STUDIES ON THE REPLICATION AND FUNCTIONS OF NEWCASTLE DISEASE VIRUS RIBONUCLEIC ACIDS

Anthony Meager

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DECLARATION

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree. The work of which it is a record has been done by myself and all sources of information have been specifically acknowledged by means of references.

Anthony Meager

A C K N O W L E D G E M E N T S

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C O N T E N T S

ABBREVIATIONS

ADP'ase	adenosine diphosphatase
AMD	actinomycin D
ATP	adenosine triphosphate
ATP'ase	adenosine triphosphatase
BPL	β-propiolactone
BSA	bovine serum albumin
CE	cytoplasmic extract
CEC	chick embryo cell(s)
c.p.m.	counts per minute
CTP	cytidine triphosphate
DOC	sodium deoxycholate
DNA	deoxyribonucleic acid
DNA'ase	deoxyribonuclease
D_2O	water - d_2
EID_{50}	(egg infectious dose)$_{50}$
EDTA	ethylenediaminetetraacetic acid (di-sodium salt)
GMP	guanosine monophosphate
GTP	guanosine triphosphate
HA	haemagglutinin or haemagglutinin titre
m.o.i.	multiplicity of infection
mol. wt.	molecular weight
mRNA	messenger ribonucleic acid
ND	Newcastle Disease
NDV	Newcastle Disease virus
OD	optical density
PBS	phosphate buffered saline
p.f.u.	plaque forming unit
p.i.	post infection
PMS	post mitochondrial supernatant
POPOP	1,4-di(2-(5-phenyloxazolyl))-benzene
PPO	2,5-diphenyloxazole
RNA	ribonucleic acid
RNA'ase	ribonuclease
RNP	ribonucleoprotein
RSB	reticulocyte suspension buffer

SDS	sodium dodecyl sulphate
SFV	Semliki Forest virus
TEMED	$NNN^{1}N^{1}$-tetramethylethylene diamine
TMV	tobacco mosaic virus
tris (Trizma)	tris-hydroxymethylaminomethane
UMP	uridine monophosphate
UTP	uridine triphosphate
UV-irradiation	ultraviolet irradiation
UV-light	ultraviolet light
vol.	volume(s)
vsi-RNA	virus specific intracellular RNA
VSV	vesicular stomatitis virus

INTRODUCTION

INTRODUCTION

Historical aspects

Two different diseases which both cause similar respiratory disorders in chickens, fowl plague and Newcastle disease, are termed "fowl pest". Fowl plague, first reported in Italy in 1878, is a rare, acute and often lethal disease of chickens, caused by an influenza virus. Newcastle disease, so named because of the first recorded incidence among chickens in the Newcastle-upon-Tyne area in 1926, was reported by Doyle (1927) to be a new disease with symptoms similar to those of fowl plague. This new disease, also shown to be caused by a virus, has since spread to all countries of the world and has proved difficult to control. It has been demonstrated to survive for long periods, spread rapidly and adapt to new hosts. At present, Newcastle disease is only known to cause conjunctivitis in man.

The infectious agent (i) classification

Newcastle disease virus (NDV) shares many biophysical and biochemical properties with other viruses, and these have been grouped together and called "myxoviruses" by Andrewes *et al.* (1955) because of their affinity for certain mucoproteins and because they contain an enzyme (neuraminidase) which removes neuraminic acid from mucoproteins. Another common property of myxoviruses, first reported by Hirst (1941), is their ability to agglutinate fowl and some other vertebrate erythrocytes. Myxoviruses have subsequently been divided into two subgroups by Waterson (1962) on the basis of size and certain other properties. Subgroup I contains all the influenza viruses, also known as the orthomyxoviruses. These are further subdivided into three antigenic types, A, B and C. Subgroup II, to which NDV belongs, contains the larger parainfluenza or

paramyxoviruses such as Sendai virus, Simian virus 5 (SV5), measles, mumps and rinderpest. Morphologically, these viruses are similar, but they are less well characterised than subgroup I myxoviruses, and some members appear to lack one or more of the characteristic myxovirus antigens, e.g. no measles neuraminidase has been found. No common antigen has been found that characterises the entire subgroup II myxoviruses, although every member is antigenically related to at least one other member (Channock and Coates, 1964). TABLE 1 lists the viruses belonging to each myxovirus subgroup, and summarises some of their important biophysical and biochemical properties.

(ii) purification, assessment of purity and morphology

In nature, there exist several strains of NDV which although differing markedly in their virulence for chickens (i.e. ability to cause death) are morphologically and serologically indistinguishable (Hanson and Brandly, 1955; Waterson et al., 1967). Each NDV strain can be grown in embryonated chicken eggs, and is obtained in high yield from the allantoic fluid. Purification of virus from allantoic fluid is usually achieved by a combination of differential and density gradient centrifugation. Unfortunately, there are few criteria for assessment of purity of the virus. This stems from the fact that the virus particles are heterogeneous in size, chemical composition and biological properties, contain cellular lipid and possibly cellular antigen, and have a low ratio of infectious to physical particles. However, preparations of NDV obtained from different batches of allantoic fluid by density gradient centrifugation usually have a reproducible infectivity to protein ratio, and appear as homogeneous populations of roughly spