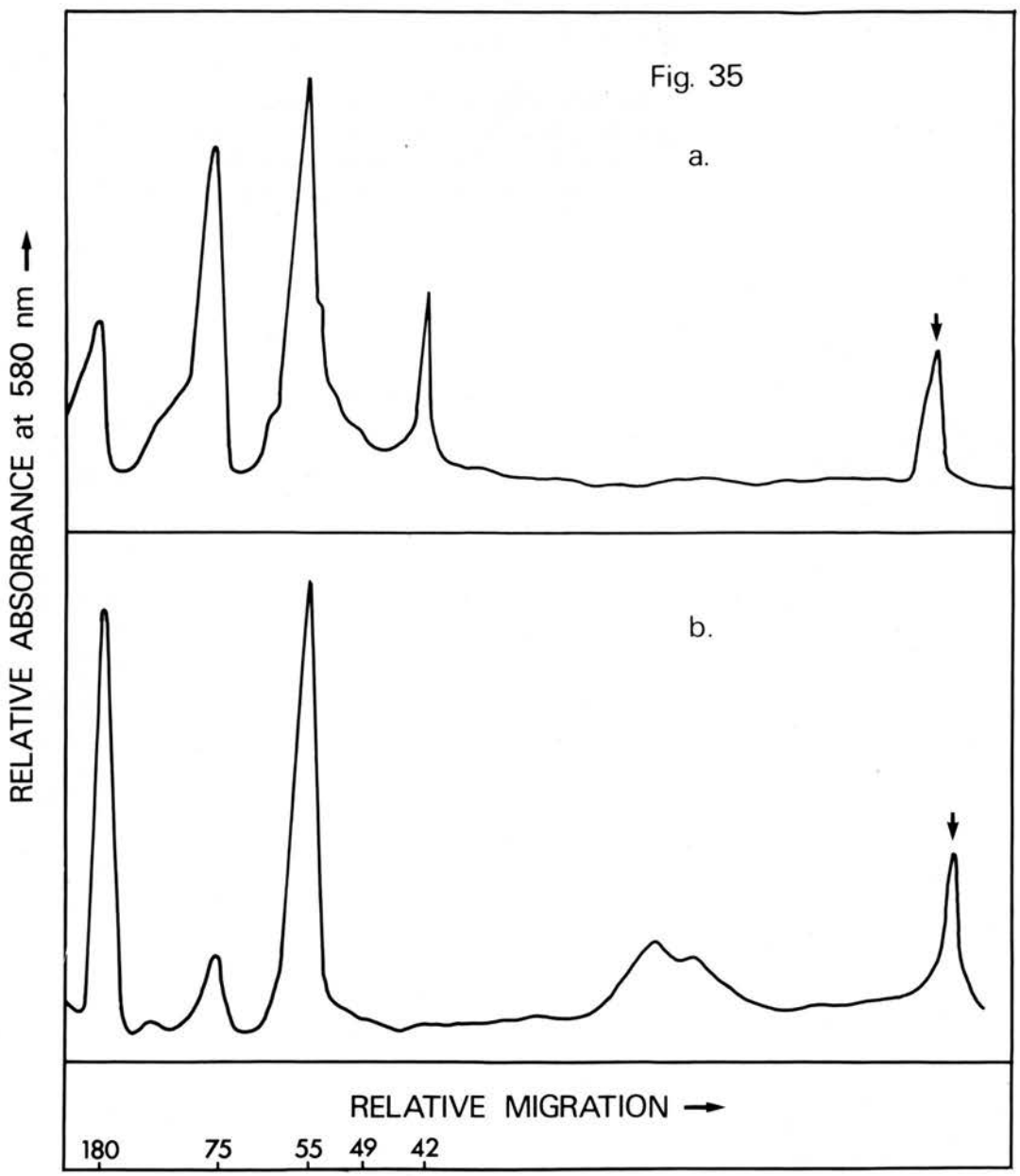


Fig. 35
a.
b.
RELATIVE ABSORBANCE at 580 nm
RELATIVE MIGRATION
180
75
55
49
42

55,000 daltons with small amounts of VGP 75, VP 62, VP 49 and VP 42 (Fig. 34, 35).

Discussion

In this work the profiles of the Herts and B1 strains of NDV grown in fertile hens' eggs closely resemble those described by Moore and Burke (21) using the same strains of virus, electrophoresed under similar conditions.

The similarity of the profiles obtained with cell-grown and egg-grown virus after electrophoresis under non-reduced conditions, suggests that the differences seen, when the viral proteins are run in a reducing environment, may be due to an increased fragility of the proteins formed in infected culture cells.

Thus, the re-appearance of VP 55 in profiles of all cell-grown viruses and of VGP 75, in the case of BK pi virus, is probably due to the maintenance of weak disulphide bonds under non-reduced conditions.

G / THE ASSOCIATION OF BIOLOGICAL ACTIVITIES WITH SPECIFIC STRUCTURAL PROTEINS OF NDV

Introduction.

Early investigations (29) have shown that cleavage of NDV virions results in subvirionic elements and that antibody to these particles specifically inhibits particular properties of the virus.

More recent work has shown that the nucleocapsid structure seen under the electron microscope is associated with VP 55 and contains RNA polymerase activity (38, 44), whereas the structural protein of the envelope spikes (VGP 75) is closely related to haemagglutinin and neuraminidase activities (17, 18). However, VGP 55 is also associated with the envelope of the virion and acts in combination with VGP 75 to cause fusion of culture cells and the closely related phenomenon of haemolysis (170).

The importance of these observations lies in the fact that if any of these or other biological activities not as yet associated with specific viral proteins are a direct cause of virulence, protection from the pathogenic effects of NDV could be provided by antibody to the specific protein on which they are borne.

I) RIBONUCLEOPROTEIN

Introduction.

Ribonucleoprotein (RNP) has been isolated from purified NDV virions (44) and from cells infected with

with NDV (182), and has been shown to be associated with VP 55 (182) and, possibly, RNA polymerase (38, 44).

a) Extraction of RNP from purified NDV.

In the present work, the method of Scheid and Choppin (11) was used to isolate RNP from purified virions (Fig. 36, 38). Strands of nucleocapsid were seen under the electron microscope and the material had a molecular weight of approximately 55,000 daltons. In addition to the major component (VP 55) of this molecular weight, small amounts of VGP 75 and VP 53 were also present in preparations obtained from Ulster or B1 virus that had been grown in developing chicken embryos (Fig. 36). However, in the case of virus released from BK pi cells (BK pi virus) there was contamination of the nucleocapsid preparation and the predominant protein (VP 70) of entire virions was still the major protein associated with this nucleocapsid fraction (Fig. 38).

b) Extraction of RNP from persistently infected BK pi cells.

On the other hand, nucleocapsid extracted from BK pi cells, (Fig. 37 b) by the method of Compans and Choppin (182), co-migrated with VP 55, when subjected to PAGE under reduced conditions.

Discussion

The reason VP 70 is associated with the nucleocapsid fraction obtained by treating BK pi virus with Triton

Triton X-100, may be because a reduced amount of matrix protein (VP 42) is seen in this virus. Under these circumstances, the nucleocapsid and envelope proteins might be bound to one another in an abnormal manner. It is of interest that nucleocapsid of normal molecular weight can be extracted from the persistently infected BK pi cells by centrifuging it to equilibrium in caesium chloride density gradients. This tends to support the above hypothesis since if the nucleocapsid were abnormal, it would band at a different density.

In view of the findings of previous workers, it is not surprising that haemagglutinating, neuraminidase and haemolysing activities did not appear to be associated with the isolated nucleocapsid; nor was the phosphodiesterase function related to this protein. Thus, whether or not these properties are related to virulence, the nucleocapsid protein is not involved.

For the reasons given (see Section E(I)), relationships between RNA polymerase, nucleocapsid and virulence were not investigated.

II) THE LARGE GLYCOPROTEIN

Scheid and Choppin (11) isolated the large glycoprotein of NDV and showed that it bore both NA and HA activities. The method which they later adapted for studies on Sendai virus (18) was used in the present work. The technique involves treating purified NDV with Triton X-100, pelleting virus that had not been fractionated, precipitating all the viral proteins other

other than the envelope glycoproteins by means of buffers of varying ionic concentrations and running the two glycoproteins through a fetuin - Sepharose column to which the polypeptide containing neuraminidase and haemagglutinin is bound. The small glycoprotein passes unhindered through the column and the large glycoprotein is then eluted from the fetuin by raising the temperature of the running buffer.

a) Isolation of the large glycoprotein of strains of NDV, grown in fertile hens' eggs.

When Herts virus was treated in this way, phosphodiesterase activity, associated with polypeptides of molecular weights 180,000 and 55,000 was observed from the first fractions (Fig. 39, 40). Similar results were obtained with the Ulster, B1 and BK pi strains of NDV grown in developing chicken embryos. After the temperature had been raised, a single polypeptide (VGP 75) eluted from the column and was found to be associated with neuraminidase (NA) and haemagglutinin activity (HA) (Fig. 36 b, 39).

b) Isolation of the large glycoprotein of virus released from BK pi cultures.

On the other hand, the envelope fraction obtained by Triton X-100 treatment of virus released from BK pi monolayers contained only VGP 75 and VP 70 (Fig. 38). Preliminary attempts to separate these proteins on Sepharose-fetuin columns above, were unsuccessful. Moreover, although a low degree of haemagglutinating

Figure 36. Densitometer profiles obtained in a similar fashion to those in Fig. 24. The electrophoretic patterns are those obtained at various stages of the fractionation of purified Ulster strain of NDV grown in fertile hens' eggs. The viral structural proteins were separated by the method of Scheid and Choppin (11, 18).

a) The proteins in the pellet of material precipitated after treatment of the virus with Triton X-100 in the presence of 1.0 M potassium chloride ('Pellet 1'). Note the major protein of 55,000 daltons.

b) The proteins in the eluate from a Sepharose-fetuin column, which has been loaded with the final supernatant obtained by treatment of virus with Triton X-100. These proteins passed through the column at 4°C. Note that proteins of 180,000 and 55,000 daltons are present.

c) The proteins which eluted from the Sepharose-fetuin column described in Fig. 36 b, after transfer of the column to an environment at 25°C.

Note that the major protein present is one of 75,000 daltons.

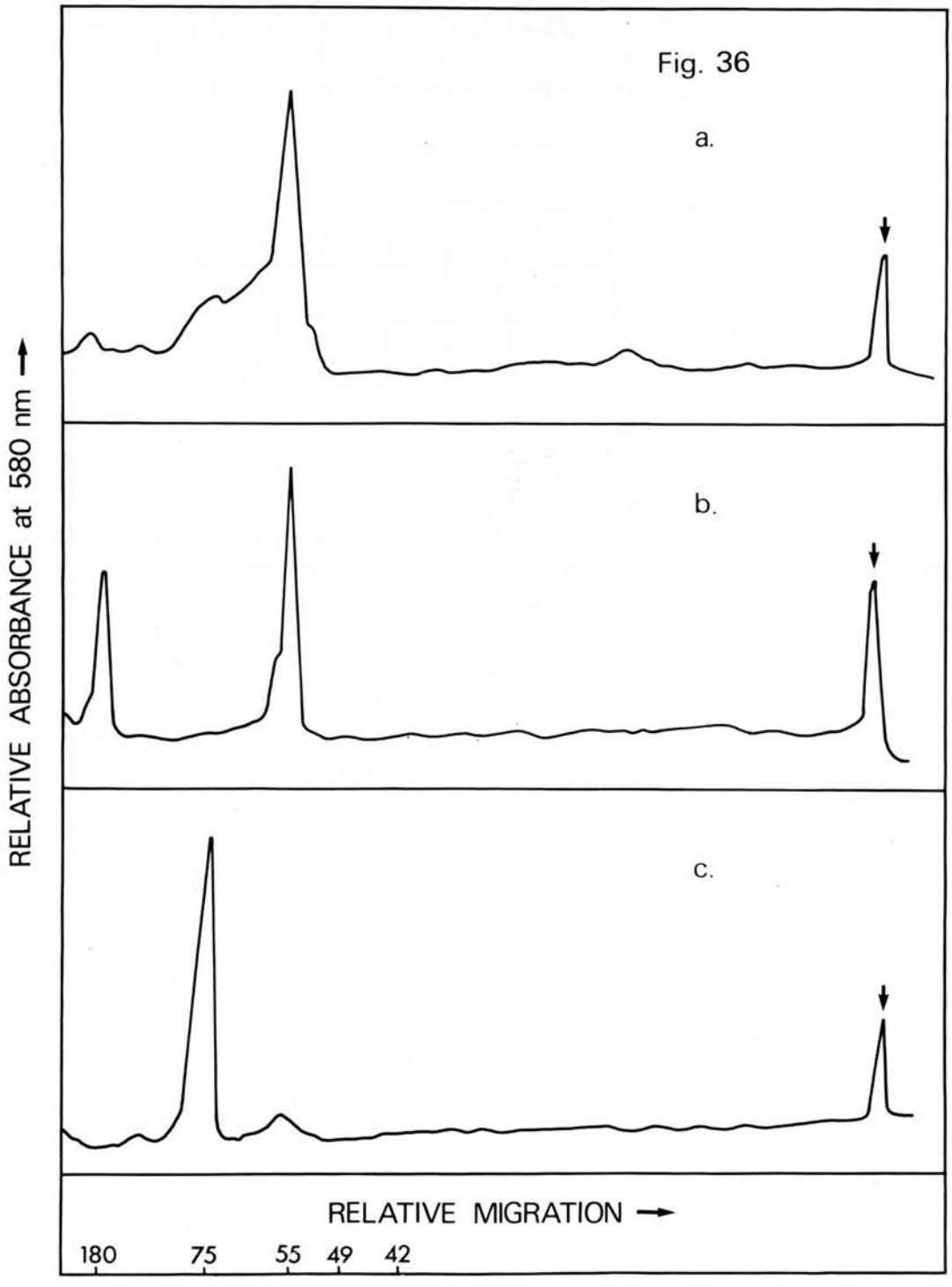


Fig. 36
a.
b.
c.
RELATIVE ABSORBANCE at 580 nm
RELATIVE MIGRATION
180
75
55
49
42

Figure 37. Densitometer profiles obtained in a similar manner to those in Fig. 24. The position of VP 70 is labelled.

a) Purified virus released from BK pi cells (similar to that in Fig. 28 a).

b) Co-electrophoresis of BK pi virus (Fig. 37 a) with purified nucleocapsid-like material extracted from BK pi cells (Fig. 37 c). Note the increase in the size of the peak of approximately 55,000 daltons compared with that in the profile of BK pi virus (Fig. 38 a).

c) Nucleocapsid-like material, extracted from BK pi cells by the method of Compans and Choppin (182).

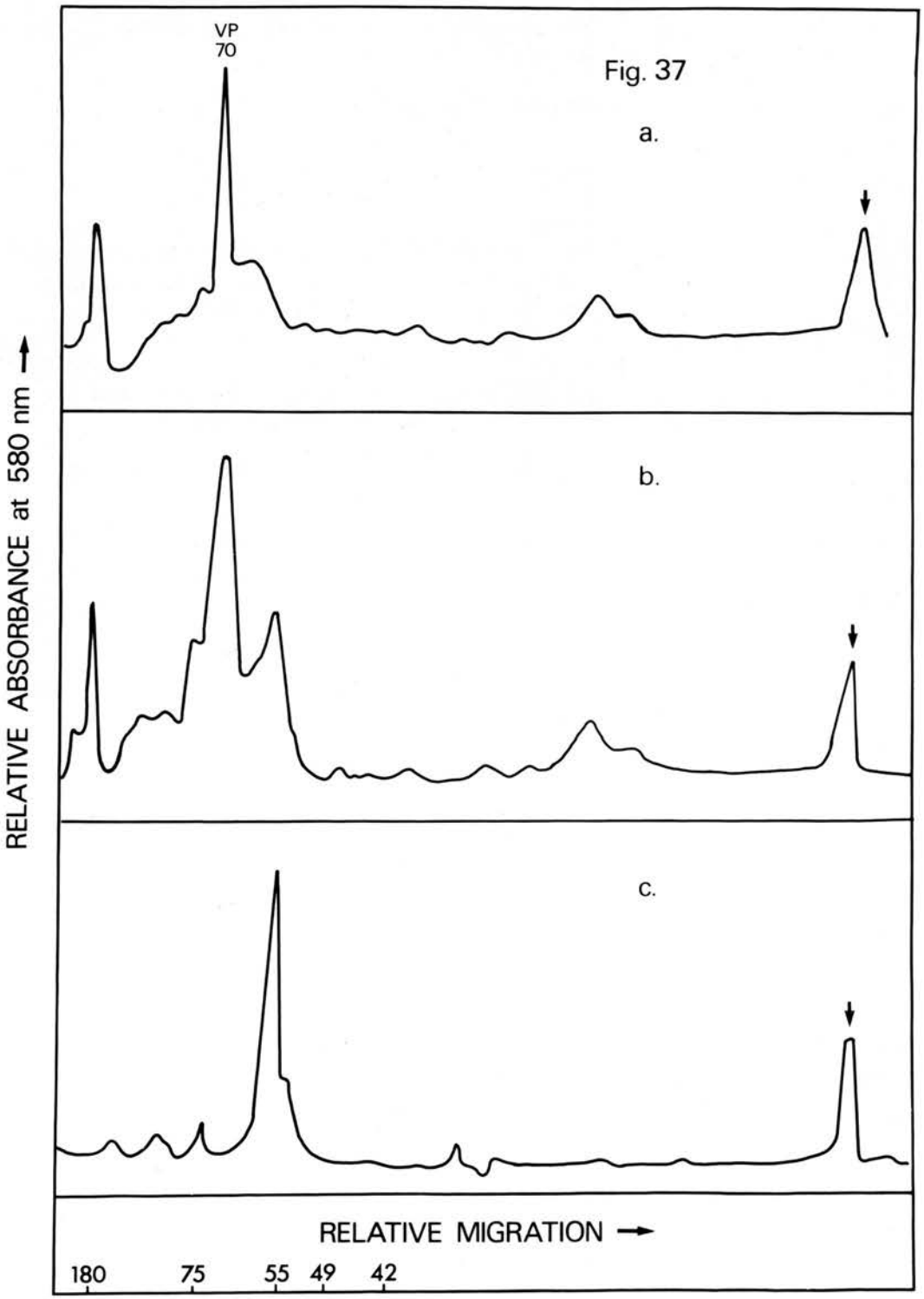

VP
70
Fig. 37
a.
b.
c.
RELATIVE ABSORBANCE at 580 nm
RELATIVE MIGRATION
180
75
55
49
42

Figure 38. Densitometer profiles obtained in a similar manner to those in Fig. 24, showing B1 virus grown in embryonated chicken eggs and virus released from BK pi cells. These viruses had been treated with Triton X-100 (as described in Fig. 36). The position of VP 70 is indicated by the labelled arrow. These profiles should be compared with the equivalent electrophoretic patterns shown in Fig. 36.

a) The proteins in the pellet of material precipitated by Triton X-100 treatment of the B1 strain of NDV in the presence of 1.0 M potassium chloride.

b) The proteins in the pellet of material precipitated by Triton X-100 treatment of virus released from BK pi cells in the presence of 1.0 M potassium chloride.

c) The proteins in the final supernatant obtained by treatment of BK pi virus with Triton X-100.

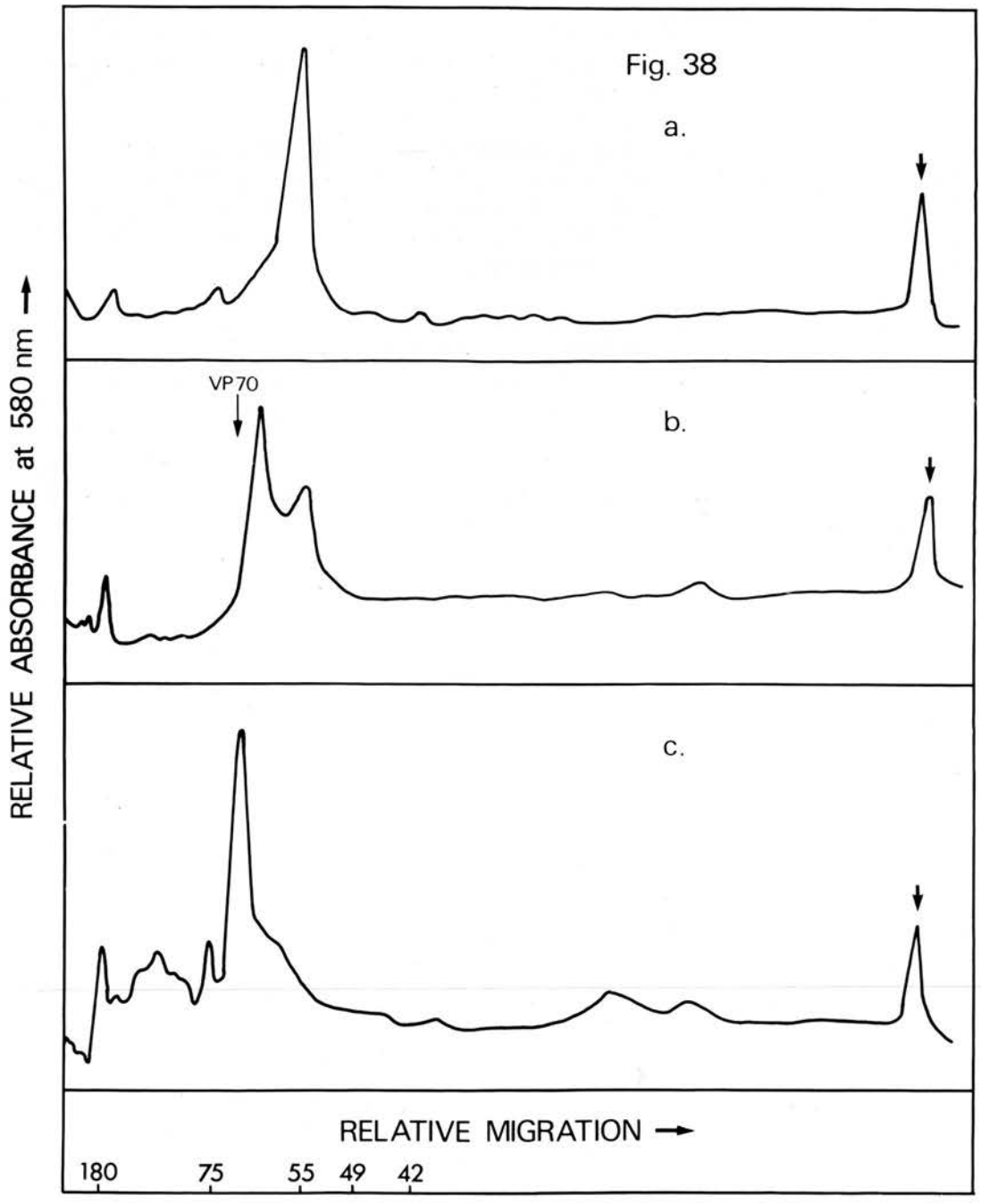


Fig. 38
a.
b.
c.
VP70
RELATIVE ABSORBANCE at 580 nm
RELATIVE MIGRATION
180
75
55
49
42

Figure 39. The association of phosphodiesterase and neuraminidase activities with fractions collected after running the envelope proteins of the Herts strain of NDV through a Sepharose-fetuin column. The viral proteins were obtained by treatment of the purified virus with Triton X-100 in the manner of Scheid and Choppin (11, 18). The column was held initially at a temperature of 4°C and, after the collection of fraction 16, was transferred to an environment at 25°C, as described.

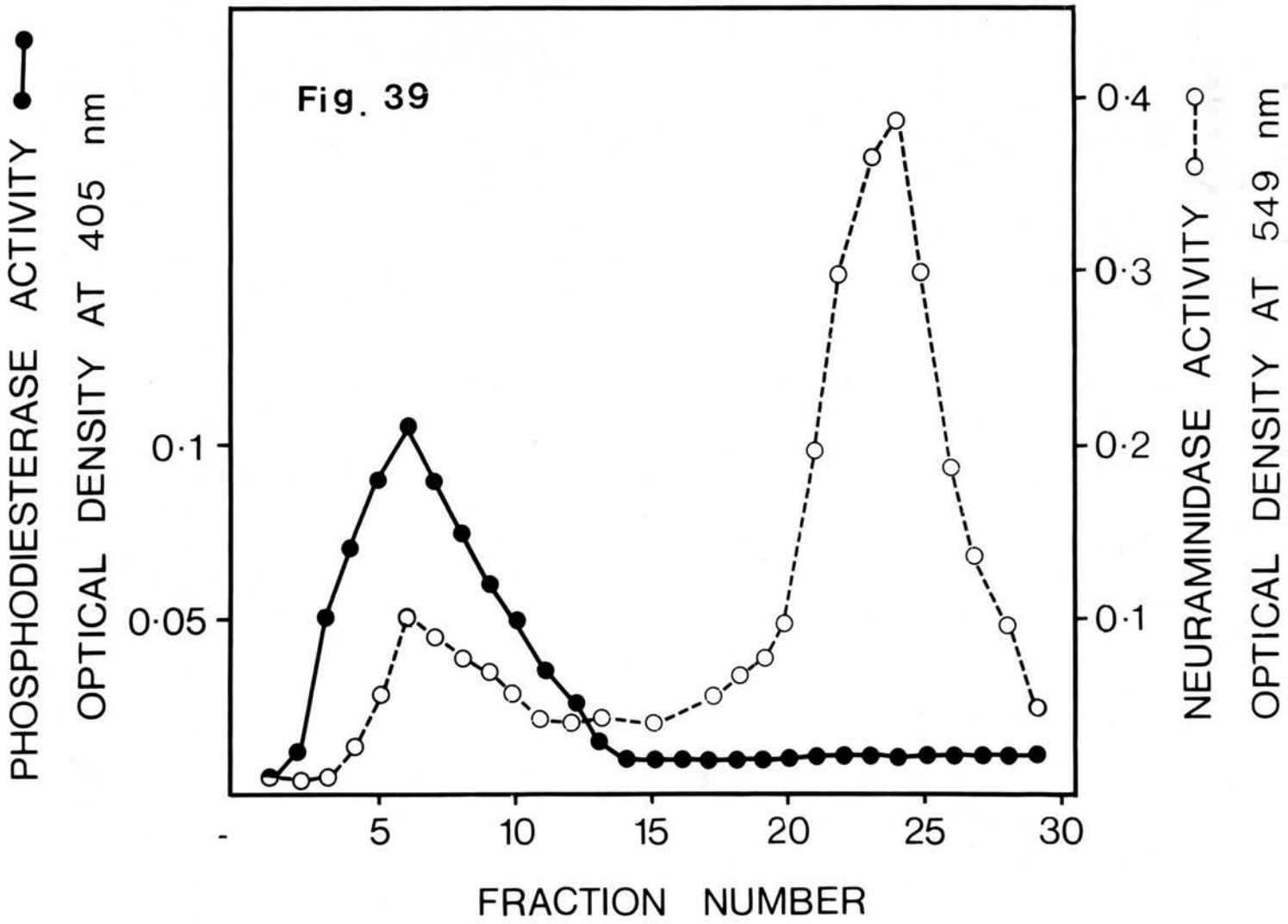


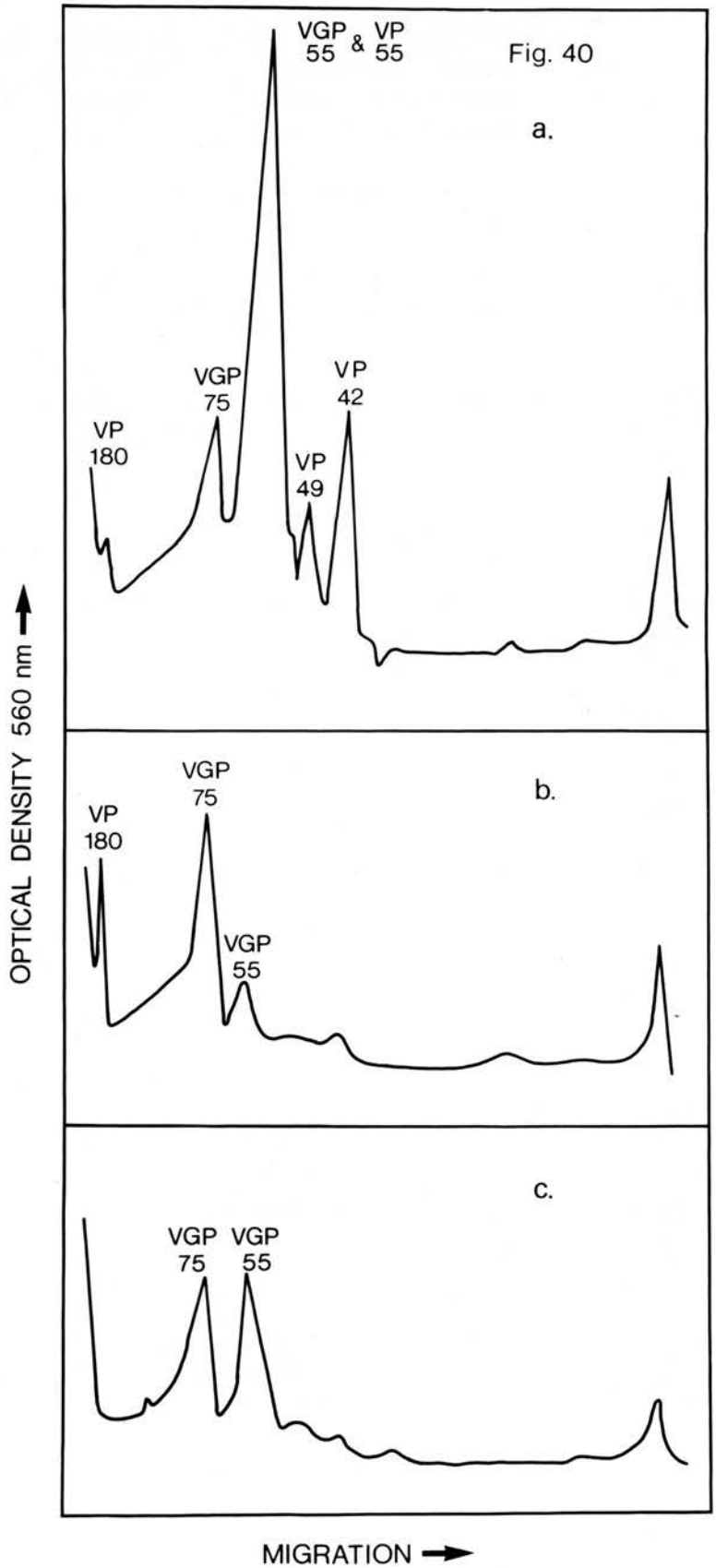
Fig. 39
PHOSPHODIESTERASE ACTIVITY
OPTICAL DENSITY AT 405 nm
0·1
0·05
-
5
10
15
20
25
30
FRACTION NUMBER
0·4
0·3
0·2
0·1
NEURAMINIDASE ACTIVITY
OPTICAL DENSITY AT 549 nm

Figure 40. Densitometer profiles obtained in a similar manner to those in Fig. 24.

a) B1 strain of NDV, grown in fertile hens' eggs and purified through tartrate density gradients (similar to that shown in Fig. 24 c).

b) The proteins found in fractions 22-24, as shown in Fig. 39.

c) The proteins found in fractions 5-7, as shown in Fig. 39.


VGP 55 & VP 55
Fig. 40
a.
VP 180
VGP 75
VP 49
VP 42
b.
VP 180
VGP 75
VGP 55
c.
VGP 75
VGP 55
OPTICAL DENSITY 560 nm
MIGRATION

haemagglutinating ability was associated with this fraction, neuraminidase activity was absent.

Discussion.

These results support the hypothesis that both HA and NA are normally associated with VGP 75.

Haemagglutinin is associated with the envelope proteins of BK pi virus and is also found in persistently infected BK pi cells but neuraminidase activity is absent in both cases. Furthermore, envelope spikes similar to those associated with haemagglutinin in normal strains of NDV (17), are borne on the surface of BK pi virions (Fig. 17), but the quantity of VGP 75 present in BK pi virus is much less than that of other cell-grown viruses (Compare Figs. 28 a and 27). On the other hand, PK pi virus apparently contains normal quantities of VGP 75 in addition to VP 70 (its PAGE profile is (Fig. 27 c) very similar to B1 virus grown in HeLa cells (Fig. 27 a), and its HA activity, like that of B1 virus, is 10 times greater than that of BK pi virus). However, like BK pi virus, PK pi also shows an almost complete lack of neuraminidase activity, while that of B1 virus remains the same, whether grown in HeLa cells or in developing chick embryos.

Thus, the extremely low level of HA activity in BK pi virus is related to the reduced quantities of VGP 75 present; but the defective NA in both persistent strains is apparently due to failure at the site of enzyme activity in this protein. The presence of VP 70

VP 70 in the envelope fraction, following Triton X100 treatment, suggests that this may be related to defective haemagglutinin. Indeed, this polypeptide is also found in both PK pi and B1 viruses released from mammalian cells, in addition to VGP 75, and, although less abnormal than BK pi virus, these strains also have reduced ability to agglutinate erythrocytes. Moreover, although the neuraminidase of B1 released from HeLa monolayers is no less active than that of the same strain grown in eggs, the infectivity of this virus is decreased. Thus, lack of virulence may be related in all these instances to a reduced quantity of VGP 75 (and haemagglutinin). On the other hand, in circumstances in which the quantity of this protein is normal, (e.g. in lentogenic strains of NDV grown in hens' eggs) the evidence suggests that reduced neuraminidase activity may cause decreased infectivity.

These observations may be summarised as follows:

1) Virulence appears to be multifactorial and a deficiency in one or more of these factors may be the cause of avirulence in a particular strain.

2) Two features of VGP 75 must be normal for the virus to be virulent:-

a) the quantity, which seems to be related to the amount of haemagglutinin.

b) the activity of the neuraminidase sites on this protein.

3) In the case of BK pi virus both haemagglutinin and neuraminidase are grossly defective. The former (2a),

(2a), is somewhat deficient in PK pi, while neuraminidase activity is again very much reduced.

B1 virus grown in HeLa cells is normal with regard to neuraminidase activity but is imperfect in regard to the amount of haemagglutinin present.

III) THE SMALL GLYCOPROTEIN

As described, the small glycoprotein of NDV grown in developing chicken embryos was separated from VGP 75 by means of a fetuin-Sepharose column. However, in addition to a protein with a molecular weight of 55,000 daltons, a small quantity of VP 180 was also found. NA activity was very low, haemagglutinin could not be detected but phosphodiesterase activity reached a peak in the fraction associated with these polypeptides (Figs. 36 c, 39, 40). It was further shown that haemolysing activity is not present in these polypeptides nor in the fractions containing VGP 75; nor in a preparation in which small and large glycoproteins were recombined.

Discussion

These results support the finding of other investigators (171, 170), who showed that the presence of phospholipid is necessary, in addition to the two envelope glycoproteins, for the cell-fusion and haemolysing properties of NDV to function. In the present case, any lipid content of the viral envelope is probably removed by the treatment with Triton-X 100.

The envelope fraction obtained by treating BK pi virus

virus with Triton X-100 apparently did not contain any protein of molecular weight, 55,000 daltons, (Fig. 38 c) and inconclusive results were gained when such material was run through a fetuin-Sepharose column. However, since haemolysin and phosphodiesterase have been demonstrated in BK pi virus, the small glycoprotein must be present, either in a reduced quantity or in an unusual form.

H / SYNTHESIS OF VIRAL PROTEINS IN CELLS INFECTED WITH NDV

I) INTRODUCTION

The results obtained in Section 1, showed that there is generally less evidence of viral antigen in cells infected with an avirulent strain of NDV than in cells infected with a virulent strain. On the other hand, it was also demonstrated that although the virus released from persistently infected cells was of low infectivity, nevertheless, over 90% of the cells in a monolayer showed definite evidence of the synthesis of viral antigen.

It was also shown that 'wild-type' virus released from mammalian cells shared several characteristics with virus produced from persistent infections. However these abnormalities were not found in the same strain of virus, when it was grown in developing hens' eggs. In summary, these were low levels of haemagglutinin, haemolysin, phosphodiesterase, and infectivity. In addition, there were alterations in the PAGE profile of virions such that there was a reduction in the quantity of the major proteins of virus grown in chicken embryos (VGP 75, VP 55, VP 42) and an extra peak, VP 70, was seen.

This suggested that infections of mammalian cells, especially those of the persistent type, might result in incomplete synthesis of certain proteins necessary for the full expression of virulence by the virus. However, it appeared unlikely that the RNA of the viral

viral genome was malformed because both PK pi and BK pi viruses regain the properties of virulent virus following passage through chicken tissue. Thus it appears that a cellular factor may prevent the formation of 'normal' viral proteins. It is of interest, therefore, that Sendai virus released from abortive and persistent infections of mammalian cells, contains the precursor of the small glycoprotein of this virus. The phenomenon is apparently due to the absence of a particular enzyme in MDBK and HeLa cells, which is necessary for the cleavage of the precursor protein. This cellular enzyme can be replaced by trypsin, which restores full infectivity and haemolysing activity to the virus (56). On the other hand, rather than the absence of a particular cellular factor, a host protein may inhibit the synthesis of virus. Northrop showed that the use of actinomycin D in a persistent infection of human conjunctival cells with mumps virus, prevented the formation of such a protein and so increased the infectivity of the virus released (146). Both these phenomena were investigated with regard to the persistent infections maintained in this laboratory and the results are reported in Sections 3 and 4. In addition, the formation of viral proteins in infected cells was investigated to find out if abnormal poypeptides were present.

Previous workers have shown that by labelling the proteins formed with two different radioactive isotopes and analysing them by PAGE, both structural

structural and precursor polypeptides can be identified within chick fibroblasts infected with NDV.

Alexander and Reeve (48) described six polypeptides, four of which may be identified with the structural proteins of NDV (VP 180, VGP 75, VP 55 and VP 42) and two others of molecular weights 200,000 and 38,000 daltons, which were thought to be possible precursors of structural proteins. On the other hand, Samson and Fox (50) identified the four structural proteins but only one additional polypeptide (m.wt. 65,000), which was a trypsin sensitive precursor of either VGP 55 or VP 55.

Therefore, in the present work, three allied techniques were used to examine for the presence of non-cellular proteins in infected cells, as follows:-

a) All the polypeptides present in infected or uninfected cells were separated on the basis of their molecular weights by PAGE under reduced conditions and were then examined by staining with Coomassie brilliant blue. In this way the appearance of a particular protein, following infection with NDV, might be detected but could be 'hidden' by a cellular protein of approximately the same molecular weight. However, even in the latter case, a relative increase in the size of the peak may be seen.

b) The proteins are again separated on the basis of molecular weight but, in this instance they are identified by the presence of a radioactive label

label in their amino-acids. By this means, only those proteins synthesised after the addition of the labelled amino-acids to the infected monolayers are educed. Thus, if the synthesis of cellular proteins is inhibited by actinomycin D, the major peaks found will be due to viral polypeptides. However, not all cellular proteins are inhibited by the dose levels of actinomycin D, which allow viral synthesis to continue undiminished.

c) The use of one isotope (e.g. ^{14}C) in infected cells and another (e.g. ^{3}H) in uninfected controls, (the double label technique) enables protein from both to be electrophoresed simultaneously. If the ratio of one isotope to the other is examined it will retain a constant value for cellular proteins but will increase where viral proteins are present.

It should be noted that due to the mechanics of slicing gels before their radioactive content can be measured, the method employing Coomassie brilliant blue is more sensitive in regard to the number and relative size of the peaks present. Thus, the cellular proteins and their molecular weights can most accurately be identified by the latter process.

Methods (a), (b) and (c) above were employed on the persistently infected cells, BK pi, the control line MDBK and MDBK cells infected 6 hours previously with the B1 strain of NDV.

II) DETECTION OF VIRAL PROTEINS

II) DETECTION OF VIRAL PROTEINS

a) Staining of gels with Coomassie brilliant blue

When PAGE was performed under reduced conditions on MDBK cells treated with sodium dodecyl sulphate (SDS) and the resulting gels were stained with Coomassie brilliant blue, no fewer than 26 polypeptides were identified and these were labelled CP1 - CP26 (Fig. 33a). The electrophoretic pattern of BK pi cells was extremely similar (Fig. 33b) with two exceptions, an additional polypeptide was observed of a higher molecular weight than CP 11 and of an identical molecular weight to VGP 75. There was also an increase in the size of the multiple peak (CP 11-14) which is of approximately the same molecular weight as the VP 70 found in released BK pi virions. The major peaks of the PAGE profile of MDBK cells infected with B1 (MDBK/B1) (Fig. 33c) were similar to those of BK pi and MDBK, but in this instance two additional peaks were found - one of much the same molecular weight as VGP 75 and the other corresponding to that of VP 42. Furthermore, the cellular protein peaks (CP 14, CP 15, CP 17 and CP 19) of an approximate molecular weight equivalent to VP 70, VP 55, VP 53 and VP 49 respectively, were all increased in size compared with those of MDBK controls. Co-electrophoresis of BK pi cells with purified BK pi virus reveals co-incidence of peaks at the molecular weights of VGP 75, VP 70 and VP 55. On the other hand, the small amount of

of VP 42 found in BK pi virus is apparently unrelated to a peak in the persistently infected cells,(Fig 32 b). This finding supports the contention that synthesis of VP 42 is reduced in this instance.

Co-electrophoresis of MDBK cells with BK pi virus produces a very similar profile (Fig. 32a). However, it will be seen that the peaks corresponding to the VP 70 and VGP 75 of BK pi virus are not related to any of the cellular proteins and thus four peaks are seen of this approximate molecular weight in place of the two found in PAGE profiles of MDBK (Fig. 33 a).

The close relationship between MDBK and BK pi cells, which justifies the use of the former cell line as a control, is borne out by the near similarity of their gel profiles under reduced and non-reduced conditions (Figs. 33 and 45) as compared with the gross differences seen in the number and molecular weights of the polypeptides found in uninfected HeLa cells (Fig. 32 c). Indeed, no significant differences could be found between BK pi and MDBK cells which had been electrophoresed under non-reduced conditions (Fig. 45). It can also be seen that one of the major cellular peaks under these conditions is of a very similar molecular weight to VGP 75. The implications of this, vis-a-vis the electrophoretic mobilities of the proteins of BK pi virus under non-reduced conditions, will be discussed below.

Discussion

Discussion

Thus, the evidence provided by staining the polyacrylamide gels with Coomassie brilliant blue suggests that VP 70 and VP 55 are synthesised in BK pi cells, in addition to the normal cellular proteins. In MDBK cells infected by B1 strain of NDV, there appears to be greater synthesis of VGP 75 and it is also possible that there is evidence of the synthesis of other proteins (VP 55, VP 53, VP 49 and VP 42) which are found in mature virions. The synthesis of VP 70, which is not found in NDV grown in embryonated chicken eggs, seems to be related to the infection of MDBK cells with NDV.

b) Estimation of labelled proteins in slices of gel.

Only twelve peaks could be found in uninfected MDBK cells, following separation of cellular polypeptides by PAGE under reduced conditions. The proteins are located by the labelled amino-acid that has been incorporated into them, during the final hour of growth. These twelve saliences correspond, as follows, to the 26 proteins found in gels of MDBK stained by Coomassie Blue (Fig. 44 b).

1) CP 2 - 4
2) CP 5 - 6
3) CP 7 - 10
4) CP 11
5) CP 12 - 14
6) CP 15
7) CP 16 - 17
8) CP 18 - 19
9) CP 20
10) CP 21
11) CP 22
12) CP 23

Two additional peaks were found in BK pi cells (Fig. 44 a). These were of approximate molecular weights 75,000 and 53,000 and in addition, the 70,000 dalton peak (within that part of the gel dominated by CP 11) was much enlarged. In the case of MDBK/B1, a large peak was found, which was between 75,000 and 68,000 daltons. Three further proteins, not found in such quantity in uninfected cells, were seen, which had the same electrophoretic mobility as VP 55, VP 49 and VP 42. Thus, the same result was obtained by this method as by staining the gels with Coomassie brilliant blue namely, the tentative identification of the three major structural proteins of NDV in MDBK/B1 cells, and the presence of VP 70 in BK pi cells.

However, following actinomycin D treatment (Fig. 43) at a dosage which inhibited 90% of cellular protein synthesis, a clear difference was noted between the proteins synthesised in uninfected MDBK cells and the two infected cell-lines. It is evident that at five molecular weights, more protein is formed in BK pi cells than (Fig. 43 a) in the control line, and these are approximately equivalent to certain of the structural proteins found in BK pi virus (i.e. VP 75, VP 70, VP 55, VP 49, VP 42). Other of the peaks may also be due to viral proteins but are small and may be caused by residual synthesis of cellular polypeptides of the same molecular weight. On the other hand, only seven peaks are found in preparations

preparations of MDBK/B1 cells (Fig. 43 c) and their electrophoretic mobilities are equivalent to the following proteins found in the B1 strain of NDV released from mammalian cells: VP 180, VP 110, VGP 75, VP 70, VP 55, VP 49 and VP 42.

Discussion

It is emphasised that although some or all of these peaks may be due to viral polypeptides, cellular proteins of approximately the same molecular weights as VP 180, VP 110, VP 70, VP 55 and VP 49 are formed in uninfected MDBK cells that have not been treated with actinomycin D. Thus, unless the cellular proteins can be eliminated by a technique such as double labelling, the relative sizes of the viral proteins synthesised within infected cells cannot be accurately determined. Nonetheless, it may be stated that while infection of MDBK cells with B1 virus results in some inhibition of cellular protein synthesis (Figs. 44 b,c) still fewer non-viral polypeptides are found following treatment of the infected cells with actinomycin D (Fig. 43 b, c). On the other hand, cellular protein synthesis is not inhibited by the persistent infection with BK pi virus, although it appears that viral protein formation is less affected than cellular protein synthesis by actinomycin D (Fig. 43).

c) Identification of viral proteins in infected cells by the double label technique.

Introduction.

Figure 41. Equal quantities (μg) of proteins from cells labelled with ^{3}H-amino acids or ^{14}C-protein hydrolysate, were co-electrophoresed under reduced conditions. The ratio of c.p.m. of $^{3}H/^{14}C$ in 0.75 mm slices of the polyacrylamide gels was corrected as described. The relative positions of the major proteins of MDBK cells and VP 70, in gels run simultaneously and stained with Coomassie brilliant blue, are indicated by the labelled arrows.

▲ ---------- ▲ the ratio of ^{3}H-labelled protein in MDBK cells infected with B1 virus to ^{14}C-labelled protein in uninfected MDBK cells.

● ———— ● the ratio of ^{3}H-labelled protein in BK pi cells to ^{14}C-labelled protein in uninfected MDBK cells.

○ - - - - - - ○ the ratio of ^{3}H-labelled protein in uninfected MDBK cells to ^{14}C-labelled protein in uninfected MDBK cells.

Figure 42. The ratio of $^{3}H/^{14}C$ in 0.75 mm slices of polyacrylamide gels obtained in a similar manner to that described opposite Fig. 41, except that all cells are treated with actinomycin D, as described. The relative positions of the major proteins of MDBK cells and VP 70, in gels run simultaneously and stained with Coomassie brilliant blue, are indicated by the labelled arrows.

▲ ---------- ▲ the ratio of $^{3}H/^{14}C$ for the cells described in Fig. 41.

● ———— ● the ratio of $^{3}H/^{14}C$ for the cells described in Fig. 41.

○ - - - - - - ○ the ratio of $^{3}H/^{14}C$ for the cells described in Fig. 41.

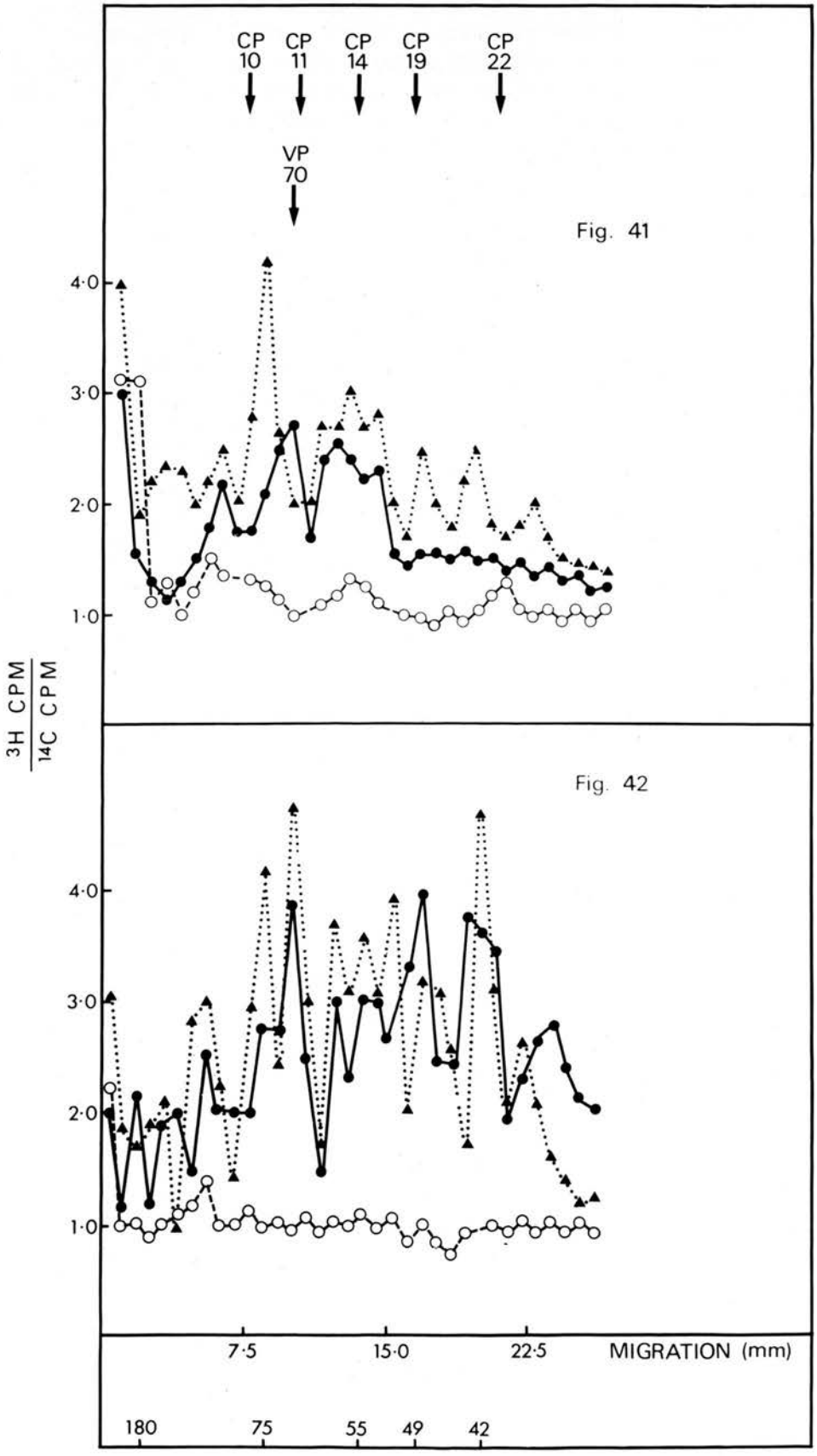

CP 10
CP 11
CP 14
CP 19
CP 22
VP 70
Fig. 41
4·0
3·0
2·0
1·0
3H CPM / 14C CPM
Fig. 42
4·0
3·0
2·0
1·0
7·5
15·0
22·5
MIGRATION (mm)
180
75
55
49
42

Figure 43. The c.p.m. due to ^{14}C in 0.75 mm slices of polyacrylamide gel after electrophoresis under reduced conditions of proteins from the cell-lines described below. The relative positions of the major proteins of MDBK cells and VP 70 in gels run simultaneously and stained with Coomassie brilliant blue are indicated by the labelled arrows.

a) BK pi cells treated with actinomycin D

b) healthy MDBK cells treated with actinomycin D

c) MDBK cells six hours after infection with the B1 strain of NDV and treated with actinomycin D.

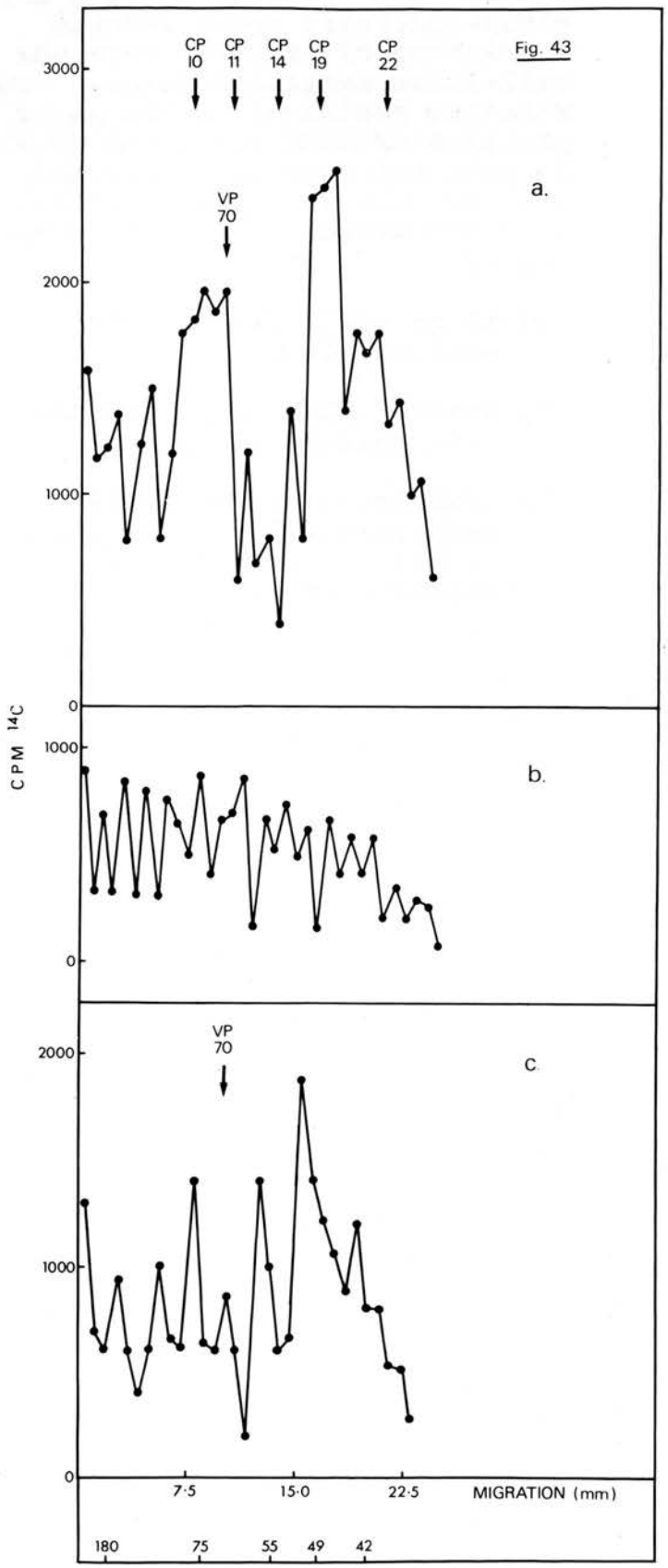


Fig. 43
CP 10
CP 11
CP 14
CP 19
CP 22
VP 70
a.
b.
c.
3000
2000
1000
0
CPM 14C
7·5
15·0
22·5
MIGRATION (mm)
180
75
55
49
42

Figure 44. The c.p.m. due to ^{14}C in 0.75 mm slices of polyacrylamide gels after electrophoresis under reduced conditions of the same cell-lines as in Fig. 43 but in the absence of actinomycin D. The relative positions of the major proteins of MDBK cells and VP 70, in gels run simultaneously and stained with Coomassie brilliant blue, are indicated by the labelled arrows.

a) BK pi cells

b) healthy MDBK cells

c) MDBK cells six hours after infection with the B1 strain of NDV.

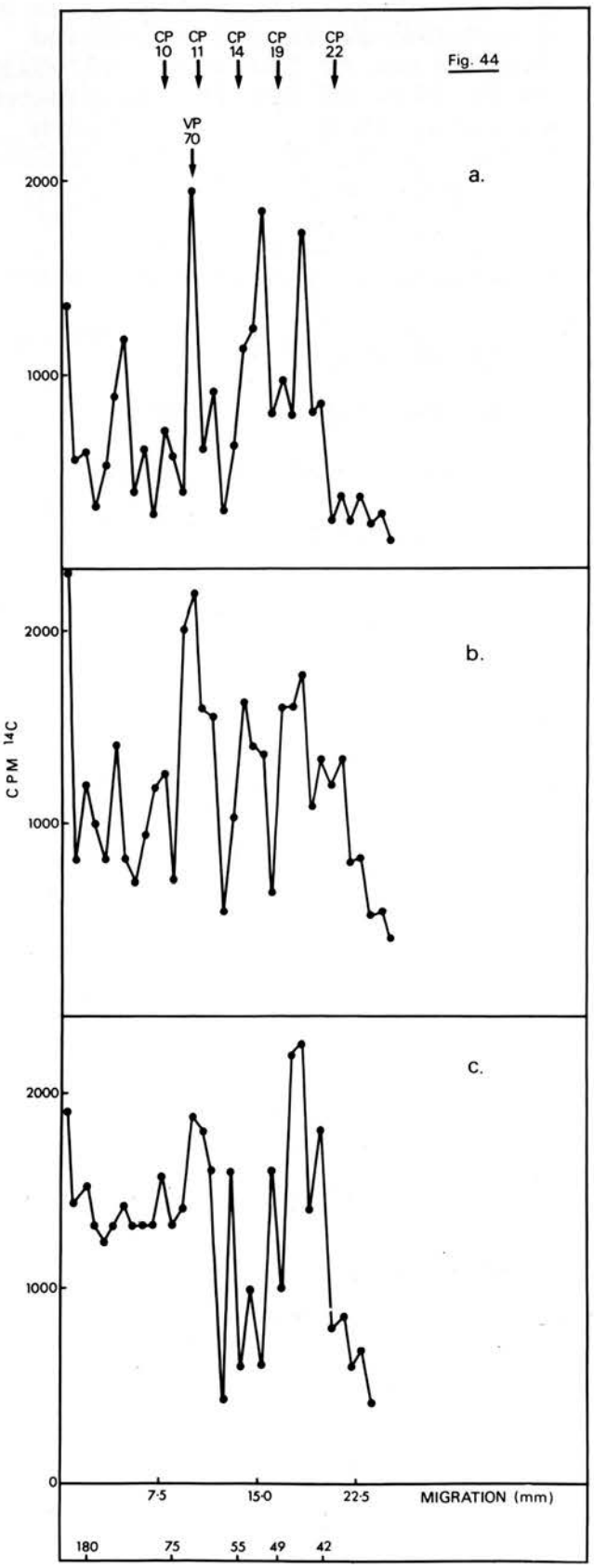
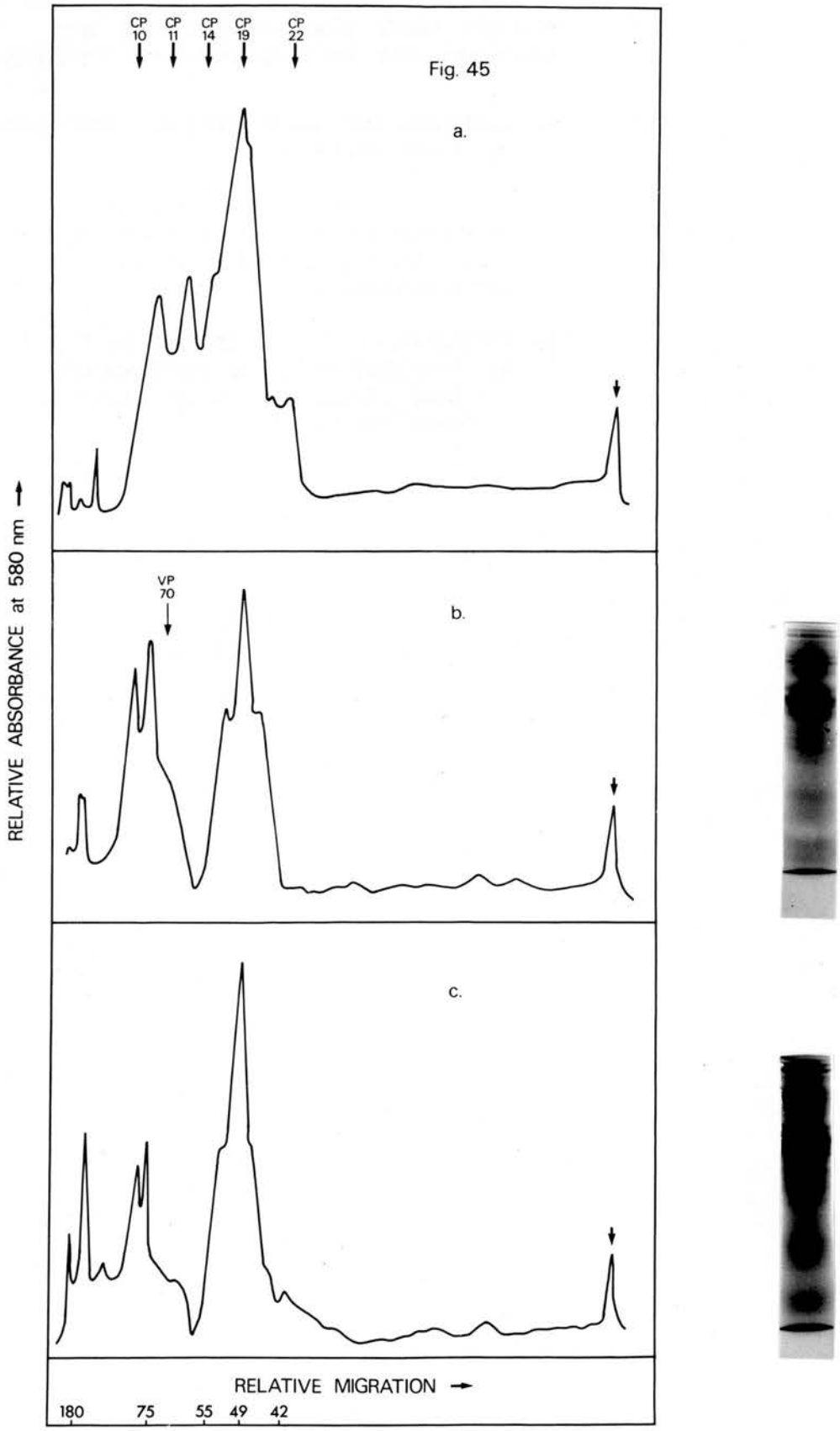

CP 10
CP 11
CP 14
CP 19
CP 22
Fig. 44
VP 70
a.
b.
c.
2000
1000
0
CPM 14C
7·5
15·0
22·5
MIGRATION (mm)
180
75
55
49
42

Figure 45. Densitometer profiles obtained in a similar manner to those in Fig. 24, except that electrophoresis was carried out in non-reduced conditions.

a) Uninfected HeLa cells, disrupted by sonication.

b) BK pi cells, disrupted by sonication (a photograph of the stained gel is shown for comparison).

c) Uninfected MDBK cells, disrupted by sonication, (a photograph of the stained gel is shown for comparison).


CP 10
CP 11
CP 14
CP 19
CP 22
Fig. 45
a.
VP 70
b.
c.
RELATIVE ABSORBANCE at 580 nm
RELATIVE MIGRATION
180
75
55
49
42

Introduction.

Proteins from infected cells labelled with ^{3}H-amino acids, were mixed with proteins from uninfected cells, that were labelled with ^{14}C-amino acids. The mixture was then electrophoresed on a polyacrylamide gel in the usual manner. The gels were sectioned and the ratio of counts per minute (cpm) of ^{3}H to ^{14}C in each slice were calculated as described. Since proteins of similar molecular weight migrate to the same distance, a high ratio of ^{3}H to ^{14}C indicates that more protein of that molecular weight is synthesised in infected than in uninfected cells. Thus, in regions of the gel where viral proteins are present, there are peaks of the $^{3}H/^{14}C$ ratio. However, since cellular protein synthesis is often inhibited by viral infection, the accuracy of the double label method may be affected. Thus, because less cellular protein may be present in infected cells, the ratio of isotope in the infected cell to that in the control will be artificially lowered and should there be a viral polypeptide of the same molecular weight as the inhibited cellular protein, it may be insufficient to raise the ratio to a significant level. Therefore, fewer viral proteins will be found in infected cells that have not been treated with actinomycin D (Fig. 41), than in those that have been treated (Fig. 42), because in the latter case, cellular proteins will be inhibited in both infected and control cells. Furthermore, cellular proteins produced in response to viral

viral infection will also be eliminated by treatment with actinomycin D.

i) The polypeptides found in MDBK cells infected with B1 virus.

Nine polypeptides are found in infected MDBK cells in the absence of actinomycin D, and their approximate molecular weights are: 180,000, 110,000, 75,000, 62,000, 55,000, 53,000, 49,000, 42,000 and 38,000 daltons respectively (Fig. 41). In the presence of actinomycin D, an additional polypeptide of 70,000 daltons is found (Fig. 42). Two very small peaks are seen in the control gels of approximately 110,000 and 55,000 daltons and it is probable that these are due to abnormal quenching in these sections of the gel (Fig. 41, 42).

ii) The polypeptides found in BK pi cells.

Six peaks of $^{3}H/^{14}C$ ratio were seen in BK pi cells in the absence of actinomycin D and their approximate molecular weights were 110,000, 75,000, 70,000, 62,000, 55,000, 42,000 (Fig. 41). In addition, four more peaks were observed in the presence of actinomycin D, with molecular weights of about 200,000, 150,000, 49,000 and 35,000 daltons (Fig. 42).

Discussion

If these peaks of $^{3}H/^{14}C$ ratios described in (i) and (ii) above are due to viral proteins, then it is clear that some peaks correspond to structural polypeptides of NDV. These are VP 110, VGP 75,

VGP 75, VP 55 (and possibly VGP 55), VP 53, VP 49 and VP 42 in the case of BK pi cells, and also VP 180 in the case of MDBK cells infected with the B1 strain of NDV. The peak of 70,000 daltons corresponds to that found in virions released from BK pi cells or HeLa cells infected with B1 strain of NDV (Fig. 28 a, 27 a). It is of interest that proteins of over 200,000 daltons (48), 62-70,000 daltons (211,50) and 38,000 daltons (48) have been reported to be formed in chick fibroblasts infected with NDV.

III) DISCUSSION OF THE RELATIONSHIP BETWEEN THE STRUCTURAL PROTEINS OF NDV AND THE POLYPEPTIDES SYNTHESISED IN INFECTED CELLS.

Since 1969 there have been several reports, in which the character and number of the proteins present in purified preparations of NDV, have been examined by means of separation on the basis of molecular weight by PAGE. Although there is general agreement on the size of the three major polypeptides (VGP 75, VP 55 and VP 42), nine minor proteins have been described but not more than eight have been recorded by any one author. Some of these minor proteins vary considerably from strain to strain (21) and it is possible that not all of them are essential to the structure of the virion. Indeed, some may be traces of precursors while other may be non-structural proteins whose synthesis is required in order that viral replication may proceed;

proceed; but the inclusion of cellular protein in the mature virion is also feasible.

a) VP 180

The largest structural polypeptide of NDV that has been described in the literature has an approximate molecular weight of 180,000 daltons (21). This may be a compound of several of the smaller proteins, since glycoprotein was present in some strains but not in others. VP 180 is a constant but relatively minor component of all strains of NDV under reduced conditions. However, when the virus is solubilised but not reduced before electrophoresis, VP 180 is not found in the majority of strains. In certain strains, including Herts, a polypeptide of this approximate molecular weight replaces VGP 75 as the third major polypeptide under non-reduced conditions, and this has been shown to be composed of proteins which separate under reduced conditions and co-migrate with VGP 75 and VP 55 (21). A polypeptide of this size was shown to be synthesised in chick embryo fibroblasts infected by the Herts strain of NDV and was demonstrated by PAGE under reduced conditions (50). The relationship of the protein which cannot be reduced by mercapto-ethanol and is synthesised in infected cells, and the compound seen under non-reduced conditions is not clear.

In the present work, VP 180 has been shown to occur, under reduced conditions, in all strains of NDV examined whether they were grown in embryonated chicken

chicken eggs or released from mammalian cell cultures. In addition, VP 180 has been shown to be synthesised in MDBK/B1 cells and possibly in BK pi cells also. However, in the latter case, two additional unidentified high molecular weight proteins are found. VP 180 appears to be related to the envelope glycoproteins of the virus, because it appears in the same fraction as VGP 75 and VGP 55 following Triton-X 100 treatment (Fig. 40). The large polypeptide found under non-reduced conditions, is a compound of viral glycoprotein and other viral material (21) found in the infected cells. In the present studies, it was confirmed that a high molecular weight protein was formed under non-reduced conditions in the case of Herts virus grown in developing hens' eggs but was not present in the B1 strain of NDV grown in a similar manner. On the other hand, in B1 virus that had been released from HeLa or BHK cells, this large protein was substituted for VGP 75 and VP 70 seen under reduced conditions. It is therefore suggested that in this instance, these proteins, in addition to VP 55, are involved in the agglomerate. Although the PAGE profile of BK pi virus is altered by running the preparation under non-reduced conditions, the high molecular weight protein is not formed. Thus it is probable that variations in VP 180 under non-reduced conditions are not significant in the normality, or otherwise, of strains of NDV, and the protein is likely to be a compound of VP 55 and VGP 75 or VP 70.

b) <u>VP 110</u>

b) VP 110

The next largest protein found in NDV has a molecular weight of 110,000 daltons. Sendai virus has been shown to closely resemble NDV in many of its properties and to contain structural polypeptides, many of which are of similar molecular weights to the equivalent proteins of NDV (11, 18). The exception is the presence of a polypeptide that has a higher molecular weight than the largest of the two glycoproteins (VGP 75 of NDV) and which is believed to assist in RNA polymerase activity (46). No representative of this protein has been identified in NDV. However, it is possible that VP 110, reported by Moore and Burke (21) may have this role. VP 110 has not previously been shown to be synthesised within cells infected by NDV.

In the present work, VP 110 was usually found as a very small peak or shoulder on the leading edge of the VGP 75 following PAGE under reduced conditions in all strains of virus grown in developing hens' eggs. This protein was difficult to distinguish in virus released from mammalian cells and was absent from any strain of virus electrophoresed under non-reduced conditions. A protein of this approximate molecular weight was synthesised in both MDBK/B1 and BK pi cells in clearly detectable quantities. In liberated virions, the comparatively small peaks due to this protein, suggest that it is mainly synthesised in cells to aid viral replication and is not an essential

essential component of the mature virion. This suggestion would not conflict with its possible role in the formation of viral RNA, which has been discussed above. Although the presence of this protein in the mammalian cells infected with NDV does not necessarily imply that it is in an active form, it seems unlikely that a defect in VP 110 contributes to a lack of virulence in this instance.

c) VGP 75

VGP 75 is a major structural protein seen in all strains of NDV under reduced conditions of PAGE (21). It is a glycoprotein and is synthesised in infected chick embryo fibroblasts (48). However, virus in a non-reduced state does not always exhibit a protein of this molecular weight. For example in the Ulster strain of NDV an aggregate protein of about 180,000 daltons is formed, which is composed of VGP 75 and VP 55 (21).

In the present work, a protein of this molecular weight was found in all strains of NDV, whether grown in eggs or released from mammalian cells. However, the size of the peak seen following PAGE of BK pi virus is much smaller under reduced conditions than that obtained with any other strain. On the other hand, the VGP 75 of the other strain of persistent NDV investigated (PK pi) was normal in this respect. Apparently this distinction is due to the small quantities of this polypeptide that are synthesised in BK pi cells compared

compared with control cells infected with the B1 strain of NDV (Fig. 42). However, the situation is made more complex by the PAGE profile obtained from viral preparations in a non-reduced state.

In these latter circumstances, most strains of NDV grown in developing chicken embryos contained a protein of 75,000 daltons. However, the VGP 75 of Herts virus forms a mixture with other viral proteins (probably by means of disulphide bonds) and this migrates with an apparent molecular weight of about 180,000 daltons (Fig. 34).

The electrophoretic pattern of B1 virus released from HeLa cells (Fig. 35), resembles that of the Herts strain of NDV under non-reduced conditions (Fig. 34) although the VGP 75 of B1 virus grown in hens' eggs is stable in a non-reduced state. Since a protein of 75,000 daltons is also found under these circumstances in a preparation of BK pi virus (Fig. 34), the binding of VGP 75 into a high molecular weight compound is probably of relatively minor importance.

d) VP 70

It is significant that VP 70, which is present exclusively in infected mammalian cells or in virus released from them (Figs. 27, 28), disappears under non-reduced conditions and is supplanted by VGP 75, in the case of BK pi virus (Fig. 34). It would appear therefore, that VP 70 is composed wholly or partly of an incomplete form of VGP 75 which regains its normal

normal molecular weight by disulphide bonds formed under non-reduced conditions. This hypothesis might also account for the defective haemagglutinin found in all virus released from mammalian cells and especially in BK pi virus, from which VGP 75 is almost completely absent in reduced preparations (Fig. 28 a). On the other hand, concomitant with the disappearance of VP 70 and the appearance of VGP 75 in BK pi virus under non-reduced conditions, is the increase in size of the VP 55 peak. This argues that VP 70 may be a compound of incomplete nucleocapsid as well as haemagglutinin. Additional evidence in favour of this hypothesis is the fact that after BK pi virus is treated with Triton X-100, VP 70 is found both in the fraction containing the envelope glycoprotein and that bearing VP 55 (Fig. 38). On the other hand, a peak of about 75,000 daltons is seen in the PAGE profiles of BK pi and uninfected MDBK cells in a non-reduced state, and thus the poypeptide of this size observed in BK pi virus electrophoresed under non-reduced conditions may be due to cellular proteins incorporated in the virion. Nevertheless, since a protein of 70,000 daltons is synthesised in infected cells in the presence of sufficient actinomycin D to cause a 90% inhibition in the formation of cellular protein (Fig. 42), this hypothesis is untenable.

It is interesting to note that a protein of this approximate weight has previously been described in chick fibroblasts infected with some strains of NDV (211).

(211). Thus, it is probable that VP 70 is a viral protein, or a mixture of viral polypeptides, that are incompletely formed and which may be related to both VGP 75 and VP 55, found in normal virus.

e) VP 60-65

A protein of between 60,000 and 65,000 daltons has been reported in association with NDV in the two following circumstances:-

i) in infected chick embryo fibroblasts under reduced conditions where it is believed to be a precursor of a viral polypeptide of 55,000 daltons which may be the nucleocapsid of small glycoprotein of NDV (50).

ii) when the purified virions are examined under non-reduced conditions, the molecular weight of the small structural glycoprotein (VGP 55) of NDV has been shown to be between 60,000 and 65,000 daltons (21). A protein of this molecular weight has not been found in liberated virions of NDV examined by PAGE under reduced conditions.

In the present work, a diffuse area of approximately this molecular weight is seen on the leading edge of VP 55 in analyses of BK pi virus under reduced conditions but this resolves, in non-reduced preparations, into two distinct peaks of which the smaller is 62 - 63,000 daltons, and the larger is 55,000 (Compare Figs. 28 a and 34). A similar effect is noted in the case of B1 virus released from HeLa cells, although, in this instance, the virus preparation was treated with trypsin and then electrophoresed in a reduced state (See Section 4,

(See Section 4, Fig. 46). Here, the diffuse area in front of the 55,000 peak seen before trypsin treatment, is removed and is compensated for by an increase in the height of VP 55. A similar phenomenon has been demonstrated in Sendai virions released from MDBK cells and has been shown to be due to the cleavage of an inactive precursor of the small envelope glycoprotein by trypsin. The incorporation of the precursor molecule into the mature virus particle is believed to be due to the lack of the necessary enzyme in MDBK cells (56) (11).

In the present case, it is possible that the small glycoprotein (VGP 55) of NDV is synthesised as a protein of 62,000 daltons, with both peptide and disulphide bonds. It is also suggested that the peptide bonds are cleaved prior to the release of NDV from fertile hens' eggs and that under non-reduced conditions, VGP 55 maintains its molecular weight of 62,000 daltons by means of the disulphide bonds. However, the latter linkages are broken when PAGE is carried out under reduced conditions and the molecular weight of VGP 55 is then found to be 55,000 daltons.

It is also suggested that cleavage of the peptide bonds of VGP 55 does not occur in mammalian cells and that therefore, the molecular weight of VGP 55 of NDV released from mammalian cells is 62,000 daltons, whether PAGE is carried out under reduced or non-reduced conditions. It is believed that VGP 55 is largely inactive so long as the peptide bonds are intact and

and that this is the cause of the reduced haemolysin activity of NDV grown in mammalian cells. Treatment of the virus with trypsin destroys the peptide bonds, thus restoring haemolysin activity and producing a molecular weight of 55,000 daltons under reduced conditions.

Thus the evidence suggests that there is a relationship between the reduction in certain biological activities of virus released from mammalian cells and the defective structure of the VGP 55 of such virions.

f) VP 55 and VGP 55

Two proteins with a molecular weight of 55,000 daltons have been consistently identified in preparations of NDV analysed by PAGE in reducing conditions. The first, VGP 55, which has been discussed previously, is a glycoprotein and so may be distinguished from VP 55. The latter protein has been identified as the nucleocapsid protein, both on extraction from cells (182) and from purified virions (18). Its synthesis in chick embryo fibroblasts has also been demonstrated (48, 50).

In the present work, it has been confirmed that VP 55 is the major peak found following PAGE of NDV grown in developing chicken embryos whether examined under reduced or non-reduced conditions (Fig. 24, 34 & 35). However, in virus released from mammalian cells, VP 55 forms a much lower percentage of the total viral protein and the small glycoprotein is probably seen as a peak of 62,000 daltons.

daltons.

On the other hand, when PAGE is performed on BK pi virus in a non-reduced state, the nucleocapsid peak is much enlarged, the VP 70 peak disappears, the VP 62 peaks is more clearly resolved and the VGP 75 peak increases in size. Because VGP 55 does not co-migrate with the nucleocapsid protein under non-reduced conditions (21), it is probable that this enlargement of VP 55 is related to the diminuition of VP 70.

A protein of 55,000 daltons was shown to be synthesised both in MDBK cells infected with B1 virus (MDBK/B1) and BK pi cells and in addition, in the former case, an equal amount of VP 53 was formed. Neither of these two proteins could be described as a major polypeptide (Fig. 42), although, in mature virions released from infected chicken embryos, VP 55 is the major peak, and VP 53 is relatively small (Fig. 24). Nor, indeed, is VP 53 detectable in virus produced from mammalian cells (Figs. 27 & 28). Therefore, it is possible that one of the causes of the diminished size of the VP 55 peak, seen in the latter type of virus, is the slow rate of conversion of a precursor (e.g. VP 53 or a part of VP 70) into normal nucleocapsid. Vorkunova et al.(61) showed that two abnormal forms of nucleocapsid were formed in abortive infections of L cells with Sendai virus, and that although both contained normal RNA, the virions released from these cultures were of low infectivity. Nucleocapsid was isolated from BK pi cells in this laboratory but was

was found to migrate as a single peak with the same electrophoretic mobility as proteins of 55,000 daltons (Fig. 37).

In summary, VP 55 is formed in BK pi cells and in MDBK cells infected with B1 virus (MDBK/B1 cells) and is incorporated into mature virions. However, it is possible that the quantity of VP 55 is reduced in comparison with that of virus released from infected chicken embryos, due to the slow or incomplete conversion of one or more precursors into normal nucleocapsid.

g) VP 49

VP 49 is found in NDV that has been grown in embryonated hens' eggs, but is less evident in virions that are solubilised in non-reducing conditions before electrophoresis (21). In the present work, VP 49 was found to be one of the major viral proteins present in the infected bovine kidney cells (Fig. 42). Moreover, its existence in egg-grown strains of NDV was confirmed (Fig. 24), and this protein was also found to be present in small quantities in virus released from mammalian cells. However, the relatively large quantities of VP 49 found in MDBK/B1 and especially BK pi cells, compared with the amount of polypeptide detected in liberated virions, suggests that VP 49 may be a precursor of one of the other structural proteins. This contention is supported by the fact that VP 49 is not present under non-reduced conditions and by the absence of any evidence that VP 49 is involved in a

a biological activity associated with NDV.

h) VP 42

It has been suggested that VP 42 is the matrix protein of the viral envelope (18), and there is clear evidence that it is one of the major proteins of purified virions released from infected chicken embryos (21). Moreover VP 42 is synthesised abundantly in infected chicken embryo fibroblasts (48, 50). This protein was evident in infected bovine kidney cells (Fig. 42), but the quantity found in the virions liberated from these and other mammalian cell- lines was extremely low (Fig. 27,28), except in the case of PK pi virus. However, electrophoresis under non-reduced conditions results in a somewhat larger peak at this molecular weight. In view of these findings it is likely that VP 42 is involved in the reduced biological activities and infectivity of NDV grown in mammalian tissues.

i) VP 34-38

The last protein considered in this present Section has a molecular weight of 38,000 daltons and has previously been reported in chick embryo fibroblasts infected by NDV (48). In the preset work, a protein of this size was found in MDBK/Bl cells while a polypeptide of approximately 34,000 daltons was synthesised in BK pi monolayers (Fig. 42). Because this protein has not been observed in liberated NDV,

NDV, either in this laboratory or by other workers, it must be regarded as a precursor of structural proteins.

IV) SUMMARY AND DISCUSSION

The results in this Section show that the two envelope proteins (VGP 75 and VGP 55), the nucleocapsid protein (VP 55) and the matrix protein (VP 42) are abnormal in NDV released from cell cultures, whether the infection be persistent or not. Moreover, the biological activities associated with two of these proteins (VGP 75 and VGP 55) are also defective. It can thus be assumed that the concomitant lack of virulence exhibited by these strains of NDV, which is especially marked in the virus produced from persistently infected cells, is due at least in part to these structural defects.

The abnormality of VGP 55 appears to be due to the incorporation of this protein into mature virions, without the severance of certain peptide linkages found in the precursor molecule. Because these bonds remain intact, the molecular weight of VGP 55 under reduced conditions is 62,000 and the haemolysin activity associated with this protein is defective. This effect may be analogous to a similar phenomenon reported in abortive infections with Sendai virus (11), and is possibly due to the lack of a trypsin-like host enzyme.

In general, VGP 75 and VP 55 were seen only in small quantities in virus released from infected cell

cell cultures and were apparently replaced by a protein of 70,000 daltons. It was generally found that the greater the amount of VGP 75 that was present, the higher was the haemagglutinin activity per µg protein and thus the strain (BK pi virus) with the lowest haemagglutinin and neuraminidase activities, was the least infectious.

The association of VP 42 with both the envelope and the nucleocapsid fractions of NDV suggests that VP 42 is the matrix protein (18). Recent work (212) has shown that the nucleocapsid of paramyxoviruses (VP 55) is contained within a sheath of VP 42 and that the latter protein is in turn bound to the envelope protein (VGP 75).

Although VGP 75, VP 55 and VGP 55 of virus released from mammalian cells are not seen in reduced conditions, they do occur under non-reduced conditions, but VP 42 is largely absent in either circumstance. However, the VP 42 of Herts virus released from chick fibroblasts is more prominent but is still defective compared with virus of the same strain grown in fertile hens' eggs.

The cause of the abnormality of the proteins of virus released from cell cultures is unknown and may be explain by several hypotheses. Thus, it is possible that :-

(i) the released virus contains VGP 75 and VGP 55 in the form of proteins with molecular weights of 70,000 and 62,000 daltons respectively, but does not

not possess VP 55 or VP 42.

(ii) the released virus is normal but is contaminated with serum proteins.

(iii) the released virus is normal but is degraded by the action of certain substances in the supernatant fluids

(iv) both the nucleocapsid protein (VP 55) and the major envelope protein (VGP 75) are each composed of two or more sub-units. The matrix protein (VP 42) separates the nucleocapsid from the envelope in normal virions but VP 42 is largely absent from the cell-grown virus, and the sub-units of VGP 75 and VP 55 become bound together in an abnormal manner. Thus, under reduced conditions, a protein of 70,000 daltons occurs, consisting of nucleocapsid sub-units combined with envelope protein sub-units. Under non-reduced conditions, coalescence of the sub-units into their respective proteins takes place, and their normal molecular weights are maintained by means of disulphide bonds.

The suggestion above that VGP 75 is replaced by VP 70, and that VP 55 is absent, as described in hypothesis (i) is unsatisfactory, since VGP 75 and VP 55 are present under non-reduced conditions and both apparently are derived from VP 70.

Contamination with serum proteins, as described in hypothesis (ii), is unlikely in view of the fact: (a) that the major polypeptides of serum are of different molecular weights from those of the proteins seen in virus preparations, and (b) that viral proteins seen

seen under non-reduced conditions are of similar molecular weights whether the virus was grown in cell culture or fertile hens' eggs.

The degradation of the viral proteins by a substance in the nutrient medium as described in hypothesis (iii), possibly occurs in the case of virus released from cell cultures, since the gel profiles are broadly similar for all cell-grown strains of NDV. In this connection, it is interesting to note that Popa et al. (213) have reported that a somewhat similar phenomenon occurs with Sendai virus. They showed that if released virions are stored in nutrient medium and kept at -70°C for periods of up to six months, degradation of both the nucleocapsid and matrix proteins occurs. Their results imply that a substance in the cell-culture medium probably causes the breakdown of viral proteins.

The absence of the matrix protein (VP 42) from gel profiles under both reduced and non-reduced conditions supports hypothesis (iv), since without this protein it is possible that VP 55 might be bound to VGP 75 in an abnormal manner. Indeed, although VP 70 is observed in Herts virus released from chicken fibroblast cells electrophoresed under reduced conditions, the relative quantities of VP 42, VP 55 and VGP 55 are higher than in other cell-grown viruses. This finding suggests that the more matrix protein there is, the less possibility there is of an abnormal combination of the nucleocapsid and envelope proteins.

proteins.

Four other results support the hypothesis that VP 70 is an abnormal combination of sub-units of VP 55 and VGP 75, namely:-

a) Following treatment of egg-grown NDV with Triton X-100, nucleocapsid proteins (VP 55) can be separated from envelope proteins (VGP 75) on the basis of their solubility in solutions of different molarities.
However, in the case of BK pi virus treated with this detergent, VP 70 is found in both nucleocapsid and envelope fractions.

b) Although both electron microscopy and PAGE show that nucleocapsid is found in reduced quantities in virions released from cell culture, nucleocapsid structures are clearly evident within persistently infected BK pi cells and the nucleocapsid material extracted from BK pi cells has a molecular weight of 55,000 daltons. Thus, nucleocapsid within persistently infected cells appears normal, whereas in the released virions it is present in an abnormal form.

c) Virus-associated proteins of 75,000, 70,000 and 55,000 daltons are present within infected mammalian cells and it thus appears that VP 70 is a viral protein and not a contaminant.

d) Bands of proteins of 75,000 and 55,000 daltons are seen following PAGE of BK pi virus under non-reduced conditions but under reduced conditions neither of these proteins are present, and

and they are replaced by a protein of 70,000 daltons.

Nevertheless, these results do not necessarily indicate that the formation of VP 70 is due to the reduced incorporation of VP 42 into the mature virion. On the other hand, these findings may also suggest that aggregation of the nucleocapsid and envelope protein sub-units into a protein of 70,000 daltons occurs following the action of a substance in the cell-culture medium which destroys the matrix protein (VP 42) completely and breaks down the nucleocapsid protein into its component sub-units. If this latter suggestion is correct, it is reasonable to suppose that since the gel profile of Herts virus released from CF cells is more normal, the proteins of virus released from mammalian cells are less stable than those of Herts virus released from CF cells.

With most strains of cell-grown virus observed under reduced conditions, there is sufficient VGP 75 to exist independently of the VP 70 aggregate, but in the case of BK pi virus, VGP 75 is only seen under non-reduced conditions. Thus, whether the formation of VP 70 takes place before or after release of the virus, it is still evident that there is less VGP 75 in BK pi virions than in any of the other strains of cell-grown virus. It is emphasised that the precise nature of VP 70 is not known and that further studies are necessary to confirm or refute the suggestions made in this present work. For example, the formation and degradation of the proteins observed here could be

be confirmed by employing the pulse-chase technique, and the synthesis of glycoproteins could be traced with labelled glucosamine. It would also be desirable to obtain radioactively labelled virions, released from BK pi cells, MDBK cells and fertile hens' eggs in order to establish whether a protein of 70,000 daltons is found in cell-grown virus, since it has not been described by other workers (18) in virus released from MDBK monolayers.

In conclusion, the present results indicate that the small envelope protein (VGP 55) of NDV grown in certain mammalian cell-lines is defective and, in consequence, not only haemolysin but also infectivity is reduced. The infectivity of the cell-grown NDV is probably also affected by certain substances in the supernatant fluids which cause degradation of the matrix protein (VP 42) and the formation of an abnormal aggregate (VP 70), consisting of sub-units of the nucleocapsid (VP 55) and the large envelope (VGP 75) proteins. The reduced haemagglutinin activity of virus grown in mammalian cell-lines is probably also attributable to this abnormality, since haemagglutination is associated with VGP 75. The lack of VGP 75, under reduced conditions, in virus released from BK pi cells corresponds with a very low haemagglutinin activity per μg protein of this strain of NDV. The infectivity, haemagglutinin activity and amount of VGP 75 all increase, following passage of this virus through HeLa cells. Since the amount of VP 70 remains constant under these

these circumstances, it is evident that haemagglutinin activity is dependent not only on the quantity of the large envelope protein which is present, but also on the form of this protein being normal.

The neuraminidase activity of the large envelope protein (VGP 75) is apparently unaffected by the combination of its sub-units with sub-units of VP 55 to form VP 70, because the neuraminidase activity of the purified B1 strain of NDV is the same whether the virus is grown in HeLa cells or fertile hens' eggs. Moreover, BK pi virus which is passaged through HeLa cells retains an extremely low neuraminidase activity, in spite of an increase in the amount of VGP 75. Thus, neuraminidase activity unlike haemagglutinin activity is probably dependent on the functional capacity of the enzyme site.

The reduced infectivity and virulence of virus released from mammalian cell-lines appears to be multifactorial. Decreased haemolysin and haemagglutinin activities are associated with low infectivity, even when neuraminidase activity is normal (e.g. B1 virus grown in HeLa cells).

Reduced neuraminidase activity, in addition to low haemolysin and haemagglutinin activities, is associated with still lower infectivity (e.g. virus released from PK pi cells, BK pi virus and PK pi virus grown in HeLa cells). Infectivity is lowest in the case of virus released from BK pi cultures where both neuraminidase and haemagglutinin activities are extremely low.

With the exception of the reduced quantity of

of VGP 75 in BK pi virus, no structural differences can be observed between virus released from persistently infected cells and virus released from other cell-cultures infected with NDV.

NDV grown in mammalian cell-lines shares several abnormal characteristics with the virus released from the carrier cell-lines. It is emphasised however, that an extremely low level of neuraminidase activity is common to the two strains of persistent virus (BK pi and PK pi) but that neuraminidase activity can be normal in NDV released from mammalian cells. This fact may be of importance since high levels of neuraminidase activity are associated with the virulent strains of NDV grown in fertile hens' eggs.

Thus persistence may be related not only to growth of virus in a non-permissive cell system but also to a mutation of the virus to an avirulent form. The different responses of the two strains of persistent virus to growth in a permissive system (Section 2 (D) II (d)) may reflect an inverse relationship between the mutation of the virus to an avirulent form and the degree of non-permissiveness of the host cell-culture.

RESULTS

SECTION 3

THE EFFECT OF ALTERING THE ENVIRONMENT OF PERSISTENTLY INFECTED CELLS ON THEIR CHARACTERISTICS AND ON THE PRODUCTION OF VIRUS

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SECTION 3

THE EFFECT OF ALTERING THE ENVIRONMENT OF PERSISTENTLY INFECTED CELLS ON THEIR CHARACTERISTICS AND THE PRODUCTION OF VIRUS

I) ENRICHMENT AND DEPLETION OF THE NUTRIENT MEDIUM

Introduction

Since viral replication employs much of the synthetic apparatus of the cell, it is not surprising to find that the formation of virus is considerably affected by the environment of the cell. This is particularly true of persistent infections; for here, cellular anabolism must co-exist with the replication of virus.

There are many reports in the literature of persistent infections in which production of virus is temperature-sensitive (t-s), including recent investigations of NDV carried in L cells (136), where synthesis of RNA polymerase is believed to be inhibited at 41^{o}C (123). In addition, it has been shown that some nutrient factors are necessary for the metabolism of both cells and virus, although a certain material may be required by one and not the other. Serine has been found to be necessary for the synthesis of the protein or lipid contained within the viral envelope of NDV but the requirements of HeLa cells or poliomyelitis virus for this amino acid are much less (183).

(183). Thus, low concentrations of serine in the medium will inhibit synthesis of NDV within HeLa cells but will permit poliomyelitis virus to replicate and cellular metabolism to continue.

Furthermore, it has been shown in Section 1, that some of the characteristics of the persistently infected cells PK pi, BK pi and OK pi, appear to have altered following the change from EYL medium to MEM. Therefore, it was decided to study the effects on the growth of these cell-lines of medium (BME) containing amino acids at lower concentrations than in MEM and also by supplementing the latter medium with different amounts of calf serum.

In these experiments, three effects had to be distinguished:

1) diminished growth of cells due to lack of adequate nutrient,

2) reduced production of virus due to a low concentration of an essential substance,

3) increased release of virus due to the absence of an inhibitory factor contained in the serum or synthesised by the cells under normal conditions.

a) <u>The effect on BK pi cells of MEM with different concentrations of serum</u>.

In a preliminary experiment, it was shown that BK pi could be seeded and grown for periods of up to two weeks in MEM without serum, However, MDBK cells were incapable of growth under these circumstances, although they survived for as long as a week. (This

(This result confirms observations made in Section 1, that there was a greater degree of cellular transformation in persistently infected cells than in the controls.)

BK pi cells were seeded in MEM containing 0, 5, 10 and 20% serum, and the number of viable cells, the cell-associated haemagglutinin (CAHA) and released haemagglutinin (RHA) were measured on days 3, 7 and 14 (Table XXXI). It was found that there was little difference between the growth of cells in medium without serum and in MEM containing 5% serum. However, relatively greater growth was observed in cultures fed with MEM, supplemented with 10 or 20% serum. Furthermore, it was noticed that these latter cells contained far more CAHA and it might thus be concluded that the rate of synthesis was faster. On the other hand, the amount of haemagglutinin released from monolayers of BK pi cells did not appear to be dependent on the concentration of serum in the medium, although the highest titre per cell was produced after 14 days growth of cultures in the absence of serum (Table XXXI). However, when the ratio of RHA to CAHA is examined (Table XXXI col. 4), it is evident that the lower the percentage of serum in the medium, the greater is the proportion of the total HA that is present in the supernatant. (It should be noted that generally less than one-fifth of the haemagglutinin was found in the form of RHA).

Discussion

T A B L E XXXI

THE EFFECT OF SERUM CONCENTRATION ON THE GROWTH OF BK pi CELLS AND ON THE PRODUCTION OF HAEMAGGLUTININ

SERUM CONCENTRATION IN MEM (%)	VIABLE CELLS/ML x10^{-5}			CELL ASSOCIATED HAEMAGGLUTININ (CAHA)/CELL x10^{6}			RELEASED HAEMAGGLUTININ (RHA)/CELL x10^{6}			RHA/CAHA x10^{2}		
	DAY			DAY			DAY			DAY		
	3	7	14	3	7	14	3	7	14	3	7	14
0	3.1	6.0	10.5	5.2	10.7	6.0	0.6	1.7	15.5	12.5	20.0	66.0
5	4.3	5.2	9.5	7.6	24.6	30.0	0.8	0.9	7.5	12.2	12.5	15.0
10	6.5	7.0	17.0	20.0	73.1	25.0	1.3	1.5	3.5	6.2	6.2	8.4
20	4.9	6.1	21.0	12.8	85.0	17.0	0.8	3.3	3.7	6.2	12.5	16.7

All cells were seeded at 1.0 x 10^{5} cells per ml.

Discussion

There are several possible causes for this difference; namely:-

1) There is a limiting lactor, so that whatever quantity of HA is synthesised, no more than a certain number of mature virions containing a restricted amount of HA may be released from the BK pi cells. However, in view of the fact that the greatest amount of RHA was obtained from cells grown for 14 days in medium containing 5 or 10% serum, this hypothesis is untenable.

2) There is an increased yield of mature virions per HAU synthesised in the absence of serum.

3) A relatively larger proportion of cells fed on low amounts of serum die and become detached from the glass. Thus the CAHA in the membranes of such cells causes an increase in the quantity of HA in the supernatant fluids.

The last hypothesis (3) is unlikely because a great number of dead cells was observed in cultures grown in the presence of large quantities of serum, as well as in cultures fed on medium containing low percentages of serum.

Therefore, it may be concluded that although a higher rate of cellular and viral protein synthesis is promoted by growth in MEM containing a high percentage of serum, restriction of the quantity of serum allows a greater amount of virus to be released. The effect of the serum may be indirect, in that virus release in inhibited by a fast rate of mitosis or by the increased cellular metabolism with which it is associated. On the other hand, it may be direct,

direct, because Cohen has shown that non-specific inhibitors of haemagglutinin are found in rabbit and horse serum (184, 185) and similar substances may be found in calf serum. Although the effect of these inhibitors may be reduced by periodate treatment before assay for haemagglutinin, their action on the haemagglutinin incorporated into the cell surface may be sufficient to prevent the release of virus

b) A comparison of the properties of the carrier cell-lines grown in BME in place of MEM.

The increased quantities of released haemagglutinin observed in the previous experiment when BK pi cells were grown in medium containing low percentages of serum, could be caused in two ways, namely:-

i) the absence of the non-specific inhibitors of haemagglutinin that are found in serum.

ii) the increased production of virus in cells with a slow rate of metabolism.

It was decided therefore, to assay the replication of virus in cultures of all three persistently infected cell-lines, when these were grown in a medium with lower concentrations of amino acids. This medium (BME) produced a slower rate of mitosis in uninfected controls than MEM and thus simulated the effect of growth in MEM containing low concentrations of serum. However, since the percentage of serum for cultures grown in MEM was the same as that for cultures grown in BME, the effect of the non-specific inhibitors of haemagglutinin

haemagglutinin could be disregarded.

It was found that cell growth (whether measured by the rate of mitosis (Column 1, Table XXXII or the total volume of cellular material (Column 8), was less in BME than MEM. However, the volume and surface area of the individual cells was larger in the former medium (Column 7).

Thus some of the increased number of erythrocytes per haemadsorbing cell was due to this property (Column 3). However, in PK pi, and to a lesser extent in OK pi and BK pi respectively, there were still significantly more red blood cells per mm^2 of the affected cells, when these were grown in BME (Column 6).

Furthermore, in both OK pi and PK pi cultures, but to a smaller degree in BK pi monolayers, a greater proportion of the cells were shown to haemadsorb, if the nutrient medium were BME (Column 2). The release of haemagglutinin was more easily detected in this medium in the case of BK pi and OK pi cultures, while fusion of cells in the latter cell-line also increased, when grown in the poorer medium.

Discussion

It may be concluded that the effects noted in the serum-depleted cultures of BK pi are due, not to the absence of serum, but to the poverty of the nutrient medium. Thus, the incorporation into the cell membrane of the envelope proteins of NDV, which are responsible for the properties of syncytial formation and haemagglutination (including haemadsorption), is reduced

T A B L E XXXII

DIFFERENCES IN GROWTH, CELL SIZE, CELL FUSION, HAEMADSORPTION AND HAEMAGGLUTININ RELEASE BETWEEN THE THREE PERSISTENTLY INFECTED CELL-LINES, GROWN IN BME and MEM

CELL LINE	MEDIUM	NUMBER OF CELLS $x10^{-5}$	HAEMADS-ORPTION (%)	ERYTHRO-CYTES PER HAEMADS-ORBING CELL	SYN-CYTIA (%)	RELEASED HAEMAGG-LUTININ PER ML	ERYTHRO-CYTES PER mm^2 OF HAEMADS-ORBING CELLS	VOLUME OF CELL (μm^3)	TOTAL VOLUME OF CELLS PER ML $x10^{-5} \mu m^3$
PK pi	MEM	3.4	48	6.1	1.4	Nil	150	2723	9260
	BME	2.3	64	9.3	1.5	Nil	210	3650	8396
BK pi	MEM	3.0	81	9.2	1.5	Nil	440	2916	8748
	BME	2.5	83	11.8	1.5	4	460	3423	8566
OK pi	MEM	2.7	31	13.3	2.1	Nil	180	7263	19611
	BME	1.7	50	19.8	3.9	4	220	9197	15636

In all cases figures refer to the third day after seeding at 100,000 cells/ml. Both types of media were supplemented with 5% calf serum.

reduced in conditions which favour a high rate of mitosis. This may be due to the increased synthesis of an anti-viral factor or, more likely, the unsuitable nature of the surface of a cell in division for the budding of mature virions. It is notable, in this respect, that the cell-line (BK pi) whose mitotic rate was least affected by growth in BME (Table XXXII Column 1) showed the smallest increase in viral activity.

II) THE EFFECT OF ALTERING THE TEMPERATURE OF INCUBATION OF THE CARRIER CELL-LINES.

Introduction

It has been shown in the preceding paragraphs that the release of virus (but not the synthesis of viral protein) from the three carrier cell lines is enhanced by nutritional conditions that reduce the rate of mitosis and that this is probably a consequence of the slow metabolic rate of host cell under these circumstances.

Another important factor which influences the growth of cells, is the temperature of incubation. Other workers have described a persistent infection of L cells with NDV (137), in which the persistence was interferon mediated and the virus released was of reduced virulence. It was further shown that virus was liberated from the carrier L cells only at permissive temperatures (136) and that viral RNA

RNA polymerase was inhibited by interferon at the higher, non-permissive temperature. Moreover, in order to induce persistence with NDV, the virus had first to be adapted so that it was incapable of replication at raised temperatures (that is; a mutation to a temperature-sensitive form) (124). However, a second type of persistent infection has been described with a paramyxovirus (conjunctival (C-M) cells carrying mumps virus) in which a reduced yield of virus at 41°C was ascribed to the increased metabolic rate of the cells at this temperature (146).

Thus, when the effects of altering the temperature of incubation of our persistently infected cells are considered, it must be remembered that these changes may be due to alterations in the cellular growth rate or to a temperature-sensitive step in viral replication. In the latter case, the portion of the synthetic pathway affected can vary widely. Semliki Forest virus infections were shown to be temperature-sensitive for the formation of either RNA polymerase or viral envelope protein (186). Temperature-sensitive mutants of Sendai virus may be inhibited from synthesising viral messenger RNA, from forming RNA polymerase, or they may have a haemagglutinin that does not function at the non-permissive temperature (121).

a) Growth of carrier cell-lines at 31°C.

In the present work, the number of live cells per

per ml was less when the persistently infected monolayers were cultured at 31°C or 41°C, instead of 37°C (Tables XXXIII and XXXIV). In the former instance, the reduced rate of growth is thought to be due to a slower rate of metabolism, while in the latter, it is probable that the viability of the cells is reduced because of the inability of certain enzymes to function at high temperatures.

It is clear that the amount of haemagglutinating material released into the medium is greater in all three carrier lines when they are incubated at 31°C (Table XXXIII); OK pi cells being the most and BK pi cells the least affected. Moreover, it will be noticed that when a comparison is made of the amount of the semi-purified virus released, (as assayed by haem-agglutinin activity - Table XXXIII) almost 14 times the amount of virus is produced in OK pi and PK pi cells at the lower temperature than at 37°C, but, the quantity of BK pi virus liberated from each cell is unaltered.

Discussion

These results suggest that in the case of all three cell-lines, some of the haemagglutinin found in the supernatant is due to that contained within cellular debris and some is due to released virus particles. With regard to OK pi and PK pi cultures, the proportion of the total activity contributed to by the mature virions is greater at 31°C than at 37°C. On the other hand, although more dead cellular material is found in the supernatant of BK pi

TABLE XXXIII

THE PRODUCTION OF VIRUS FROM THE PERSISTENTLY INFECTED CELL-LINES INCUBATED AT 31°C AND 37°C

CELL-LINE	TEMPERATURE OF INCUBATION (°C)	CELLS PER ML (AVERAGE)	AVERAGE YIELD (HAU/CELL) x10^6		RATIO OF YIELD OF HAEMAGGLUTININ at 31°C to that at 37°C		INFECTIVITY PER HAU ($TCID_{50}LOG_{10}$)
			IN SUPERNATANT	SEMI-PURIFIED VIRUS	IN SUPERNATANT FLUID	IN PELLETED VIRUS	
PK pi	31	400,000	31.4	2.56	4.3	13.9	0.95
PK pi	37	700,000	2.6	0.18	1.0	1.0	0.87
BK pi	31	550,000	22.2	0.32	2.9	0.9	$\bar{1}$.81
BK pi	37	750,000	7.6	0.37	1.0	1.0	1.80
OK pi	31	250,000	58.5	5.12	6.0	13.8	$\bar{1}$.23
OK pi	37	350,000	9.7	0.36	1.0	1.0	$\bar{1}$.15

Cells were grown in 5% MEM, and reseeded every two weeks.
The supernatant was collected every three days.
Infectivity assays were performed in 6" x $\frac{5}{8}$" tubes.

BK pi monolayers grown at 31°C, the amount of virus released per cell is not affected.

Although more virions are liberated from PK pi and OK pi cells cultured at 31°C, the infectivity per HAU does not change. For this reason, it is unlikely that the defects of these persistent viruses, which were discussed in Section 2, are reduced. Thus, the increase in released virus is not due to a lower temperature being more permissive for viral replication but is caused by the slower rate of mitosis of the host cells, in a similar manner to that discussed above with regard to growth in BME. It appears that the reduction in cell growth in the case of BK pi monolayers cultured at 31°C is insufficient to allow more virus to be liberated.

(b) Growth of carrier cell-lines at 41°C

The evidence presented in Tables XXXIV a, b, c, d suggests that a reduction in viral synthesis is observed when the carrier cell-lines are incubated at 41°C and that this may be due to the temperature-sensitivity of one or more steps in the replicative process.

Because of the poor ability of BK pi cells to withstand a temperature as high as 41°C, the experiments were mainly performed with the other two cell-lines. In the case of PK pi cultures, haemadsorption was not seen in cells that had been incubated for three days at 41°C, but measurement of the cell-associated haemagglutinin (CAHA) indicated that this took one

T A B L E XXXIV a

INCUBATION OF PK pi CELLS AT 41°C AND 37°C.

TEMPERATURE OF INCUBATION (C°)	DAY	BIOLOGICAL ACTIVITIES ASSOCIATED WITH PK pi CARRIER CELLS				
		RELEASED HAEMAGGLUTININ PER CELL ($x10^6$)	CELL-ASSOCIATED HAEMAGGLUTININ PER CELL ($x10^6$)	PERCENTAGE OF CELLS SHOWING HAEMADSORPTION	PERCENTAGE OF CELLS STAINED BY IMMUNOFLUORESCENCE	NUMBER OF CELLS PER ML
37	1	nil	21.2	50	91	120,000
	2	nil	22.2	70	90	230,000
	3	nil	23.2	80	89	320,000
	4	nil	24.9	80	93	410,000
	5	nil	N.D.	80	91	N.D.
41*	1	nil	21.2	50	91	120,000
	2	nil	7.1	<1	90	180,000
	3	nil	2.3	nil	90	170,000
	4	nil	nil	nil	85	210,000
	5	nil	nil	nil	86	N.D.

*The cells were transferred to 41°C on DAY 0.

Nucleocapsid structures were seen on all days in 37°C cells, but not after day 3 in the 41°C cells. Budding virus was not detected at the latter temperature, except on day 1.

Positive fluorescence was normally observed until the cells had been incubated at 41°C for about 28 days. N.D. = Not Done.

T A B L E XXXIV b

INCUBATION OF OK pi CELLS AT 41°C AND 37°C

TEMPERATURE OF INCUBATION (°C)	DAY	BIOLOGICAL ACTIVITIES ASSOCIATED WITH OK pi CARRIER CELLS				
		RELEASED HAEMAGGLUTININ PER CELL x10^6	CELL-ASSOCIATED HAEMAGGLUTININ PER CELL x10^6	PERCENTAGE OF CELLS SHOWING HAEMADSORPTION	PERCENTAGE OF CELLS STAINED BY IMMUNOFLUORESCENCE	NUMBER OF CELLS/ML
37	1	8.0	N.D.	60	90	100,000
	2	N.D.	N.D.	90	89	N.D.
	3	N.D.	N.D.	90	91	N.D.
	4	7.0	25.6	90	89	250,000
	5	N.D.	N.D.	90	92	N.D.
	6	N.D.	N.D.	90	90	N.D.
	7	6.0	80.0	90	91	320,000
41*	1	-ve	N.D.	60	90	100,000
	2	-ve	N.D.	70	89	N.D.
	3	-ve	N.D.	10	90	N.D.
	4	-ve	15.0	5	87	120,000
	5	-ve	N.D.	<1	92	N.D.
	6	-ve	N.D.	-ve	88	N.D.
	7	-ve	1.1	-ve	93	240,000

N.D. Not done. *The cells were transferred to 41°C on DAY 0.
Nucleocapsid and budding virus were seen in 37°C cultures.
In monolayers held at 41°C the nucleocapsid is degenerate but persists till the seventh day, while budding virus is not found after the second day.
Fluorescence was seen until the sixteenth day of incubation at 41°C.

T A B L E XXXIV c

INCUBATION OF BK pi CARRIER CELLS AT 37°C and 41°C

TEMPERATURE OF INCUBATION (°C)	DAY	BIOLOGICAL ACTIVITY ASSOCIATED WITH THE BK pi CARRIER CELLS	
		PERCENTAGE OF CELLS SHOWING HAEMADSORPTION	PERCENTAGE OF CELLS STAINED BY IMMUNOFLUORESCENCE
37	1	50	91
	2	70	89
	3	70	90
	4	90	92
	5	90	90
	6	90	91
41*			
	1	30	91
	2	20	90
	3	2	89
	4	<1	88
	5	nil	91
	6	nil	90

*The cells were transferred to 41°C on Day 0.

These cultures collapsed on the seventh day of incubation at 41°C.

T A B L E XXXIV d

RE-APPEARANCE OF EVIDENCE OF INFECTION AFTER INCUBATION OF THE CARRIER CELL-LINES PK pi AND OK pi AT 41°C.

CELL-LINE	EXPERIMENT	TIME HELD AT 41°C (DAYS)	THE TIME OF RE-APPEARANCE OF EVIDENCE OF INFECTION AFTER RETURN TO 37°C (DAYS)
PK pi	A	72	*
	B	93	30
	C	77	*
	D	29	7
OK pi	A	8	8
	B	49**	N.A.
	C	28	8
	D	7	5
	E	14	4
		21	*
		28	1
		35	*
		56	*

After incubating the cultures at 41°C for the periods indicated, the cells were returned to 37°C and maintained at this temperature for two months or until evidence of infection re-appeared.

Haemadsorption and immunofluorescence were employed as indicators of renewed synthesis of viral protein.

* On these occasions, evidence of virus protein synthesis was absent, 9 weeks after the return of the cells to 37°C.

** On this occasion, viral protein synthesis recurred on the 49th day of incubation at 41°C.

Note: If the period of incubation at 41°C was less than 7 days, virus protein synthesis recommenced within 7 days of the return of the cultures to 37°C.

one day longer to disappear. Nucleocapsid, which is visible in control cells viewed with the electron microscope became degenerate and disappeared within four days, whereas specific immunofluorescence was obtained till the twenty-eighth day of incubation at the higher temperature, thus indicating that some viral antigen was still present within the cells up till this time. However, in two of the four experiments, haemadsorption reappeared after the cells were returned to 37°C, although in one case 30 days elapsed before the percentage of cells that could be shown to be infected equalled that of controls. In the other two experiments, haemadsorption did not recur even after two months of incubation at 37°C. Somewhat similar results occurred, in OK pi cells, but here, haemadsorption was found for up to five days after the cultures were transferred to 41°C and CAHA persisted for seven days. Immunofluorescence was usually absent from monolayers by the sixteenth day of incubation and there was eventual degeneration of the nucleocapsid. In a similar way to PK pi, evidence of infection normally reappears when OK pi monolayers are returned to a 37°C incubator. The results in experiment 'B' show that there was a spontaneous recurrence of haemadsorption, while the cells were still at 41°C and, in the final series of experiments (E), viral synthesis only restarted in certain clones, irrespective of the time the cells had been held at 41°C.

Immunofluorescent studies indicated that viral

viral antigen was present in BK pi monolayers for the first week of incubation at 41°C but that, in a similar manner to the other two cell-lines, haemadsorption was absent by the fifth day. (In this respect, it is of interest that BK pi cells studied by Edwards (93) in 1970-72 could, at that time, be maintained for long periods at 41°C without ill effect and that, although haemadsorption initially was lost, its eventual reappearance occurred spontaneously at 41°C.)

Discussion

The effect of lowering the temperature of incubation of our carrier cell-lines is to allow more virus to be released per cell. In contrast, culture at 41°C inhibits viral synthesis to a far greater degree than cellular synthesis. Thus, this temperature may be described as non-permissive to viral replication. It should be noted that wild-type strains of NDV must be capable of replication at 41°C, because this is the body temperature of its natural host - the chicken. However, the initial point of infection is the respiratory system, where the ambient temperature is likely to be lower.

In the case of two of the persistently infected lines, OK pi and PK pi, (and probably BK pi also), synthesis of both haemagglutinin and nucleocapsid is temperature-sensitive. Because it is known in addition that the haemagglutinin of BK pi virus is temperature-stable (see Section 2), it is likely that inhibition of viral replication at 41°C occurs at an early stage in

in the growth cycle. This step could involve the formation of either viral RNA polymerase or viral messenger RNA. Although haemagglutinin (CAHA) is still present within the cell, the incorporation of haemagglutinin into the cell membrane, as measured by haemadsorption, is also inhibited at the higher temperature. Thus the reduced incorporation of haemagglutinin into mature virions at 41°C is probably due to a temperature-sensitive step in the synthesis of another protein essential to the assembly of new virions.

Although neither haemagglutinin nor nucleocapsid can be identified in any of the three cell-lines after one week of incubation at 41°C, the greater sensitivity of the immunofluorescent staining technique enables remnants of viral material to be detected for periods of up to one month. However, the eventual failure of even this method to show the presence of viral antigen in cells at 41°C indicates that synthesis of new viral protein is unlikely to take place after transfer of cells to the non-permissive temperature.

The reappearance of signs of viral infection, which frequently occurs after the cultures are returned to 37°C, suggests that the viral genome is usually maintained at 41°C. In view of the fact that viral RNA-dependent DNA polymerase has been described in cultures persistently infected with NDV (45) and the preliminary work which suggests that this enzyme is also present in some of our carrier cell-lines (Section 2), it is possible that the viral genome is

is integrated into the cellular DNA. Since viral synthesis does not always recommence on the return of carrier cultures to a permissive temperature, the integrated viral genome may be absent from that clone of cells or present in an inactive form. The latter possibility is more likely because of the variation in the length of time between the cells being brought back to 37°C and the reappearance of haemadsorption. When haemadsorption did recur, it was initially confined to discrete areas of the monolayer but after incubation for a further two days, the majority of cells haemadsorbed. In view of the low infectivity of persistent virus, this observation suggests that replication of viral RNA must recur in most of the cells in the monolayer.

CONCLUSION TO SECTION 3(II)

All three cell lines are persistently infected by strains of virus that have a temperature-sensitive step early in the replicative cycle. This probably involves the synthesis of viral RNA, since synthesis of both of the major structural proteins is prevented, and their incorporation into the cellmembrane ceases. Furthermore, accumulated viral material gradually decreases until within two to four weeks at 41°C, no viral antigen can be detected in any of the cells. At this time, since it is probable that the viral RNA has been transcribed into DNA, as described by other workers (45), and since formation of viral RNA

RNA appears to be the temperature-sensitive step, the viral genome must be carried within the cellular genetic material. However, on occasion a mutation gives rise to a form capable of synthesising new viral RNA at 41°C. On the other hand, renewed production of virus usually takes place when the carrier cells are returned to 37°C, although some clones either contain viral genome that is incapable of expression or may have eliminated the persistent virus altogether.

Although carrier cell-cultures grown at 31°C produce more virus than at 37°C, neither the cytocidal capacity nor the infectivity of the released virus is increased at 31°C. Thus, while restriction of viral replication occurs at 41°C, low infectivity of released virus at 37°C is unlikely to be due to temperature-sensitivity and the increased synthesis of virus at 31°C is probably due to the decreased metabolic rate of the host cell.

III) THE EFFECT OF ANTISERUM ON THE PERSISTENTLY INFECTED CELL-LINES

Introduction

Certain carrier cell cultures have been described, where the spread of infection largely occurs through the supernatant medium. Usually these persistent infections involve strains of virus which are of reduced virulence and only a low percentage of the cells are infected at any one time. The addition of antiserum

antiserum to HeLa cells infected in this manner with Cocksackie A 9 virus results in elimination of the virus and cells cured by this method are susceptible to reinfection by related and unrelated strains of virus (116).

It has been demonstrated (93) that the BK pi carrier cell-line cannot be cured by growing the cultures for four weeks in medium containing antibody to NDV. Although haemadsorption is absent during this period of treatment, it reappears 24 hours after the removal of anti-NDV serum from the supernatant medium.

Results

In the present work, it was shown that anti-NDV serum (prepared in rabbits) diluted 1:256 was sufficient to neutralise the infectivity of $10^{4.0}$ $TCID_{50}$ (or four HAU) of Herts virus, which is the same titre as that found to inhibit haemagglutination by the same amount of virus.

Using this antiserum at 1:8 dilution, it was confirmed that haemadsorption disappeared two days after it was incorporated in the culture medium of BK pi, OK pi and BK pi cells. However, when the antibody was removed from the medium following seven days of incubation, haemadsorption rapidly reappeared. Furthermore, immunofluorescence was undiminished throughout the monolayers on the seventh day of the treatment.

It was also found that if a BK pi culture was

was treated with antiserum for 24 hours and then refed on medium free of antibody, 4 HAU/ml were yielded from these cells two days later. A similar quantity of haemagglutinin was released from untreated carrier culture controls.

Discussion

The only effect antiserum has on the three carrier cell-lines is to neutralise the haemagglutinin on the cell surface. Thus, after removal of the antiserum from the supernatant fluids, there is no consequent effect on the release of virus or the incorporation of haemagglutinin into the cell membrane. Since the percentage of haemadsorbing cells is not affected by treatment of the carrier cell-cultures with antiserum, it is unlikely that transmission of infection within the persistently infected monolayers takes place through the nutrient medium and viral material is probably passed to daughter cells at mitosis. Transference of virus from adjacent cell to cell is also unlikely in view of the low percentage of the infected cells which exhibit fusion and also the low infectivity of the released virus.

IV) THE EFFECT OF TREATMENT OF MONOLAYERS OF BK pi WITH ACTINOMYCIN D AND CYCLOHEXIMIDE.

Introduction.

Actinomycin D and cycloheximide together with several other antibiotic and chemical agents have

have frequently been used to investigate the replicative pathways of viruses.

Actinomycin D prevents the formation of messenger RNA from the DNA replicate and, at a sufficient concentration, will halt cellular RNA synthesis. Since it has little effect on the translation of the messenger RNA at the ribosome, there is very little direct inhibition of protein synthesis. However, since the duration of the replicative cycle of cellular messenger RNA is approximately one hour, the formation of cellular protein will eventually be prevented also (187).

Mithramycin and camptothecin are similar in activity to actinomycin D but have even less effect on the translation of messenger RNA (188, 189), while the major influence of puromycin and cycloheximide is the inhibition of protein assembly from the ribosomal template. It has been shown in the case of orthomyxoviruses, that synthesis of viral messenger RNA is inhibited by the same agents that also prevent the translation of cellular DNA, although synthesis of the nucleic acid of the viral genome is relatively unaffected. Messenger RNA formed in this way, is employed in the synthesis of viral proteins and genome RNA within the cytoplasm

cytoplasm (188, 189). Although there is evidence that the production of neuraminidase and haemagglutinin is strongly inhibited in cells treated with actinomycin D or similar agents, the synthesis of viral RNA-dependent RNA polymerase and ribonucleocapsid is less affected (190). Actinomycin D acts in a similar way to prevent the replication of the messenger RNA of Rous sarcoma virus, where once again, the RNA genome of the infecting virus is translated into a DNA form, which, in this case, is incorporated into the genetic material of the host cell (191). Nevertheless, such substances have little effect on the replication of other RNA viruses including poliomyelitis virus, where the genome of the invading virus particle can act as messenger RNA (192).

Because paramyxoviruses form a RNA replicate of the genome RNA which acts as messenger RNA (36), they may be described as intermediate between these two groups of RNA viruses. However, since actinomycin D exerts its major influence on the translation of DNA, it has much less effect on the synthesis of either the genome or messenger RNA of NDV than on those of orthomyxoviruses (187, 188).

The formation of cellular protein and viral protein of both myxo- and paramyxoviruses is inhibited by such compounds as cycloheximide and puromycin, which act by blocking the translation of messenger RNA, in the cytoplasm. Thus, the formation of viral RNA polymerase will be prevented; consequently, the synthesis of viral RNA and the release of virus (51)

will be inhibited indirectly by these agents. It has also been shown that the synthesis of the RNA of measles virus, whose replication otherwise resembles that of paramyxoviruses, is relatively unaffected by cycloheximide (193). Furthermore, it has been demonstrated that following treatment with puromycin or actinomycin D, more infectious virus is released from monolayers of human conjunctival (C-M) cells that are persistently infected with mumps virus than untreated control cultures (126).

a) Effect of actinomycin D and cycloheximide on RNA and protein synthesis in the BK pi carrier cells.

In the present work, actinomycin D and cycloheximide were used to treat BK pi and control MDBK cells. Actinomycin D was employed at several dose levels, and it will be seen from the results in Table XXXV, that at concentrations of one µg and five µg per ml, over 75% of total RNA synthesis was inhibited both in persistently infected BK pi cells and in uninfected MDBK controls. It is evident that the higher dosage of actinomycin D caused greater inhibition in the uninfected cells and thus, the additional RNA formed in the carrier cells is possibly of viral origin. Similarly, the reduction in protein synthesis in cultures treated with cycloheximide, ranges from 88% to 91% for MDBK monolayers but during the same 24 hour period, there was slightly less inhibition in BK pi cells. It may also be significant that although RNA

T A B L E XXXV

INHIBITION BY ACTINOMYCIN D AND CYCLOHEXIMIDE OF SYNTHESIS OF RNA AND PROTEIN IN BK pi AND CONTROL MDBK CELLS

CELL LINE	AGENT	DOSE LEVEL per ml	LENGTH OF TIME OF INCORPORATION OF LABEL (hours)	% INHIBITION	
				PROTEIN (^{14}C amino acid)	RNA (^{3}H uridine)
BK pi	Actinomycin D	1µg	2	N.D.	75
			8	N.D.	70
			24	N.D.	60
BK pi	Actinomycin D	5µg	2	N.D.	N.D.
			8	N.D.	75
			24	N.D.	70
MDBK	Actinomycin D	1µg	2	N.D.	80
			8	N.D.	75
			24	N.D.	71
MDBK	Actinomycin D	5µg	2	N.D.	N.D.
			8	N.D.	85
			24	N.D.	89
BK pi	Cycloheximide	25µg	2	86	50
			8	85	53
			24	84	56
MDBK	Cycloheximide	25µg	2	88	54
			8	90	56
			24	91	87

RNA production, in BK pi cells treated with cycloheximide is reduced to 50% of that of untreated cells within two hours and to 56% twenty-two hours later, the relevant figures for MDBK monolayers are 54% and 87% respectively.

Discussion

These results indicate that not only does cycloheximide have an immediate inhibitory effect on RNA synthesis in both the carrier cell-cultures and the healthy control cells, but that after a time-lag of at least eight hours, this cytotoxic agent also causes additional inhibition of RNA synthesis in the uninfected MDBK cells but not in the persistently infected BK pi cells. This difference may be due to the continuing synthesis of RNA in the carrier cells due to the presence of viral RNA polymerase, while the formation of cellular RNA polymerase will itself be inhibited by the direct action of cycloheximide. The lack of effect of cycloheximide on the replication of the RNA of the BK pi strain of NDV may thus resemble that described for measles virus (193).

b) The effects of cytotoxic agents on the production and release of haemagglutinin and infectious virus from BK pi cells.

Table XXXVI shows that the amount of haemagglutinin synthesised and released is reduced over the 24 hour period following treatment with either cycloheximide or actinomycin D and, although cycloheximide produces an

T A B L E XXXVI

PRODUCTION OF RELEASED HAEMAGGLUTININ (RHA), CELL ASSOCIATED HAEMAGGLUTININ (CAHA) AND INFECTIOUS VIRUS FROM BK pi CELLS TREATED WITH ACTINOMYCIN D OR CYCLOHEXIMIDE.

AGENT	LENGTH OF TIME OVER WHICH PRODUCTION WAS OBSERVED (hours)	RHA/CELL $\times 10^6$	CAHA/CELL $\times 10^6$	INFECTIOUS VIRUS RELEASED PER CELL***
Actinomycin D*	2	-ve	6.5	-ve
	8	0.25	6.0	$\bar{5}.30$
	24	0.12	5.0	$\bar{5}.93$
Cyclo-** heximide	2	N.D.	6.5	-ve
	8	N.D.	3.5	$\bar{5}.11$
	24	N.D.	3.2	$\bar{6}.48$
Cyclo-** heximide with Actinomycin D*	2	N.D.	N.D.	-ve
	8	N.D.	3.4	$\bar{5}.53$
	24	N.D.	2.1	-ve
None	2	-ve	6.5	-ve
	8	0.25	6.0	$\bar{5}.0$
	24	0.93	12.0	$\bar{5}.54$

Assay of infectivity performed in microtitre plates.

* 1 µg/ml

** 25 µg/ml

***expressed as $\log_{10}(TCID_{50})$.

an initial increase in the number of infectious particles released per cell, it causes a reduction in yield by 24 hours. On the other hand, the yield of infectious virus per cell is greater both eight and 24 hours after overlaying the cells with actinomycin D. When the two agents are used in combination, synthesis of cell associated haemagglutinin is again diminished. Although no infectious virus could be detected one day after treatment with the mixture of actinomycin D and cycloheximide, the quantity released at eight hours appears to be increased above that yielded by either cycloheximide or actinomycin D treatment on its own. This observation is similar to the results reported by Northrop (126) with a persistent mumps virus infection. The carrier cell system he described had in many respects many features, resembling those of BK pi cells, but although 90% of the C-M cells contained mumps virus antigen, less than five per cent of the cells showed haemadsorption. In cultures of BK pi cells, over 90% of the monolayer can be stained by specific immuno-fluorescence and 70% adsorbed red blood cells. Although Northrop observed an increase in the percentage of C-M carrier cells showing haemadsorption, following treatment with actinomycin D, no increase or decrease in these parameters was observed on treatment of BK pi cells with actinomycin D.

Discussion

In the present work, the results obtained by

by treating BK pi cells with actinomycin D and cycloheximide indicate that viral RNA and protein synthesis are less affected by these agents than are cellular RNA and protein synthesis. Although treatment with actinomycin D causes reduction in the synthesis and release of virus from BK pi cells, the infectivity of the virus is increased. Compared with actinomycin D, cycloheximide causes greater reduction of viral protein synthesis in BK pi cells and virus production is eventually completely inhibited. However, treatment of BK pi cells with both of these agents initially causes a synergistic increase in the quantity of infectious virus released.

Unfortunately, no increase could be detected in the levels of neuraminidase, phosphodiesterase and haemagglutinin activities per μg protein of purified virions from cultures treated with actinomycin D and these activities remained approximately 10, 3 and 100 times less than the respective activities of the wild-type virus. This is not surprising, since the infectivity of BK pi virus is at least 10^5 lower than that of wild-type virus. Thus, the 2-3-fold increase in infectivity observed in this experiment, implies that particles of normal virulence account for less than one per cent of the total yield of virus from the persistently infected cells. Therefore, any rise in biological activity associated with the occasional virions with increased infectivity, will not be evident since the great majority of the virus particles

particles liberated from persistently infected cells do not possess these properties. Although treatment of BK pi cells with sufficient actinomycin D to prevent cellular RNA and protein formation, causes less inhibition of viral synthesis, haemagglutinin production and release from treated BK pi cells are considerably reduced. Since the capacity of NDV to synthesise viral protein is not normally affected by these amounts of actinomycin D (187, 188), it is therefore postulated that synthesis of RNA in the persistently infected cells may involve a DNA intermediate, in addition to the normal RNA replicative complexes (36). This is supported by the results of other workers who have described a viral RNA-dependent DNA polymerase in L cells carrying NDV (45) and DNA replicates of measles virus RNA in the nucleus of persistently infected cells (150).

The increase in infectivity may be due to two possible effects of actinomycin D, as follows:

i) the blocking of a DNA pathway for viral RNA synthesis. Normal replication of the persistent virus in BK pi cells may be through a pathway involving DNA translation, which results in defective virus. The blocking of this step by actinomycin D may result in the employment of the normal method of replication of NDV, which proceeds via an RNA transcription complex and may yield virus that is not defective.

ii) the prevention of the cellular synthesis of a viral inhibitory factor. There may be a cellular

cellular factor which inhibits viral replication and which cannot be synthesised when the replicative mechanisms of the cell are interrupted by the activity of actinomycin D. The lack of this factor could also account for the increased yield of virus observed when the metabolism of the cell is slowed by incubation at low temperatures. However, in the latter example the total amount of released haemagglutinin is increased.

On the other hand, further support for hypothesis (i) being the major cause for the increased infectivity of virus released from BK pi cells is the fact that in the case of orthomyxoviruses ~~(which are known to replicate messenger RNA from a DNA template)~~, actinomycin D inhibits the synthesis of haemagglutinin, but has less effect on the synthesis of genome RNA or RNA polymerase. A similar phenomenon appears to occur in BK pi cells treated with actinomycin D since infectious virus continues to be released although less haemagglutinin is formed.

The effect of cycloheximide may likewise be explained either by the blocking of a cellular inhibitor or by the independence of viral RNA formation from the cellular system. This would enable viral RNA to be formed by the mediation of viral RNA polymerase, while cellular RNA polymerases cannot be synthesised because of the action of cycloheximide. However, the formation of viral protein will itself be inhibited by this agent so that, following an initial rise in the amount of virus released from each cell (due to the lack of

of competition from cellular synthetic mechanisms) there will be a decline in the amount of virus produced.

In conclusion it may be stated that the experiments with these cytotoxic agents and the results obtained from incubation of the carrier cells at 41°C support the hypothesis that replication of these strains of NDV can employ a DNA transcript of the viral messenger RNA. Furthermore,this could be one of the causes of the production of defective, avirulent virus and the means by which the carrier state is maintained.

V) THE EFFECT OF SEEDING BK pi CELLS AT LOW DENSITIES

Introduction

In the previous Section dealing with the effects of incubation at 41°C on the three carrier cell-cultures, it was shown that although haemadsorption disappeared after several days at 41°C, it generally recurred when the cells were returned to 37°C. The persistence of the virus at 41°C may be explained by the translation of the viral genome into an inactive DNA replicate which is incorporated into the genetic material of the host cell. It was suggested that the failure of some clones to resume synthesis of haemagglutinin when replaced in the 37°C incubator, was due to one of the following reasons:-

i) the inability of the virus to form a DNA replicate of the genome RNA.

RNA.

ii) the viral genome remaining in the form of the inactive DNA replicate, after the cells had been returned to 37°C.

iii) the greater viability at 41°C of the minority of cells in the carrier cell-cultures, which show no evidence of viral infection.

In any of the three cell-lines under examination here, FAS reveals that never more than 90% of the cells contain NDV antigen. The absence of viral proteins from the remaining cells in the monolayer may be due either to the complete absence of virus or to the presence of the viral genome in an inactive form. Therefore, if, as suggested above, growth at 41°C were to favour this type of cell more than that in which viral protein was being synthesised, the result of such incubation would be to select the uninfected cells or those in which the viral nucleic acid was in a dormant state. This phenomenon would act in sympathy with the established inability of persistent virus to replicate itself at the non-permissive temperature.

If the carrier cultures consisted of two such populations, it would, in consequence, be expected that clones could be established at 37°C in which a smaller or greater proportion of the cells would be shown to be infected. Indeed, to a certain extent, the fluctuations in the proportion of cells in the monolayer showing haemadsorption (as described in

in Section 1) may be due to random selection of cells at the time of re-seeding.

Unfortunately, attempts to sub-culture single cells from the persistently infected monolayers by the methods of Puck (194) and Simon and Fleischmann (195) were unsuccessful. On the other hand, the reseeding of cells at low concentrations, although not free from the possibility of cross-infection through the supernatant medium, provided some evidence to support the hypothesis described above.

a) Seeding of BK pi cells at low concentrations.

BK pi cells were seeded in either 60 mm petri dishes or microtitre tissue culture plates at such concentrations that 30 cells attached per plate or per well. In both instances, after two days' incubation, 60% of the cells were haemadsorbing, which is similar to the proportion observed in monolayers seeded in the normal way. Moreover, although mitosis produced groups of up to ten cells, it was found that either every cell in the colony adsorbed erythrocytes or none did.

Discussion

In view of the fact that infection in any one of the cells could be expected to spread to any or all of the remaining 30 cells in the culture, it is not clear whether all of the haemadsorbing cells were able to synthesise haemagglutinin and incorporate it into the cell-membrane.

membrane.

b) Co-cultivation of low numbers of BK pi cells with large numbers of CF cells.

Although BK pi cells could not be grown in microtitre plates at seeding densities of less than thirty cells per well, co-cultivation with chick fibroblasts (CF) enabled lower concentrations of the carrier cells to be employed. When a mixture of BK pi and CF cells were seeded in microtitre plates in certain proportions, approximately one BK pi cell could be identified in a well containing a complete monolayer of CF cells. The same percentage of BK pi cells haemadsorbed under these circumstances as in a complete monolayer of BK pi cells.

c) Seeding of BK pi cells on to complete monolayers of CF cells.

When BK pi cells were overlaid on to one-day-old complete monolayers of uninfected CF cells, haemadsorption was confined to 10% of the BK pi cells on the first day, rising to 40% by the third day and 80% by the sixth day of incubation. The proportions were the same whether one or thirty BK pi cells were added to each well with a complete monolayer of chick embryo fibroblasts. Furthermore, it was again shown that for each clone the presence of haemadsorption was an all or none phenomenon. Moreover, in addition to the attachment of erythrocytes, by the sixth day 70% of such groups of cells also exhibited the ability to fuse with the neighbouring chicken fibroblasts and

and haemadsorption was frequently observed in cells of the latter type, adjacent to the clones of BK pi cells.

In all of these experiments the BK pi cell suspensions were obtained by treatment of monolayers of this cell-line with a mixture of trypsin and versene (STV). In order to assess the influence of these two components on the amount of haemadsorption seen after reseeding, the procedures outlined in the previous paragraphs were repeated using either trypsin (0.05%) or versene (0.007%) for dispersing the cells in stock carrier cultures. Closely similar results were obtained using clones treated in either way, whether the carrier cells were grown alone or were co-cultivated with chick embryo fibroblasts. However, when versene was employed to subculture BK pi cells on one-day-old CF monolayers, the proportion of haemadsorbing cells did not exceed 10% on the fifth day of incubation. Moreover, over 50% of the haemadsorbing clones contained BK pi cells that did not exhibit this phenomenon.

Discussion

i) The replicative cycle of persistent virus in BK pi cells.

On the basis of these results, it is suggested that the viral replicative cycle in BK pi cells passes through the following four stages:-

(Since these stages are independent of cellular mitosis, the ability of daughter cells to haemadsorb will be the

the same as the parent cells. Thus, haemadsorption by clones of these cells will be largely an all or none effect.) It is emphasised that this is put forward only as a hypothetical model to explain certain features of the persistent infection of BK pi cells.

A) Inactive

The virus is either absent or the viral genome is present in an inactive form. Less than 10% of the cell population is in this state and the cells cannot be stained by immuno-fluorescence (See Section 1).

B) Synthesis of viral proteins

Viral antigens are detectable by immunofluorescence and cause physiological changes in the cell membrane resembling those of cellular transformation. It is possible that at this stage red blood cells adsorb to undifferentiated cell membranes (see Section 1) in a similar manner to that described for mumps virus (60). This effect has not been described in normal infections with NDV.

C) Incorporation of haemagglutinin into the cell membrane

Cells in this state stain with fluorescent antibody and haemadsorption takes place at differentiated portions of the plasmalemma. Probably not more than 10% of the population is in this state at any one time and these are the only cells capable of adsorbing erythrocytes following sub-culture on to one-day-old CF monolayers.

D) Mature virions are attached to the cell surface

This is probably due in part to the defective

defective neuraminidase of the persistent virus and in part to the defective small envelope glycoprotein, which is trypsin-sensitive (see Section 4). Therefore, haemadsorption and fluorescence are seen in these cells. (It is probable that in mature cultures, some cells in stages (B) and (C) will also have virions attached to their surface.)

The trypsin or versene employed to remove cells from the glass of the culture bottle may also detach the mature virions from cells in stage D and, additionally, the trypsin (but not the versene) will alter the small envelope glycoprotein to an active form. In consequence the cells in stage D will be incapable of haemadsorption immediately after reseeding and because the observed reduction is about 30%, it may be concluded that this is the proportion of cells which neither actively synthesise viral proteins nor possess differentiated membranes. It is evident that in normal circumstances a sufficient number of cells have passed through the complete viral replicative cycle in two to three days to produce haemadsorption in 80% to 90% of the monolayer. (The remaining 10% of cells are those in stage A).

ii) <u>The relationship of certain features of BK pi cells to cellular transformation</u>

It has been shown that contact inhibition and suppression of mitosis, which occur in normal overcrowded untransformed monolayers, do not take place

place in BK pi cells (Section 1). Thus this cell-line is capable of colony formation in semi-solid agar. In addition, several authors have shown that the closely related phenomenon of cell agglutination due to lectins, such as concavallin A (196) (96), takes place both in transformed cells and in those infected with NDV. In the latter case, the physiological change in the cell membrane occurs after synthesis of viral proteins has begun, but does not require incorporation of these polypeptides into the plasmalemma (96). It is also probable that the adsorption of red blood cells to undifferentiated membranes of BK pi cells, is due to a similar phenomenon. However, the one-day-old monolayers of chick embryo fibroblasts to which BK pi cells were added, were both crowded and untransformed, and were likely to be emitting the chemical agent which inhibits mitosis (174). Therefore it is possible that this transmitter might be present in sufficient quantities to conteract the transforming action of the viral proteins which, it is postulated, causes haemadsorption at stage 'B'. If this hypothesis were correct the only cells still capable of haemadsorption would be those in stage 'C', with haemagglutinin incorporated into the cell membrane but without budding virus. The proportion of haemadsorbing cells was 10% and, since 30-40% of the cells are in stage 'D' with about ten per cent in stage 'A', the remaining 40-50% will be in stage 'B'. Therefore, during the period of four to five days which is necessary for cells in stage B to proceed through stages 'C' and 'D', the

the proportion of clones showing haemadsorption will gradually rise. This indeed was the case in cultures where the overlying BK pi cells had been treated with trypsin.

VI) SUMMARY OF SECTION (3)

1) Growth of carrier cells in conditions that slow the rate of mitosis, results in decreased synthesis of viral proteins but this is accompanied by an increase in the incorporation of haemagglutinin into the cell membrane and in the amount of virus released. These features are observed in cultures fed on lower than normal concentrations of amino acids and serum, or in monolayers incubated at 31°C.

2) The replication of virus is sensitive to a temperature of 41°C at an early point in the synthetic pathway. Following prolonged incubation at this temperature, viral antigen becomes undetectable but when the cultures are returned to 37°C sometimes reappears after a variable period of incubation.

3) Specific antibody inhibits the release of virus for as long as it is in contact with the infected monolayer. However, when the antiserum is removed, haemadsorption and the release of haemagglutinin return. The percentage of cells containing viral antigens is unaltered by treatment with antibody.

4) Actinomycin D and cycloheximide inhibit the synthesis and release of BK pi virus but cause an increase in the infectivity of the material that was liberated.

liberated.

5) The percentage of BK pi cells absorbing erythrocytes remains the same whether the cells are grown in isolation or as a complete cell-sheet. However, overlay of carrier cells on to one-day-old monolayers of CF cells, causes a reduction in the number of haemadsorbing cells. After six days' incubation, the proportion of haemadsorbing cells reaches normal levels, provided they are prepared for sub-culture by trypsinization but remains at 10% of the total in cases where versene had been used to detach the cells from the glass.

VII) DISCUSSION AND CONCLUSIONS

From the results reported in Section '1' and '2' and those described in the present Section, the following conclusions have been drawn concerning the nature of the carrier cell state.

Specific immunofluorescence shows that 90% of the cells in a carrier cell culture are synthesising viral proteins, and it is extremely rare for haemadsorption to exceed this percentage in any of the carrier cell-lines. It is unlikely that different cell populations were responsible for the 10% of cells that did not show either of these effects; and the absence of these properties would suggest that they are completely free of virus or contain the viral genome in an inactive form. Had it been possible to clone some of these 'inactive' cells it is postulated that they would behave

behave in a similar manner to clones of cells in which viral synthesis had been halted by incubation at the higher non-permissive temperature of 41°C. It is believed that, in both these cases, the viral genome is in an inactive form and is contained, as a DNA transcript, within the genetic material of the cell. Viral replication, accompanied by haemadsorption, recommences when cells, which have been cultured at 41°C, are returned to 37°C. It is probable therefore, that many clones of the 'inactive' minority (10%) of cells in a carrier cell monolayer, would eventually demonstrate signs of viral infection. This hypothesis is further supported by the presence of an RNA-dependent-DNA-polymerase in another case of persistent infection with NDV (45) and the sensitivity of BK pi virus replication to actinomycin D. However, both this antibiotic and cycloheximide, although reducing the quantity of viral material that is synthesised and released, also increase the infectivity of the liberated virus. Therefore, it is suggested that the dependence of the persistent virus on a DNA template for viral messenger RNA replication is a cause of some of the defective biological activities observed.

In the great majority of the cells (90%) with immunological evidence of the synthesis of viral proteins, the occurrence of haemadsorption is believed to be due to three different mechanisms:

i) haemadsorption to undifferentiated membranes

ii) haemadsorption to differentiated areas of the

the cell membrane

iii) haemadsorption to liberated virus still attached to the cell surface.

Type (i) has been shown by electron microscopy to take place in BK pi cells (Section 1). Results obtained in this present study suggest haemadsorption in this case is due to a process akin to that causing the alteration in membranes of cells, infected with NDV (96). These features of cells, infected with NDV, together with the formation of 'colonies' in sloppy agar by the carrier cell-lines (Section 1) are suggestive of cellular transformation.

Transformation is believed to be brought about by 'jamming' in the 'on' position of the 'switch' or receptor on the cell surface which controls mitosis (174). Cultures which are overcrowded may release an agent that switches the receptor 'off' but this process does not take place in transformed cells. Therefore, it is suggested that in the case of BK pi cells overlaid on to a complete CF monolayer, sufficient transmitter substance is released from the CF cells to 'unjam' the mitosis 'switch' of BK pi cells and prevent haemadsorption of type (i).

Haemadsorption of type (ii) is the normal process by which the phenomenon occurs. However, because of the results obtained when BK pi cells were overlaid on CF monolayers, it is believed that it usually takes place in only 10% of the cells at a time. Conditions that discourage cell growth and mitosis result in

in increased incorporation of haemagglutinin into the cell membrane and the greater release of virus. Under these circumstances, which include reduced quantities of serum or amino acids in the nutrient medium, or incubation at 31°C, the synthesis of viral proteins, is inhibited. Furthermore, because haemadsorption of sparsely seeded cells was either all or none, depending on the clone of cells, virus within daughter cells is in the same stage of the replicative cycle as in the parent cells. Thus, although most cells in a monolayer of BK pi are actively synthesising viral protein, only ten per cent release haemagglutinin at any one time. Moreover, except after actinomycin D treatment, very few of the viral particles are fully infectious. These results correspond to those of a persistent infection of C-M cells with mumps virus (126).

The third type of haemadsorption (iii) which is due to released virus particles adhering to the surface of the infected cell is probably more prevalent in the carrier cells than in normal infections with NDV because of the lack of neuraminidase in the defective virus. These virions are believed to be detached from the cells when they are treated with STV prior to their transfer to another culture vessel. Moreover, although haemadsorption is neutralised by specific antibody, the same percentage of cells are shown to be infected before and after treatment with antiserum. Therefore intercellular transmission of infection, through released

released viral particles in the supernatant medium, is not the means whereby the persistent infections are maintained.

It is probable that the fluctuations in the percentage of cells shown to haemadsorb during a series of sub-cultures is due to variations in the rate of synthesis of viral proteins. For this reason, insufficient haemagglutinin would be formed and incorporated into the cell membrane. Therefore, fewer cells would haemadsorb by mechanisms (ii) and (iii). It is probable that the extremely low proportions of haemadsorbing cells that are occasionally found in OK pi cultures, are caused in a similar manner. In addition, because of the poor ability of OK pi cells to form colonies in semi-solid agar, it may be inferred that the degree of transformation in the OK pi cell-line is lower than in BK pi. Therefore, it is unlikely that type (i) haemadsorption ever occurs in OK pi cultures, and for this reason an even lower percentage of haemadsorbing cells will, occasionally, be found than in the other two persistently infected cell-lines.

RESULTS

SECTION 4

ATTEMPTS TO RESTORE BIOLOGICAL ACTIVITIES TO THE PERSISTENT VIRUS

RESULTS

SECTION 4

ATTEMPTS TO RESTORE BIOLOGICAL ACTIVITY TO THE PERSISTENT VIRUS

INTRODUCTION

Several studies on persistent infections have indicated that defects in the released virus may be corrected by treatment with certain enzymes (56, 114, 139, 61). In addition, it has been shown that sera used to supplement growth media generally contain mucoproteins which inhibit the neuraminidase and haemagglutinin activity of the virus liberated into the supernatant fluids of infected monolayers (184, 185).

Virus produced by our three carrier cell-lines is notable not only for its extremely low infectivity but also for its deficient capacity to cause haemolysis, hydrolysis of neuraminic acid and agglutination of erythrocytes. In the case of haemagglutination, one strain (BK pi) is much less active than the other two (Table XXIII).

There may be four reasons for these imperfections:

1) Certain of the structural proteins may be absent or incompletely synthesised.

2) One or more of the viral proteins may be incorporated into the virion as the inactive precursor molecule but may be activated by treatment with the

the appropriate cleavage enzyme (11, 42).

3) A deficiency in neuraminidase activity may cause the virions to bind to each other, and to the cell surface, by means of their haemagglutinin. Thus apparently less virus will be released, and the free virus will be in the form of large aggregates; consequently there will be fewer particles capable of adsorbing red blood cells (67). Treatment of liberated virions with neuraminidase should result in the breaking up of these aggregates with a consequent increase in HA titre.

4) The surface of the virions may be coated with serum proteins, which bind to the glycoproteins thereby preventing full expression of the haemagglutinin and neuraminidase activities. These non-specific inhibitors of haemagglutinin may be removed by periodate treatment (185). Experiments were therefore carried out to see if the measures outlined in (2), (3) and (4), were able to increase the infectivity and haemagglutinating ability of the viruses released from persistent infections.

A / TREATMENT OF VIRUS PARTICLES WITH TRYPSIN

Introduction

Reports by several authors, but notably those of Homma *et al.* (56, 114, 139), have shown that incomplete Sendai virus is released following both abortive and persistent infections of certain mammalian cells. This virus has reduced infectivity, haemolysin and fusion activities. Furthermore, analysis of the proteins of the progeny virions of the abortive infection, have revealed that the small envelope glycoprotein is in the form of its precursor. Normally, the antecedent polypeptide is only isolated from infected cells and has a higher molecular weight than the active constituent of mature virus particles. Following treatment of the infected cells with trypsin, there is an increase in the yield of infectious virus (56). Moreover, it has been reported that when purified virus is subjected to this enzyme, its infectivity (56), cell fusion (139) and haemolysing (114) functions are restored and the small glycoprotein is cleaved to its normal size (11, 42).

In Section 3, the effect of trypsin on cloned BK pi cells has been described. Compared with cells treated with versene, there was enhancement of the ability of trypsin-treated cells, to act as a centre of infection for the surrounding CF monolayer. However, haemadsorption was confined to the chick embryo fibroblast cells immediately adjacent to the clone of BK pi cells.

cells.

Since haemolysin, haemagglutinin and infectivity of the B1 strain of NDV (B1/HeLa) were impaired, in comparison with those of the same strain grown in CF cells or chicken embryos, it was decided to carry out parallel experiments with the latter virus, in addition to purified virus from BK pi cells. However, it should be noted that, compared with BK pi, the B1/HeLa virus has relatively greater haemagglutinating ability and more infectivity, besides having normal neuraminidase activity. In Table XXXVII, a comparison is made of the effects of two concentrations of trypsin on the biological activities of the virus released from BK pi monolayers as well as the B1 strain of NDV grown in HeLa cells (B1/HeLa) or developing chicken embryos (B1/E).

Results.

The results of this experiment show that the untreated BK pi and B1/HeLa viruses both have only 12% of the haemolysing activity capacity of B1/E virus. However, following treatment with the low concentration of trypsin, the activity of B1/E virus is unaltered, whereas those of BK pi and B1/HeLa are raised by 69% and 32% respectively. On the other hand, in neither of the defective viruses does the ability of the defective strain to lyse erythrocytes approach one-fifth of that of the egg-grown virus. When the higher dose of trypsin is employed, the haemolysin activity of all

T A B L E XXXVII

THE EFFECT OF TWO CONCENTRATIONS OF TRYPSIN ON THE HAEMAGGLUTININ, HAEMOLYSIN AND INFECTIVITY OF BK pi VIRUS AND THE B1 STRAIN OF NDV GROWN IN HELA CELLS AND EMBRYONATED HENS' EGGS.

STRAIN OF VIRUS	CONCENTRATION OF TRYPSIN (%)	HAEMOLYSIN ACTIVITY** PER µg PROTEIN	HAEMAGGLUTININ ACTIVITY PER µg PROTEIN	INFECTIVITY* PER µg PROTEIN
BK pi	none	1,600	0.18	4.45
	0.0004	2,700	0.18	6.82
	0.0160	1,350	0.72	5.66
B1 / HeLa	none	1,700	1.15	Not done
	0.0004	2.250	1.15	Not done
	0.0160	1,400	2.30	Not done
B1 / Egg-grown	none	13,300	15.0	12.15
	0.0004	13,300	15.0	13.15
	0.0160	12,600	30.0	12,15

* Infectivity assays were performed in microtitre plates and expressed as $\log_{10}TCID_{50}$.

** Number of fowl red blood cells lysed per ml.

all three viruses tested is reduced but a greater haemagglutination titre is produced in each case.

Following treatment with the lower concentration of trypsin, BK pi virus is 2.3×10^2 times more infectious, whereas the infectivity of B1/E shows only a ten-fold increase. Although the infectivity of the latter strain is unaffected by high concentrations of this enzyme, that of BK pi virus is slightly raised.

Analysis of the polypeptides of B1/HeLa virus by polyacrylamide gel electrophoresis (PAGE) (Fig. 46) reveals that the peak, corresponding to VP 55 and VGP 55 of egg-grown virus, is small but shows a relative increase in size following treatment of the virus with 4 p.p.m trypsin. In addition, the diffuse band of polypeptides of approximately 60,000 to 65,000 daltons seen in PAGE profiles of untreated B1/HeLa virus disappears after trypsin treatment. When higher concentrations of trypsin are employed, there is general destruction of all the viral polypeptides, whether the B1 virus is grown in HeLa cells or developing chicken embryos. On the other hand, the gel profile of the egg-grown virus is totally unaffected by the lower doses of trypsin.

The effect of trypsin on the haemolysing capacity of BK pi virus is confirmed by the experimental data recorded in Table XXXVIII. These show that maximum activity occurs when the enzyme makes up 4 p.p.m. of the reaction mixture and that fewer erythrocytes

T A B L E XXXVIII

THE EFFECTS OF DIFFERENT CONCENTRATIONS OF TRYPSIN ON THE HAEMOLYSIN AND HAEMAGGLUTININ OF BK pi VIRUS

CONCENTRATION OF TRYPSIN (ppm)	HAEMOLYSIN ACTIVITY PER µg PROTEIN (Number of erythrocytes lysed)	HAEMAGGLUTININ ACTIVITY (HAU) PER µg PROTEIN
0	1,600	0.18
0.25	1,600	0.18
1	1,600	0.18
2	2,400	0.18
4	2,700	0.18
10	2,100	0.36
20	1,800	0.36
160	1,350	0.72
320	1,050	0.72

Figure 46. Densitometer profiles obtained in a similar manner to those in Fig. 24, showing the effect of trypsin treatment on the proteins of completely purified B1 virus released from HeLa cells or grown in fertile hens' eggs. For comparison, the electrophoretic pattern of untreated virus is shown in Figs. 27 a and 24 c, respectively.

Purified B1 virus released from HeLa cells treated with:

a) 4 p.p.m. trypsin

b) 160 p.p.m. trypsin

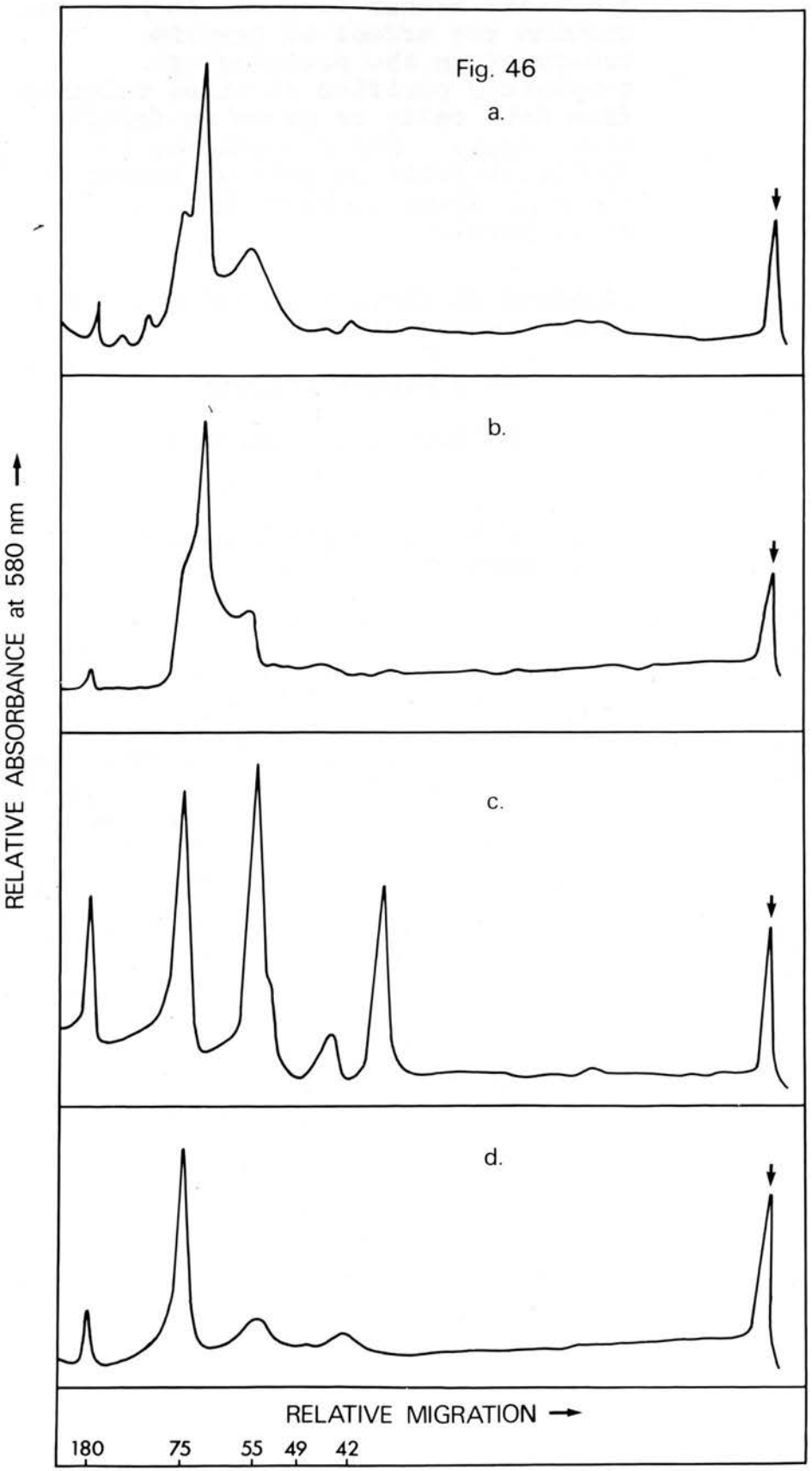
Purified B1 virus grown in fertile hens' eggs treated with:

c) 4 p.p.m. trypsin

d) 160 p.p.m. trypsin

Note the destruction of most viral proteins of both forms of B1 virus by the higher concentration of trypsin. It may also be seen that the profile in Fig. 46 c does not differ from that in Fig. 24 c, but there is a clearer separation of the two major peaks in Fig. 46 a, compared with those in Fig. 27 a.

Fig. 46


a.
b.
c.
d.
RELATIVE ABSORBANCE at 580 nm →
RELATIVE MIGRATION →
180
75
55
49
42

erythrocytes are lysed by virus treated with higher concentrations of trypsin. On the other hand, the higher the percentage of trypsin used within the limits of this experiment, the greater is the haemagglutinin activity of the virus. It is clear from Table XXXVII that this phenomenon occurs with all three viruses tested and is probably related to the general destruction of virions as implied by the diffuse bands of protein seen in electrophoretograms of virus treated in this manner. (Fig. 46b, d).

Discussion

First, it has been confirmed that treatment of NDV with high concentrations of trypsin, results in the destruction of the virion and is accompanied by the release of fragments of the viral envelope containing the large glycoprotein of NDV. The consequent increase in the number of particles bearing spikes of haemagglutinin is probably the cause of the rise in haemagglutinin titre. However, haemolysin activity falls because it requires the presence of both the large and small glycoproteins of the viral envelope. (A similar phenomenon has been reported in respect of Sendai virions disrupted by sonication (214)).

Second, although 4 p.p.m. of trypsin have little effect on strains of NDV grown in embryonated hens' eggs, the abnormal peptide bonds of the small glycoprotein (VGP 55) of BK pi and B1/HeLa viruses are destroyed at this concentration. As a result, the

the VGP 55 of these cell-grown viruses returns to a normal molecular weight of 55,000 daltons under reduced conditions of electrophoresis. The low infectivity and haemolysin activity of B1/HeLa and BK pi viruses are also partially restored by treatment with 4 p.p.m. of trypsin, and it appears therefore that functional haemolytic activity is associated with the presence of normal VGP 55. A similar phenomenon has previously been described with Sendai virus released from MDBK or HeLa cells (11) and in the latter case, cell-fusion is also restored by trypsin treatment (139). In view of these results, it is unfortunate that the cell-fusion index was not assayed in the present work.

The amount of trypsin required to destroy the abnormal peptide bonds in the small glycoprotein of cell-grown NDV is only slightly less than that required to cause breakdown of the virus particle, as indicated by the increase in haemagglutinin activity shown in Table XXXVIII. Thus it is apparent that the dose of trypsin required to raise haemolysis and infectivity to their normal levels, would be greater than that which causes destruction of the virion. Moreover, since BK pi virus is clearly deficient in many other properties, it is unlikely that full virulence can be restored by trypsin treatment alone.

B / THE EFFECT OF RECEPTOR DESTROYING ENZYME (RDE) DERIVED FROM VIBRIO CHOLERAE (BACTERIAL NEURAMINIDASE) ON THE HAEMAGGLUTININ OF BK pi VIRUS

Introduction

In view of the extremely low neuraminidase specific activity of the virus released from the three carrier cell-lines, it is interesting to compare the present findings with those of Palese et al. (67), who investigated a neuraminidase deficiency in persistent temperature-sensitive mutants of the WSN strain of influenza A virus.

This infection was distinguished by the minimal capacity of the liberated virus to hydrolyse neuraminic acid, by its low haemagglutinin activity and by the poor release of virus from the persistently infected monolayers. Since the last two properties were restored to normal after treatment with neuraminidase, it was concluded that in the absence of neuraminidase, neuraminic acid remains in the cell membrane adjacent to budding viruses. The haemagglutinin of the released virus then becomes bound to the cellular glycoproteins present in the cell surface or the envelope glycoproteins of other budding viruses. Thus much of the liberated virus is either bound to the cells or is in aggregates with other virions. Therefore, the presence of neuraminidase in the envelope of normal myxoviruses allows the release of virus from infected cells and prevents clumping with other virus particles.

particles.

In view of the similarity of many of the features of the three carrier-cell-lines investigated in the present work with those of the persistent infection described by Palese *et al.*, virus released from BK pi cells was treated with the neuraminidase of *Vibrio cholerae*, employing the method described by these workers (67).

Results

No alteration in haemagglutination titres occurred, whether the erythrocytes were added to a) the virus alone, b) virus plus N-acetyl neuraminic acid (NANA), c) virus and RDE or d) virus, NANA and RDE (Table XXXIX).

Discussion

It appears therefore, that the haemagglutinating capacity of BK pi virus is low because haemagglutinin is defective and is not due to the mechanism described by Palese *et al*. Moreover in orthomyxoviruses, neuraminidase and haemagglutinin are found on two separate proteins so that deficiencies in one are less likely to be associated with imperfections in the other, whereas, in NDV, both activities are borne on the same polypeptide.

Furthermore, while the polyacrylamide gel electrophoretograms of the persistent WSN influenza virus showed that its constituent polypeptides were

T A B L E XXXIX

THE EFFECTS OF N-ACETYL-NEURAMINIC ACID (NANA), VIBRIO CHOLERAE NEURAMINIDASE (RDE) AND SODIUM PERIODATE ON THE HAEMAGGLUTININ TITRE OF BK pi VIRUS.

EXPERIMENT 1 TREATMENT	H A U PER ML	
	UNPURIFIED VIRUS (in medium containing serum)	PURIFIED VIRUS
Control (untreated)	not done	1024
NANA*	not done	512
RDE**	not done	256
NANA* with RDE**	not done	512

*10 μg/ml **200 Units/ml

EXPERIMENT 2 TREATMENT	H A U PER ML	
	UNPURIFIED VIRUS	PURIFIED VIRUS
Control (untreated)	8	32
Periodate (0.01 M)	12	32

were normal (67), the proteins of BK pi virus vary considerably from those of 'wild-type' NDV (see Section 2).

On the other hand, the results suggest that the dense haemadsorption seen in mature BK pi monolayers may be due to binding of the haemagglutinin of the released virions to the neuraminic acid of the cell membrane since this constituent is not removed from areas of the plasmalemma adjacent to budding virus in the absence of neuraminidase.

C / TREATMENT OF BK pi VIRUS WITH SODIUM PERIODATE

Introduction

It has long been known that certain of the elements found in normal serum contain neuraminic acid, which can bind to viral haemagglutinin and reduce the apparent haemagglutination titre of the virus. Since glycoproteins of this character have been isolated from rabbit and horse serum (184, 185), it is possible that they are present in the calf sera used to supplement the growth media in the current experiments.

Strains of influenza virus vary in their susceptibility to these non-specific inhibitors (197), depending on which prosthetic group is attached to neuraminic acid (198). However, such differences have not been observed between strains of NDV, despite the fact that the haemagglutinin of the latter virus is inhibited by these serum proteins (199).

When neuraminic acid is oxidised by periodate, its capacity to bind to viral haemagglutinin is generally destroyed, and the non-specific inhibitory effect of a serum can be removed by this method.

In the present work, a large amount of a protein of 70,000 daltons is observed in purified NDV released from monolayers of mammalian cells (Figs. 28, 27, 29), but this polypeptide is not found when the virus is grown in embryonated chicken eggs (Fig. 24). Therefore, a possible source of this extraneous element might be the mucoproteins of serum in the nutrient medium, which

which become bound to the envelope of the virus. If this were the case, oxidation of the serum glycoproteins with periodate, would bring about their removal from the surface of the virion, and hence the disappearance of VP 70 from the polyacrylamide gel electrophoretograms of purified cell-grown virus.

Results

A comparison of the results shown in Figs. 47 and 28 indicate that treatment with periodate has no discernible effect on the presence of VP 70. Moreover, although the haemagglutination titre of the supernatant fluid from a monolayer of BK pi cells increased by up to 50% fdlowing treatment with periodate, this effect was not obtained with purified BK pi virus (Table XXXIX). This result apparently confirms that non-specific inhibitors of haemagglutinin are present in the nutrient media but are eliminated by the process of purification. Furthermore, because the haemagglutinating activity of BK pi virus is less than one-hundredth of that of the Herts strain of NDV, while the increase in titre consequent on the addition of periodate is only 50%, it is concluded that the serum glycoproteins are not responsible for the diminished capacity of BK pi virus to agglutinate red blood cells.

Figure 47. Densitometer profiles obtained in a similar manner to those in Fig. 24, showing virus released from BK pi cells, completely purified and treated with periodate as described.

a) Proteins run under non-reduced conditions (compare with Fig. 34 a).

b) Proteins run under reduced conditions (compare with Fig. 28 a).

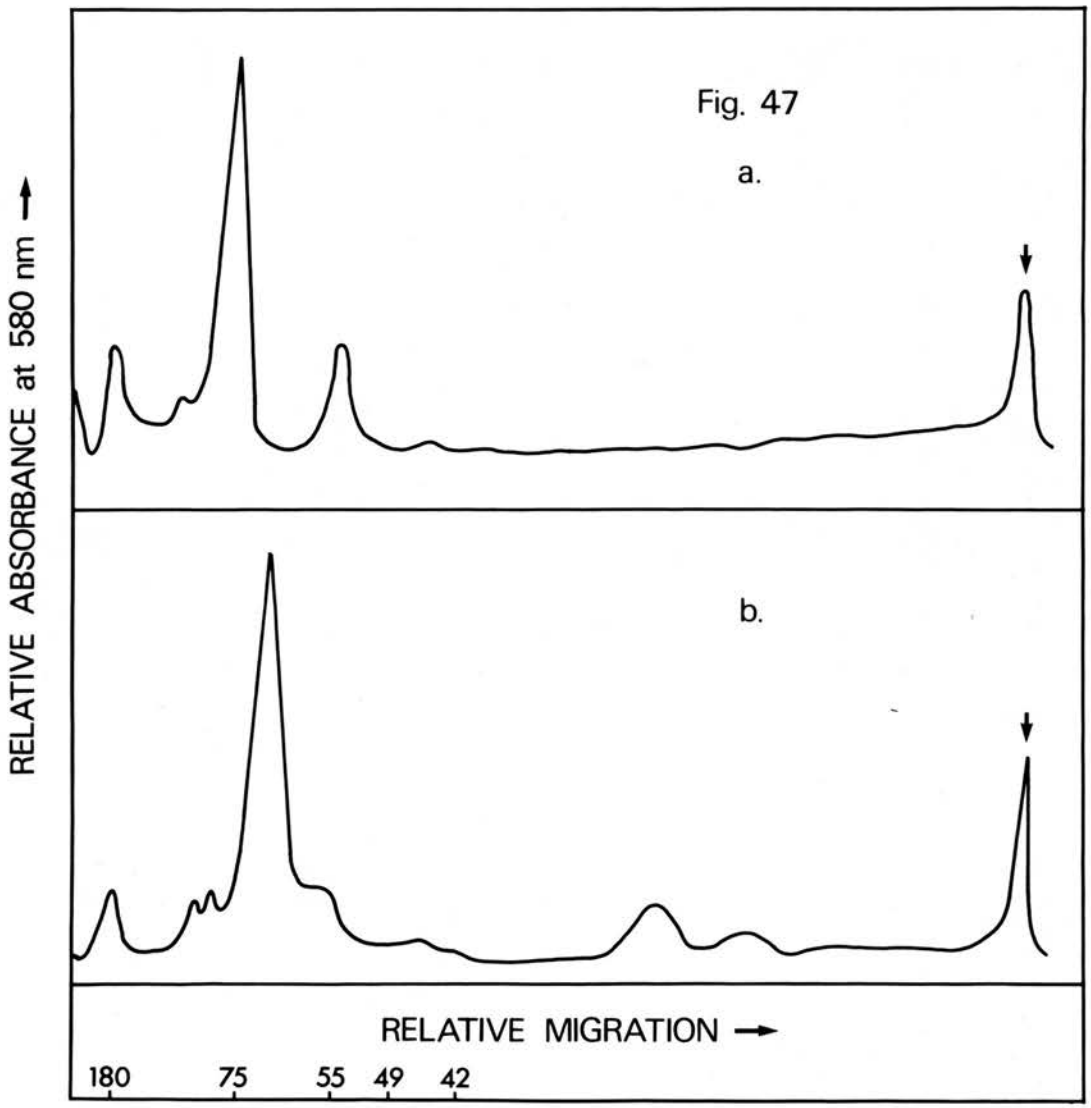


Fig. 47
a.
b.
RELATIVE ABSORBANCE at 580 nm
RELATIVE MIGRATION
180
75
55
49
42

D / SUMMARY OF SECTION 4

The reduction in activity of the haemolysin produced by strains of NDV grown in certain mammalian cells is due to the incorporation of a trypsin-sensitive precursor into the mature virion. On the other hand, the low capacity of BK pi virus to agglutinate red blood cells is not caused by defective neuraminidase. Since periodate treatment of purified BK pi virus neither alters the haemagglutination titre nor the PAGE profile, it is clear that the abnormalities of BK pi virus in these respects are not due to the adsorption of serum mucoproteins to the envelope of the virion, during its liberation into the supernatant medium.

In conclusion, the reduced biological activities of the virus released from the three carrier cell-lines are apparently caused by defective formation of their structural polypeptides or destruction of the viral proteins after their incorporation into the mature virion as discussed in Section 2.

DISCUSSION

DISCUSSION

THE VIRULENCE OF NDV

Naturally occurring NDV infection in chickens may be almost asymptomatic or it may be extremely severe, and this variation in the clinical syndrome is mainly associated with the virulence of the infecting strain. For these reasons, it is interesting to attempt to determine not only what significant factor or factors are present in virulent strains that are not found in avirulent strains but also to clarify the role that they play in the pathogenesis of the velogenic disease in chickens. There is general agreement that the virulence of a strain of NDV may be assessed *in vitro* by measuring either its intracerebral pathogenicity index in one-day-old chicks or its capacity to cause a cytopathic effect in a monolayer of chick embryo fibroblasts.

From the results in this present work, it is believed that a distinction can be drawn between the avirulence of lentogenic wild-type strains of NDV and the low infectivity of virus released from abortive or partially abortive infections. It has been shown in Section 2 (D) I of the Results and by other workers (151), that the proportion of cells in monolayers of chick fibroblast cells infected with a lentogenic strain of NDV may be the same as that in a monolayer infected with a velogenic strain. However, not only is it usually necessary to employ larger amounts of

of the lentogenic strain but also the number of infected cells destroyed by the velogenic strain is always greater. It seems likely therefore that the avirulence of a lentogenic strain is closely related to the low infectivity of all the virus particles and that both features are associated with an inability to destroy infected cells. On the other hand, additional evidence suggests that the avirulence of virus released from partially abortive infections is due to the presence of small amounts of normal virions together with a large number of incomplete virions that are incapable of a normal replicative cycle. Thus the avirulence of such virus could be brought about in two ways. First, the absence from the majority of virus particles of the factor or factors that cause cell death and, second, interference by these incomplete virions with the replication of the small number of normal virions. Three phenomena obtained with purified virus (Results, Section 2(D) I) support this proposition, namely:-

i) whether the infecting virus is grown in mammalian cells or chicken tissue, its infectivity titre is greater in chick fibroblasts than in HeLa or PK(W)K6 cells. This suggests that viral replication is partially abortive in the latter cell types.

ii) although the infectivity in virus released from mammalian cells is less than that of the parent velogenic strain, certain properties of the progeny virus are typical of the virulent strains of NDV.

NDV. For example, each chick embryo fibroblast infected with progeny virus, adsorbs large numbers of erythrocytes, which is characteristic of virulent virus (113). Avirulent wild-type strains, on the other hand, remain incapable of causing dense haemadsorption both before and after passage in mammalian cell-lines. This supports the hypothesis that the virulence of a strain resides in the individual virus particle, and that a partially abortive infection results in progeny with reduced ability to produce infection, but of the same virulence as the parent strain.

iii) the infectivity for chick embryo fibroblasts of mammalian cell-grown virus is restored following serial passage in chicken tissue. This observation suggests that in a permissive system the progeny of a partially abortive infection of mammalian cells are capable of undergoing a complete cycle of replication and that the virions released are of normal infectivity.

Several other workers have demonstrated the ability of the host cell system not only to reduce the cytopathic effects of the infecting virus (74, 89, 90) but also to diminish the virulence and biological activities of the progeny virus (215). It has also been demonstrated that a single passage in a permissive cell system is sufficient to re-establish virulence (215). Indeed Drake has shown that NDV grown in chick embryo fibroblasts is more thermo-labile than the same strain grown in chorio-allantoic membrane (215),

215, but replication of the fibroblast-grown virus in chorio-allantoic membrane results in the restoration of virulence and thermostability. Similarly, 100 times more progeny virus is released from chick embryo lung cells infected with Sendai virus than from chick embryo fibroblasts infected with the same virus (220). Matsumoto (216) and other workers have demonstrated that the reduced infectivity of Sendai virus released from MDBK or L cells is due to the malformation of the haemolysin and it is interesting to note that full haemolysin activity and infectivity are restored by a single passage in chick embryo fibroblast cells (216). Thus although it is the genotype of lentogenic strains of NDV which renders them incapable of virulence, virus grown in certain mammalian cells probably acquires a phenotype of low infectivity.

It is also possible that the virulence of a strain of NDV is attributable to the presence of a single property and that the absence of this feature might lead to avirulence. Such a defect could be phenotypic or genotypic in origin. On the other hand, virulence might be dependent on the presence of a number of activities, and a defect in one of several properties could reduce the pathogenicity of the strain.

In the present work, the relationship of virulence to various biological activities of NDV was also investigated. It was found that strains of virus with low virulence are generally defective in more than one property. In addition, no single biological

biological activity was found to be defective in all the avirulent strains examined. For example, (i) virus released from persistently infected BK pi cells has defective neuraminidase, haemagglutinin, haemolysin and phosphodiesterase activities and is also of extremely low infectivity in chick embryo fibroblasts. The low virulence in this case is phenotypic, since passage through chicken tissue resulted in progeny virus with normal levels of biological activity and normal virulence. (ii) The B1 strain of NDV has a lentogenic genotype and thus certain of its properties, including neuraminidase and haemagglutinin temperature stability, were found to be lower than those of virulent strains whether the B1 virus was obtained from chicken tissue or HeLa cells.

Other workers have shown that most biological activities of NDV, including haemagglutinin, haemolysin and cell fusion, vary with the strain but that these differences are apparently unrelated to virulence (111). Even the immediate cause of cell death cannot be attributed to a single effect of the infecting virus. Thus, neither inhibition of cellular RNA and protein synthesis (80, 81) nor rupture of cellular lysosomes (107), have been shown to be directly and exclusively caused by virulent strains of NDV.

Many features of NDV that have been associated with virulence are the consequence of cytopathic effects, for example plaque size (106), while others such as

as neuraminidase activity (108, 112),cannot be directly related to cell death.

In view of the lack of success in finding a single causative factor for virulence and in view of the results of the present work, it is suggested that virulence is the consequence of the complementary effects of several viral properties. An example of the relationship between a single biological activity and the virulence of a strain of NDV is provided by a study of haemolysin activity. It has been shown in Section 4 of the Results that the haemolysin of NDV, like that of Sendai virus (11, 42), can be defective when grown in certain cell-types and that a reduction in haemolytic activity is associated with diminished infectivity in chick embryo fibroblasts. However, since both virulence and haemolysin activity are restored by treatment of the virus with trypsin, it would appear that haemolysin activity is directly related to virulence. On the other hand, since it has been clearly shown that lentogenic strains of NDV grown in fertile hens' eggs can have a higher haemolytic activity than virulent strains (111), it is suggested that in normal circumstances haemolysin is one of the many factors that contribute to the virulence of a strain. Thus, when it is possible for variation in haemolysin activity (or any other biological phenomena) to be studied in conditions that are stable for other activities, its contribution to virulence can be established. In this connection, it should be noted

noted that since a high level of neuraminidase activity has been associated with virulent strains of NDV (112, 108), this property may be of greater importance in determining the relative virulence of a strain than other biological activities. However, it should be noted that even this relationship is not valid in all circumstances and a reduction in virulence is not always related to a decline in neuraminidase activity. For example, although the low infectivity of the B1 strain of NDV grown in HeLa cells is associated with a fall in haemagglutinin and haemolysin activities, the neuraminidase content of such virus is comparable with that of egg-grown virus of the same strain. Therefore, in conclusion, it is proposed that virulence of a strain of NDV is related to the presence of several properties and that the absence of one property or the maintenance of another has only a greater or lesser contributory effect to virulence.

Strain differences in the number and quantity of viral proteins may be related to virulence but it is also possible that the process of synthesis of viral proteins in infected cells may be of still greater significance. Other workers have studied the non-cellular proteins formed in chick fibroblast cells infected with various strains of NDV. In general, the three major structural polypeptides, VGP 75, VP 55 and VP 42, have been found (48, 49, 50, 211, 217) and, in addition, VP 180, VP 53 and VP 49 have been

been observed in cells infected with the virulent Victoria strain (217). Non-structural viral proteins with molecular weights varying between 60,000 - 70,000 daltons have also been described by several authors (217, 50, 211). No clear relationship has been established between virulence and the appearance of these non-structural proteins, but it is believed that a polypeptide of about 65,000 daltons may be the precursor of the small glycoprotein of NDV (50, 217). In addition, a further non-structural protein of about 38,000 daltons was reported by most of these workers.

Largely similar results were obtained in the present work with MDBK cells infected with the B1 strain of NDV (Results, Section 2(H)). However, as well as the non-structural polypeptide of 38,000 daltons, it is clear that two non-structural proteins were present with molecular weights in the range of 60,000 - 70,000 daltons. One of these with a molecular weight of approximately 62,000 daltons, was believed to be the precursor of VGP 55 and the other (VP 70) to be associated with VGP 75. It is interesting to note that Alexander and Reeve (48) consider that a protein of this molecular weight (70,000 daltons), which is synthesised in chicken embryo fibroblast cells infected with the Herts strain of NDV, corresponds to VGP 75.

Infection of MDBK cells with NDV generally results in a productive replicative cycle and much of the virus released has a normal complement of viral proteins (18, 218). However, both the present results

results and those of other workers indicate that a proportion of the virus released is non-infectious and contains reduced quantities of nucleocapsid (VGP 55) and matrix (VP 42) proteins. Since a small amount of the virus particles released from chick embryo fibroblast cells is similarly non-infectious (218), it is reasonable to suppose that similar viral proteins are synthesised both in infected chick embryo fibroblasts and in infected MDBK cells. Indeed, the proportions of the viral polypeptides found in MDBK cells infected with the B1 strain of NDV (Results, Section 2(H)) correspond closely to those found in chick embryo fibroblast cells infected with the lentogenic F strain of NDV (211). Since the formation of viral proteins in MDBK cells infected with B1 virus is apparently normal, the absence of VGP 75 from persistently infected MDBK cells (BK pi cells) may suggest that the malformation of this protein is related to the extremely low infectivity of the virus released from BK pi cells (Results, Section 2(H)). It is therefore possible that, when the synthesis of viral polypeptides of sufficient strains has been studied, differences may be found between the proteins formed by lentogenic and velogenic strains. It is suggested that in avirulent strains, both decreased production of structural polypeptides and increased synthesis of precursor proteins might be observed. On the other hand, while the proportions of the viral proteins synthesised in infected chick fibroblasts have

have been shown to vary with the infecting strain, no relationship to virulence has been observed.

Other workers (20,21) have examined the structural proteins of NDV released from the chorio allantoic membrane of fertile hens' eggs and although the proportions of the viral polypeptides varied with strain, the differences could not be related to virulence. However, significant differences were found in virus released from partially abortive infections. The reduced virulence of such virus has been shown to be associated with loss of certain biological activities and the absence of one or more viral polypeptides (219, 42, 11, 218). In the present work, an abnormal polypeptide (VP 70) and decreased amounts of other structural polypeptides were found to be related to the reduced infectivity of virus released from cell culture. The presence of VP 70 was thought to be due in part to the action of serum in the nutrient medium and also to defective incorporation of VP 42 into such virions. The proportions of most of the viral proteins were similar to those observed in non-infectious particles released from MDBK cells (218). In addition, the B1 strain released from HeLa cells probably contained VGP 55 in the form of its precursor and the virus released from BK pi cells contain only very small amounts of normal VGP 75. The abnormalities of VGP 55 and VGP 75 were associated with reduced haemolysin and haemagglutinin/neuraminidase activities respectively.

respectively. A very similar situation was demonstrated by other workers employing MDBK cells infected with a temperature-sensitive Sendai virus. They showed a relationship between the defective haemagglutinin/neuraminidase activity, the abnormal profile seen after polyacrylamide gel electrophoresis of the viral proteins and the low infectivity of the virus (219).

In most cases, an increase in biological activity and infectivity was associated with a restoration of the proportions of the viral polypeptides seen following polyacrylamide gel electrophoresis under reduced conditions. Thus the avirulence of such virus may be supposed to be related to the malformation or absence of certain structural proteins. Although there is no evidence that any particular protein is consistently diminished in wild-type lentogenic strains of NDV, the proportion of structural polypeptides vary from one field strain to another. Therefore, the avirulence of a wild-type lentogenic strain could possibly be due to reduction in quantity or to malformation of one of several of the viral polypeptides.

THE RELATIONSHIP OF THE CARRIER CELL TO THE PERSISTENT VIRUS

Carrier cell systems have been described in which a normal viral replicative cycle takes place in a small proportion of cells. The remaining cells are protected either by interferons induced in such cultures (118) or by the addition of antibody to the nutrient medium (117).

The persistence of the virus in the present system is not due to such mechanisms but is believed to be associated with the transfer of the viral genome at cell division. The replicative cycle of the persistent virus is considered to be relatively incomplete, since the virus released is of very low virulence and is thus incapable of destroying the carrier cells. However, most of the viral RNA synthesised in the carrier cells must be normal, because not only is viral protein formed in daughter cells after mitosis but also growth of the persistent virus in a permissive system (such as chick embryo fibroblast cells) results in the production of virus with full virulence. It has been shown in addition that when control cells, of the same type and species as the carrier cell-lines, are infected with a wild-type strain of NDV, the replicative cycle is largely incomplete and virus of low virulence is released. Therefore, it is suggested that the establishment of a persistent infection is at least partially due to the non-permissive nature of the host

host cell. It is interesting to note that many of the descriptions of persistent paramyxoviruses involve a cell system in which replication of the virus of is normally incomplete (150, 141, 61, 49). However, in at least one instance (49), the defective stage in the replicative cycle of the abortive infection has been shown to be different from that of the persistent infection. Indeed, in the present work, the virus released from the persistently infected BK pi and PK pi cultures differed from the progeny virus of partially abortive infections of their respective control lines (MDBK and PK(W)K6). It is therefore suggested that under circumstances where the normal replicative cycle is not possible, mutation of the virus to a form capable of an abnormal replicative pathway is favoured and that such mutant forms are persistent.

The results of the present work (Results, Section 3, II and IV), when considered with those of other authors (45, 150) suggest that the alternative pathway of viral replication in some cells persistently infected with paramyxovirus may involve a DNA intermediate for the transcription of viral messenger RNA. The unusual characteristics (including temperature-sensitivity and reduced biological activity) may be due to the abnormality of such a replicative pathway. Alternatively, they may be caused by the same mutation which enables the virus to synthesise viral RNA-dependent DNA-polymerase. Since other workers have shown a close relationship between the temperature-sensitivity of a strain and

and its ability to establish persistent infections (124), it is suggested that temperature-sensitivity is likely to be due to the latter possibility. The results obtained in the present work support this hypothesis. Although the haemagglutinin of BK pi virus was more thermostable than that of PK pi virus, the replication of both strains is similarly temperature-sensitive. This suggests that temperature-sensitivity is associated with the replicative pathway of the virus rather than the thermolability of the viral structural proteins. On the other hand, the low biological activities especially neuraminidase, of these persistent viruses may be consequent on the abnormal replicative cycle because they are eliminated following passage of the virus through chicken tissue.

The survival of a persistent infection may be dependent on the ability of the virus to undergo further mutations. In this context, it is interesting to note that Perekrest et al. (221) and Haspel et al. (122), have recorded changes in the nature of persistent infections over a period of serial culture. The characteristics of the three carrier cultures studied in the present work have also altered since their original isolation fourteen years ago, in 1962, and since the time of Edwards' investigations, completed in 1972 (93). It is emphasised however, that the virulent nature of the original strain has been maintained and may be demonstrated after the virus has been passaged in a permissive cell system (Results, Section 2, D(III)).

(III)).

It has been postulated that the changes in the surface of cells persistently infected with paramyxoviruses can give rise to an auto-immune response in the host animal (223). The results obtained in the present work (Results, Section 1) together with those of other workers (96), suggest that certain features of cells infected with NDV resemble those of neoplastic cells.

It is considered that the growth of a normal cell is initiated and controlled by polypeptide hormones such as insulin and adreno-corticotrophic hormone as well as by similar hormones produced locally by normal cells. Their action is thought to be mimicked by proteolytic enzymes including trypsin (222). The effect of these hormones is to lower the level of cyclic AMP within the target cells and raise the ratio of cyclic GMP to cyclic AMP. In a transformed cell culture the cells lose their requirement for normal growth controlling factors (222) and cyclic AMP remains at a low concentration. The cause may be the disruption of normal control of DNA synthesis, or alternatively, a product may be formed in transformed cells which alters the sensitivity of cell membrane to the growth control factors.

Certain features of cells infected with paramyxoviruses have been shown to be shared with those of cells infected with oncogenic viruses, These include the agglutination of infected cells with lectins (96)

(96) and the production of virus-specific RNA-dependent-DNA-polymerase (45). In addition, it has been suggested (223) that the altered characteristics of cells persistently infected with paramyxoviruses may explain the auto-immune response associated with long-term infection of an animal with a paramyxovirus. The best documented example of such a phenomenon is the relationship between sub-sclerosing pan-encephalitis and infection with measles virus (223).

Transformed cells in vitro exhibit other features in addition to uncontrolled growth, including low serum requirements (222), binding with lectins (174), loss of 'contact inhibition' leading to 'colony' formation in semi-solid agar (102), and chromosomal abnormalities. The phenomenon of transformation may be reversed by certain substances. Thus succinylated concanavalin A apparently restores the sensitivity of the transformed cell to growth control factors (224). Since low levels of cyclic AMP apparently mediate a fast growth rate, it is not surprising that the addition of high concentrations of this nucleotide to cultures of transformed cells also reduces their mitotic rate (225).

Oncogenic RNA viruses may exert their effect by directly altering the membrane of infected cells. Alternatively, since viral DNA is incorporated into the host genome of infected cells, the ability of the cell to form normal cell membrane may be affected by this process.

The transformed state of the cell systems

systems persistently infected with NDV described in the present work has been demonstrated. Thus BK pi cells have lower serum requirements than control MDBK cells and also form 'colonies' in semi-solid agar (Results, Section 1). Alexander et al. have shown that cells infected with NDV agglutinate lectins (96), and it is suggested, in the present work, that it is a related phenomenon that permits BK pi cells to adsorb red blood cells to areas of cell membrane which do not contain viral components (atypical haemadsorption). When BK pi cells are grown on complete monolayers of uninfected chick embryo fibroblasts, atypical haemadsorption is apparently inhibited. Since the normal quiescent cells contain high concentrations of cyclic AMP as well as producing other factors that regulate cell-growth, it is possible that these substances may reverse the transformation of BK pi cells. In consequence, the atypical haemadsorption of BK pi cells would also be prevented.

If cylic AMP is destroyed, the concentration of this nucleotide is lowered and the cell becomes transformed. It has been shown that for this reason, cells treated with cyclic AMP diesterase become neoplastic (226). It may therefore be of significance that phosphodiesterase of unknown substrate specificity has been demonstrated to be associated with virions of NDV (Results, Section 2(E)VII) and Sendai virus (70). The phosphodiesterase activity of NDV is probably borne on the small envelope glycoprotein. It is

is known that it is the small glycoprotein of paramyxoviruses which is largely responsible for inducing the changes in the membranes of infected cells that cause the lysis of erythrocytes (171) and the fusion of culture cells (74). It is proposed that the phosphodiesterase of NDV may reduce the amount of cellular cyclic AMP to a level which permits the transformation of the cell membrane, and also that the altered cell membrane is then capable of fusion with other cells but that the consequence of such changes to erythrocyte membranes is lysis.

On the other hand, cells persistently infected with NDV may become transformed due to irregular synthetic processes caused by the addition of viral genetic material to the host cell genome. This hypothesis is supported by the following observations:-

(i) An enzyme, RNA-dependent-DNA-polymerase, which is capable of producing DNA replicates of viral RNA, has been reported in NDV released from persistently infected cells (45).

(ii) A similar enzyme is also present in respiratory syncytial virus (a metamyxovirus), which is not known to be oncogenic <u>in vivo</u> (227), while the genome of cells persistently infected with measles virus have been shown to contain DNA transcripts of viral RNA (150).

(iii) Moreover, chromosomal changes have been demonstrated in cells persistently infected with a paramyxovirus (103).

(103).

Two features of the carrier cell systems examined in the present work, support the concept that cellular transformation may be due to the presence of a DNA transcript of viral RNA within the host cell genome. First, treatment of BK pi cells with actinomycin D reduces the amount of virus released but increases its infectivity. One explanation of this phenomenon is that defective virus is normally produced in persistently infected cells through a replicative pathway involving DNA and that this pathway is blocked in the presence of actinomycin D. Second, the persistence of the viral genome at temperatures which do not permit the synthesis of viral proteins, suggests that the viral genetic material must exist in a stable form in the majority of the carrier cells and that it is transmitted in this form at cell division. Both the persistence of the viral genome and the inhibition of viral protein synthesis at non-permissive temperatures could be explained if it is assumed that a persistent paramyxovirus acquires a thermo-labile RNA-dependent-DNA-polymerase when the carrier cell state is initiated. It is thus of interest to note that the ability of a strain of NDV to establish a persistent infection is associated with a temperature-sensitive replicative cycle (124). It may also be significant that the inability of certain strains of an oncogenic RNA virus (Rous sarcoma virus) to cause cellular transformation at non-permissive temperatures is due to their

their possession of a thermo-labile RNA-dependent-DNA polymerase (228). Thus elimination of the transformation-like features of the cells carrying NDV might be expected when the cells are grown at non-permissive temperatures. Unfortunately this possibility was not investigated in this present work but is supported by some circumstantial evidence. It has been suggested that the atypical haemadsorption of BK pi cells is associated with transformation of the membrane of these cells. When BK pi cells are cultured at 41°C, haemadsorption disappears several days before viral antigen ceases to be detectable by immunofluorescence (Results, Section 3 (II b)). Therefore, the absence of haemadsorption at this non-permissive temperature may indicate that cellular transformation has been reversed. In conclusion, it is possible that the transformation of cells infected with NDV may either be related to the presence of viral phosphodiesterase causing a reduction in cellular cyclic AMP or to the activity of a viral thermo-labile RNA-dependent-DNA-polymerase.

Zhdanov has suggested that the ability of persistent measles virus to induce cell membrane changes, leads to an auto-immune response <u>in vivo</u> (223). In the present work, the similarity between certain features of cells persistently infected with paramyxovirus and those of neoplastic cells has been discussed. In view of the possibility that paramyxoviruses can induce auto-immune and neoplastic responses in infected

infected tissues, the use of live strains of such virus for prophylactic immunisation may be questioned. Furthermore, it is probable that the viral envelope proteins of even avirulent strains play an important role in producing pathological changes in cell membranes. Therefore, the employment of isolates of purified viral envelope proteins in place of vaccines composed of whole virus may also be potentially hazardous.

SUGGESTIONS FOR FURTHER WORK

In this thesis, a variety of phenomena associated with cells persistently infected with NDV have been considered. Unfortunately, a number of these require much further investigation in order that firm conclusions may be drawn as to their role in pathogenesis.

It is essential in any further work to eliminate the confusion concerning the abnormal proteins found in virus released from cell cultures. In many respects the polyacrylamide gel electrophoresis profiles of such viruses are similar to those obtained by Lamontagne *et al*. (218) for incomplete virions released from MDBK cells. However, the extent to which these abnormalities are due to the release of defective virions or are caused by the action of serum in the nutrient medium would be shown by the following procedure:-

by incorporating radioactive label into the nutrient medium and comparing the proteins of virions released from infected cells grown in serum-free medium with those of virions released into serum-rich supernatant fluids.

Should VP 70 and VP 62 prove to be defective viral proteins and not artefacts due to the action of serum on normal virions, their role in viral metabolism may be studied by 'pulse-labelling' the infected cells with radioactive amino acids and 'chasing' with non-radioactive nutrients for variable periods before

before harvesting. In this manner, the possible function of these proteins as normal precursors of other viral polypeptides could be established.

The results obtained in the present work suggest that for a given strain of NDV, there is a positive correlation between virulence and full expression of the biological activities of the released virus. Virulence also appeared to be dependent on the cell-type in which the virus has been grown. To confirm these effects, more strains of NDV and further cell-types must be studied. Features of special interest in such experiments would be the neuraminidase, haemagglutinin, haemolysin and phosphodiesterase as well as virulence for chicken embryo fibroblasts of the released virions. It is also possible that investigation of the viral structural proteins found within the affected cell and in the released virions might reveal a common defect in the synthesis of viral polypeptides in lentogenic strains. Although the results of other workers have shown that the RNA (but not the ribonucleocapsid protein) of persistent virus is generally of the same size as that of normal virus, an investigation of the species of RNA found in PK pi, BK pi and OK pi cells may prove of interest.

It is unfortunate that the existence of viral RNA polymerase and RNA-dependent-DNA-polymerase has not been investigated in this present thesis because the thermolability of these enzymes may be related to the temperature-sensitivity of the persistent virus. In

In addition, it would have been of interest to ascertain whether the transformation-like properties of persistently infected cells, including 'colony' formation in semi-solid agar, were also temperature-sensitive. In the case of such experiments, concanavalin A would probably be more useful for the assessment of transformation, since the cells are generally of poor viability at 41°C.

A suggestion has been made in the present work that the establishment of a persistent infection with a paramyxovirus may largely be due to the non-permissive nature of the cell-type employed. It would therefore be of interest to attempt to induce a persistent infection of chick embryo fibroblast cells with NDV. Since uninfected chick embryo fibroblast cells cannot be induced to form a continuous cell-line, the establishment of a persistent infection would be further evidence of the ability of paramyxoviruses to transform infected cells.

The results obtained in this present work suggest that phosphodiesterase activity is associated with the small glycoprotein of NDV. Such a relationship could be confirmed by preparing antibody to purified samples of this polypeptide and by demonstrating that such antibody inhibited the phosphodiesterase activity of NDV. Further work is required to determine whether the phosphodiesterase of NDV is more active against substrates of RNA, DNA, cyclic AMP or other organo-phosphate diesters. The relationship between phosphodiesterase and virulence

virulence has been briefly examined but can only be confirmed by studying the activity of this enzyme in other strains of NDV. Only by such investigations will it be possible to gain an understanding of the role of phosphodiesterase in viral replication.

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PUBLICATIONS

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Accidental Persistent Infection of Cell Lines by Newcastle Disease Virus, Showing Three Unusual Features — Defective Neuraminidase, Temperature Sensitivity and Intranuclear Inclusions

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Summary

A persistent, defective infection by an unknown strain of Newcastle disease virus (NDV) appeared accidentally in established lines of pig, ox and sheep kidney cells. Virus particles released from the persistently infected cells were not infectious and were deficient in neuraminidase activity. Synthesis of some of the virus-specified proteins in the persistently infected cells was temperature-sensitive. Co-cultivation of mixed populations of carrier cells and healthy chick embryo cells induced cell fusion with the formation of multinucleate heterokaryons and intranuclear inclusions. The development of inclusions in the chicken nuclei was not accompanied by 'rescue' of infectious NDV.

Introduction

It is becoming increasingly clear that persistent virus infections are responsible for a wide range of human and animal diseases, and that many viruses can establish persistent infections in a variety of virus-cell culture systems (3, 5, 8, 22).

This paper describes the general characteristics of three lines of pig, ox and sheep kidney cells that have been maintained continuously in this laboratory for 14 years, and which became persistently infected with a strain of Newcastle disease virus. The carrier state must have been established in the absence of specific antibody, and evidence will be presented that the virus released from the persistently infected cells is defective.

Materials and Methods

Cell Cultures

Cell lines of pig kidney (Stice-2a), ox kidney (MDBK) and sheep kidney (MDOK) were kindly provided, in 1959, by Dr. S. H. Madin, Berkeley, California (9). They were

received in good condition and formed confluent monolayers without visible abnormalities. Subcultures were made at weekly intervals by inoculating 2×10^5 cells in Earle's saline containing 0.5 per cent lactalbumin hydrolysate, 0.1 per cent yeast extract and 5—10 per cent heat inactivated calf serum. For maintaining the cells, the same medium was used, but with the calf serum reduced to 2 per cent. Two hundred units/ml of penicillin, 100 μg/ml of streptomycin and 25 units/ml of nystatin or fungazole were incorporated in all media. Cells were removed from the glass with 0.05 per cent bovine trypsin in 0.02 per cent versene at 37° C. In 1962, the 3 cell lines were found to be infected with NDV, and have carried the infection until the present time.

In this paper, the persistently infected or carrier cultures of pig, ox and sheep kidney are designated PK_{pi}, BK_{pi} and OK_{pi}, respectively. Cells used in controls included the continuous line of pig kidney (PK 15) and a second strain of Madin's bovine kidney cells (MDBK) purchased from Flow Laboratories Limited, Irvine, Scotland. Secondary cultures of rabbit kidney, ox kidney, chicken kidney and chick embryo fibroblasts (CEF) were prepared by trypsinization in Earle's medium by standard methods.

Unless otherwise indicated, all cell cultures were incubated at 37° C.

Preparation of Antisera

Immune sera against NDV were obtained from rabbits 7 days after the last of 3 weekly intramuscular inoculations with the Herts 33 strain of NDV. The sera were inactivated at 56° C for 30 minutes and stored at —20° C until required for use.

Haemagglutination

Haemagglutination tests were performed in WHO perspex plates at room temperature against a 1 per cent suspension of freshly drawn fowl, horse or guinea-pig red blood cells in Dulbecco's phosphate buffered saline 'A' (PBSA; Oxoid Ltd.), using 0.25 ml of each reagent and reading the test by the pattern of agglutination 1 hour after the addition of red cells. Titres were expressed in agglutinating units (HAU). Haemagglutination-inhibition tests were carried out by standard methods (23).

Haemadsorption

During this investigation, monolayers of healthy and persistently infected cell lines were examined frequently for their ability to haemadsorb red blood cells (20). The proportion of haemadsorbing cells in 24—48 hour coverslip cultures was obtained by counting at least 500 cells in randomly chosen fields following exposure to 0.4 per cent guinea-pig red blood cells, fixation and staining. In some instances, counts of individual haemadsorbing cells were made in a haemocytometer by the method of Rustigian (17).

Neuraminidase Assay

Virus-containing fluids harvested from cells grown in serum-free medium, were clarified by slow speed centrifugation, concentrated by ultrafiltration, dialysed against PBSA and then tested for neuraminidase activity. Assay conditions were as described previously (10), except that a 30 minute incubation was used. Released sialic acid was measured by Aminoff's procedure (1), and results were expressed as optical density readings at 549 nm.

Cytology

Coverslip cultures were fixed in ethanol or Bouin's fluid and stained by Giemsa's method or with haematoxylin-eosin. Ethanol fixation was used in acridine orange staining (12). For immunofluorescence microscopy, coverslips were removed at intervals, fixed unwashed in acetone for 10 minutes at room temperature, air dried for 30 minutes and stained immediately. Viral antigen in the persistently infected cells was identified by the direct fluorescent antibody technique (4), using two rabbit immune sera which had anti-haemagglutinin titres of ca. 1280 and 2000, respectively, against 4 HAU of NDV.

Electron Microscopy

Virus obtained from fluids of carrier cultures was clarified by centrifugation at $5000 \times g$ for 20 minutes and then pelleted at $60{,}000 \times g$ for 2 hours. Resuspended pellets were then mounted by negative staining with 4 per cent potassium phosphotungstate, at pH 6.5, on 'Formvar' carbon support films and examined in an A.E.I. (EM 6 B) electron microscope.

Superinfection of Carrier Cultures

Growth of homologous and unrelated viruses in cell lines persistently infected with NDV was examined by titrating the viruses on monolayers of PK_{pi} and BK_{pi} cells, with Stice-2a and MDBK cells as controls. Inoculated cultures were examined daily for cytopathic effects (CPE), and virus titres after 4 days were expressed in TCD_{50}/ml calculated by the methods of Reed and Muench (16).

Co-Cultivation

Equal volumes of secondary chick kidney cells or CEF (5×10^5 cells/ml) and BK_{pi} (2.5×10^5 cells/ml) or PK_{pi} (1.0×10^5 cells/ml) were mixed thoroughly, held in growth medium for 2 hours at 4° C, and dispensed in 1 ml amounts in culture tubes containing glass coverslips. The coverslips were removed daily and stained with Giemsa, haematoxylin-eosin, acridine orange and fluorescein-labelled anti-NDV serum.

Cultivation of Carrier Cultures at 41° C

Cultures of BK_{pi} cells were incubated at 41° C and subcultured by trypsinization at fortnightly intervals for a period of 10 weeks. The cultures were refed daily for the first 28 days, and at weekly intervals thereafter.

Results

General Considerations

Three cell lines, Stice-2a, MDBK and MDOK, obtained in 1959, were carried successfully by weekly subculture for 3 years, when syncytial formation and large numbers of acidophilic intracytoplasmic inclusions were observed in MDBK and MDOK. Further examination revealed that, despite the absence of overt cellular changes in Stice-2a, all 3 cell lines were continuously producing NDV antigens and releasing virus particles. Nevertheless, release of infectious virus was never demonstrated during the next 10 years of study.

At the time when the 3 cell lines became infected, NDV had never been grown in cell cultures in the laboratory, although one strain was being maintained in eggs elsewhere in the building. All sera for cell cultures were obtained from a part of the United Kingdom free from NDV. Thus the source of the infection remains unknown, and we believe that the carrier state must have been established in the absence of antibody against NDV.

Before being fully investigated, the cells were carried as a laboratory curiosity for some years, largely because of their value in demonstrating virus-specific haemadsorption.

General Properties of the Persistent Infection

Identity of the Infecting Virus

Both haemadsorption shown by all 3 infected cell lines and agglutination of red blood cells by their culture fluids were inhibited by anti-NDV serum, but not by immune sera prepared against other myxoviruses including influenza A and B, mumps, parainfluenza types 1 and 3, measles, canine distemper and rinderpest. Fluorescent antibody staining revealed the presence of antigen in the persistently

infected cells only when anti-NDV serum was used. It was therefore concluded that the infecting virus was NDV.

The possibility that the PK_{pi} cells were persistently contaminated with swine fever virus (18) was ruled out by immunofluorescence staining.

Cytology of the Persistently Infected Cells

Large numbers of acidophilic intracytoplasmic inclusions and many small syncytia were present in the BK_{pi} and OK_{pi} monolayers. These abnormalities were not observed in the cell cultures before they became infected with NDV, nor were they seen in Stice-2a, PK 15 or PK_{pi} cells.

The growth rates of the persistently infected cell lines and their ability to form confluent monolayers were similar to those of uninfected controls.

Proportion of Infected Cells in Carrier Cultures

The number of infected and non-infected cells in carrier cell populations was assessed by haemadsorption and fluorescent antibody staining of lightly seeded 24-hour-old coverslip cultures. Throughout the period of investigation approximately 90 per cent of BK_{pi} and OK_{pi} cells were infected. However, PK_{pi} monolayers contained only about 40 per cent of infected cells when examined in 1966, although the number had slowly increased to approximately 60 per cent 5 years later. Haemadsorption and fluorescent antibody staining gave similar results. Immunofluorescence staining showed cytoplasmic plaques of antigen, usually perinuclear, but the nuclei were not affected.

Resistance of Carrier Cultures to Superinfection with Homologous Viruses

The ability of the carrier cultures to support growth of the infecting virus and some other viruses was compared with that of uninfected control cultures. The results of titrating these viruses by CPE (Table 1) show that the carrier cells have a high degree of resistance to superinfection with NDV, and are moderately resistant to related viruses but not to unrelated viruses.

Table 1. *Ability of control and persistently infected cell lines to support growth of related and unrelated viruses*

Virus	Virus titres[a]			
	Control cultures		Infected cultures	
Newcastle disease virus (Herts 33)	PK 15	$10^{-3.1}$	PK_{pi}	$10^{-1.0}$
	MDBK	$10^{-3.8}$	BK_{pi}	$10^{-1.0}$
Parainfluenza virus				
Type 1	MDBK	$10^{-3.5}$	BK_{pi}	$10^{-2.5}$
Type 3	MDBK	$10^{-4.3}$	BK_{pi}	$10^{-2.8}$
Enterovirus				
porcine, T 80	PK 15	$10^{-4.4}$	PK_{pi}	$10^{-3.9}$
porcine, V 13	PK 15	$10^{-5.3}$	PK_{pi}	$10^{-5.8}$
Herpesvirus				
Aujeszky's	PK 15	$10^{-5.0}$	PK_{pi}	$10^{-5.0}$
Bovine mammillitis	MDBK	$10^{-5.0}$	BK_{pi}	$10^{-5.8}$

Equal inocula of each virus were added to control and persistently infected cultures, which were examined daily and scored after 4 days for visible cytopathic changes

[a] Mean titres expressed as TCD_{50}/ml, calculated as described by Reed and Muench (1938)

Properties of Virus Released from Persistently Infected Cell Lines

Released virus was examined in the electron microscope. Large numbers of roughly spherical virus particles with typical paramyxovirus morphology were seen. The particles contained nucleocapsids measuring approximately 17 nm in diameter; likewise the quantity and distribution of spikes on the surface of the virus particle was normal.

Consistent with the visualization of virus particles in the electron microscope was the finding that culture fluids from the carrier cultures agglutinated fowl, horse and guinea-pig red blood cells. In general, the haemagglutinin titres of culture fluids seldom exceeded 8—16 HAU/ml, but were two- to three-fold higher if the carrier cultures were incubated at 31° C rather than 37° C. Unlike the Herts 33 strain of NDV, the virus obtained from all 3 persistently infected cell lines did not elute from agglutinated erythrocytes. Haemagglutination by carrier culture virus was inhibited specifically by anti-NDV sera, but the haemagglutination-inhibition titres of the sera were four-fold lower with carrier culture virus than those obtained with Herts 33 NDV.

During the 10 years' period of study covered in this paper, we were never successful in demonstrating the release from or the presence of infectious virus in any of the 3 carrier lines. Virus was concentrated by centrifugation from the supernatant fluids of carrier cultures, or obtained from carrier cells harvested by 'snap-freezing' followed by sonication. It was inoculated a) into the allantoic cavity or amniotic sac of embryonated hens' eggs, b) into newly-hatched or 7-day-old chicks intramuscularly or intracerebrally, and c) into a variety of cell cultures including MDBK, PK15 and CEF. At least 3 blind passages were made with each technique, but with negative results.

Culture of Persistently Infected Cells in Antiserum

Carrier cells cultured for 5 weeks, with weekly subcultures, in Earle's medium containing 4 per cent anti-NDV serum prepared in rabbits (anti-haemagglutinin titre 512), no longer haemadsorbed and the medium did not haemagglutinate. However, both properties were restored 18—24 hours after removal of the antibody.

Immunofluorescence staining showed that aggregates of specifically stained material in carrier cells sub-cultured in anti-NDV serum were smaller and fewer, but antibody failed to 'cure' the infection and the proportion of cells containing viral antigen was roughly the same whether or not specific antiserum was present in the medium.

Unusual Properties of the Persistent Infection

The general properties of the persistent infection outlined above, are similar to those of a regulated infection (21). The following properties are rather more unusual and the first two may be unique to our system.

Defective Neuraminidase of Carrier Culture Virus

Failure to detect infectious virus in the carrier cultures, together with the inability of haemagglutinin to elute from agglutinated erythrocytes suggested that the carrier culture virus might be deficient in neuraminidase. The neuraminidase

activity of BK_{pi} and PK_{pi} carrier culture virus was determined and compared with that of Sendai virus grown in MDBK and BK_{pi} cells and with Herts 33 NDV grown in MDBK. The results in Table 2 show that almost no neuraminidase activity was associated with BK_{pi} and PK_{pi} virus.

Table 2. *Comparison of neuraminidase activity of BK_{pi} and PK_{pi} carrier culture virus with that of infectious paramyxoviruses*

Virus	Haem-agglutinin titre[a]	Neu-raminidase activity OD 549
NDV/BK_{pi}	160	0.032
NDV/PK_{pi}	160	0.025
Herts 33/MDBK	160	0.851
Sendai/MDBK	160	1.098
Sendai/BK_{pi}	160	1.293
MDBK cell control	—	0.005

[a] HAU/ml after concentration of the viruses as described in Methods

Co-Cultivation of Carrier Cultures and Chick Embryo Cells, Resulting in Formation of Heterokaryons and Intranuclear Inclusions

The isolation of complete infectious measles virus from non-producer human brain cells by co-cultivation techniques (7) suggested that it might be possible to rescue infectious NDV from our carrier cultures with healthy chick embryo cells.

Co-cultures of healthy chick cells and persistently infected BK_{pi} or PK_{pi} cells induced extensive cell fusion after 2—3 days of incubation at 37° C (Fig. 1). The development of multinucleate syncytia was prevented by NDV antiserum but was restored within 48 hours after the anti-virus serum was removed and replaced with calf serum. In contrast to this, control cultures of mixed populations of chicken fibroblasts or renal cells and non-infected MDBK or PK 15 cells showed no abnormalities, and both types of cell produced isolated groups of actively growing cells (Fig. 2).

Giemsa and acridine orange staining clearly differentiated avian and mammalian nuclei and showed that the syncytia (heterokaryons) contained a large number of nuclei of both species (Fig. 3). Of particular interest was the fact that the heterokaryons frequently contained both intracytoplasmic and intranuclear inclusion bodies (Fig. 4), whereas the great majority of individual carrier cells showed intracytoplasmic inclusions only. Immunofluorescence staining failed to show the presence of specifically stained material in affected mammalian or avian nuclei.

Cell suspensions or supernatant fluids from co-cultures containing large numbers of multinucleate heterokaryons did not cause deaths, deformities, haemorrhages or other abnormalities when inoculated intracerebrally into 1-day-old chicks or intra-allantoically in embryonated hens' eggs, nor did they produce haemagglutinins in the allantoic fluids.

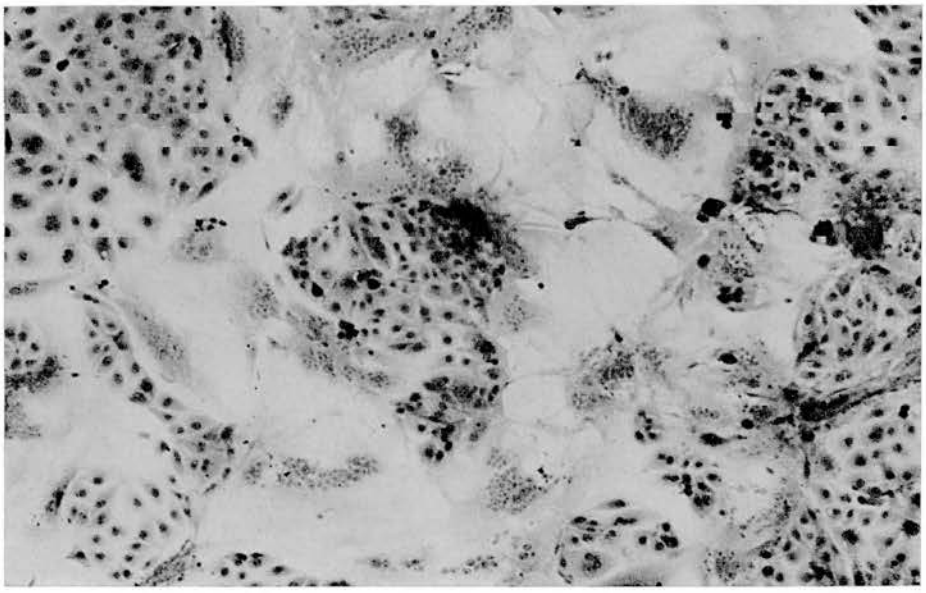

Fig. 1. Co-cultivation of CEF and BK_{pl} cells after 3 days' incubation. Cell fusion has occurred with the formation of multinucleate heterokaryons. Haematoxylin eosin $\times 80$

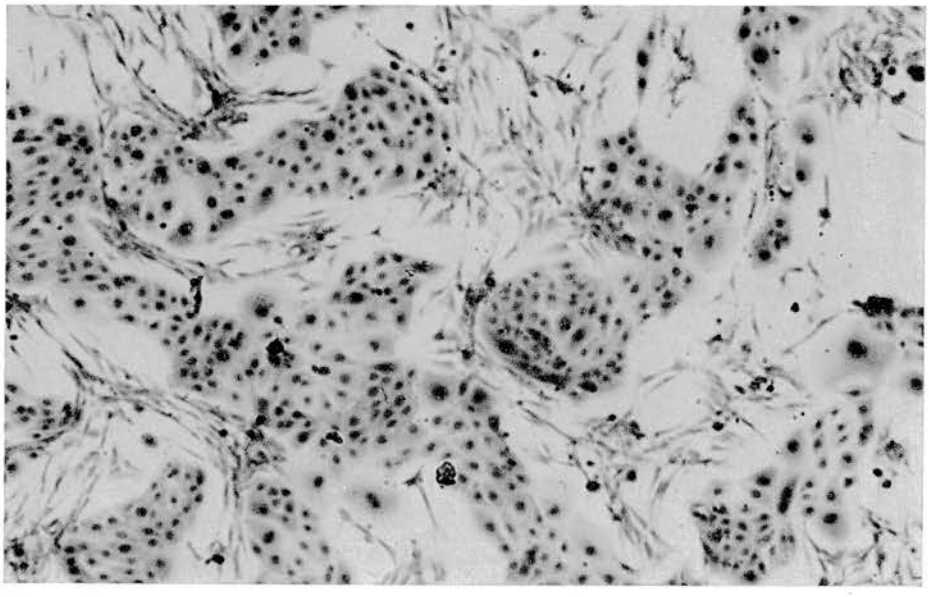

Fig. 2. Mixed culture of CEF and MDBK cells after 3 days' incubation. There is no cell fusion. Haematoxylin eosin $\times 80$

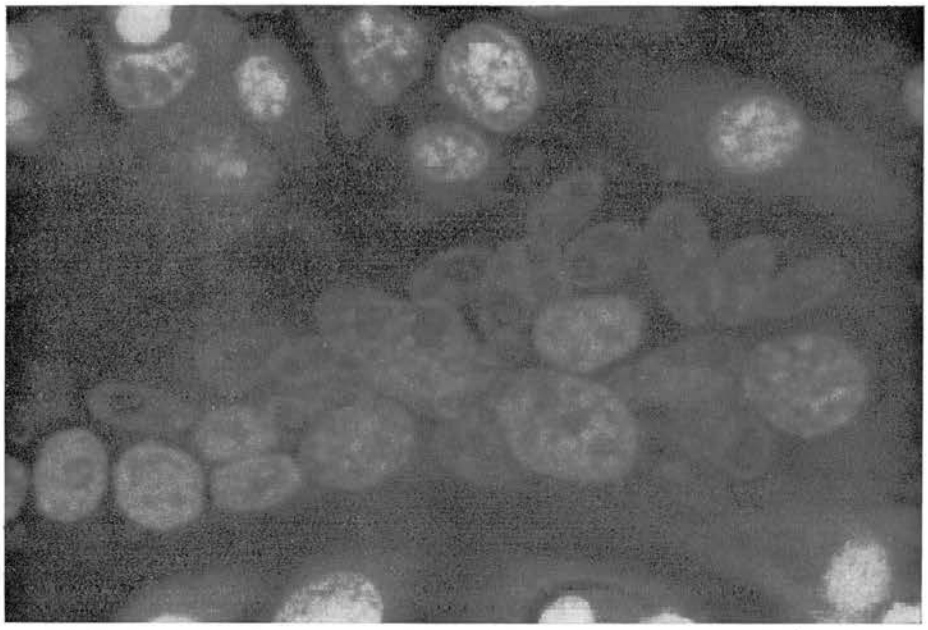

Fig. 3. Heterokaryon containing CEF nuclei (dark) and BK_{p1} nuclei (light) stained with acridine orange, 24 hours after fusion. Note inclusions in CEF nuclei $\times 1500$

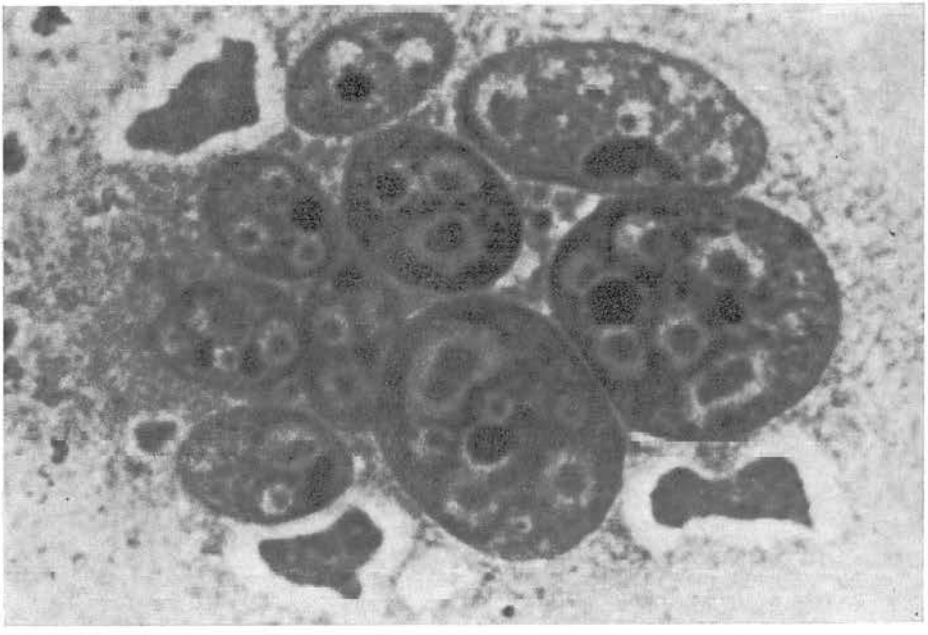

Fig. 4. Mixed culture of CEF and BK_{p1} after 7 days' incubation. The heterokaryon contains cytoplasmic and nuclear inclusions. Haematoxylin eosin $\times 2000$

Cultivation of Carrier Cultures at 41° C

Cultures of BK_{pl} cells incubated for 4 weeks at 41° C did not show haemadsorbing or haemagglutinating activity, but both effects reappaeared 2—3 days after the cultures were returned to the 37° C incubator. The amount of specifically stained material in bovine carrier cells held at 41° C was greatly reduced, but non-haemadsorbing cells were never quite free of virus antigen. The inhibitory effects of the higher temperature of incubation were not maintained indefinitely and the cultures regained the ability to adsorb and agglutinate erythrocytes after 10 weeks at 41° C.

Discussion

The main characteristics of the three carrier cultures described in this paper are in conformity with those of a regulated infection (21) in that a) the majority of the cells are infected but continue to divide and grow normally, b) the cells cannot be cured by specific antiserum, c) the cultures show a high degree of resistance to superinfection with the infecting virus, but not with unrelated viruses, and d) antibodies were apparently not required in the medium to induce and maintain the carrier state. The inability to demonstrate infectivity makes the carrier culture virus defective.

Interest in our system centres mainly around three differences from a productive NDV infection, namely temperature-sensitivity, lack of neuraminidase and the development of intranuclear inclusions in artificially induced heterokaryons.

Temperature-sensitive mutants of NDV have been isolated from a persistent infection of L cells, and experimental evidence suggests that selection of temperature-sensitive mutants plays a role in the establishment and/or maintenance of the persistent infection (14, 15). The association of virus exhibiting temperature-sensitive behaviour with persistent infections has also been described in the case of Western equine encephalitis virus (19), Sendai virus (11) and measles virus (6). It is therefore noteworthy that when BK_{pl} cells were incubated at 41° C haemadsorbing and haemagglutinating activity disappeared although some viral antigen persisted in the cytoplasm throughout these experiments, and that more virus was released from the cells at 31° C than at 37° C. This resembles the persistent infection of BHK-21 cells with Sendai virus (11) in which viral maturation was normal at 31° C but temperature-sensitive at 37° C.

The virtual absence of neuraminidase activity from the carrier culture virus is an unusual feature. As the functional significance of NDV neuraminidase is not known, it is not clear whether the virus is non-infectious because it lacks neuraminidase. Recently, temperature sensitive mutants of influenza virus defective in neuraminidase have been described in MDBK cells (13). If these mutants were grown at non-permissive temperatures they formed large aggregates of virus particles near the cell surface and were much less infectious for bovine kidney cells than virus grown at permissive temperature. This aggregation was probably due to interaction of viral haemagglutinin with neuraminic acid containing receptors on neighbouring virus particles. We have not observed such aggregation in any of our carrier cell lines, either at 37° C or at 41° C (unpublished results). However, it is possible that the virus in our carrier cultures possessed sufficient neuraminidase

activity to prevent aggregation at the time of virus release, and that this activity was inactivated subsequently; or it may be due to functional differences in NDV and influenza neuraminidase. Staining of the cell surface for neuraminic acid using colloidal iron would probably resolve this point.

Co-cultivation methods failed to rescue infectious virus from mixed populations of healthy and carrier cells, despite the fact that cell fusion with multinucleate heterokaryons readily occurred. Although the majority of virus-induced heterokaryons showed multiple acidophilic inclusions in the avian as well as in the mammalian nuclei, there was no further evidence that the defective virus had been introduced into the chicken cells because neither species of nucleus showed NDV-specific immunofluorescence, and we were unable to isolate infectious virus from the co-cultures. However, the lack of nuclear fluorescence might be due to antigenic differences between the nuclear and cytoplasmic material, as have recently been described in SSPE virus infections (2).

The failure to isolate infectious virus from both co-cultures and carrier cultures is not unique to our system, as persistent infections involving measles virus (17) and Sendai virus (11) characterised by the absence of infectious virus have also been described.

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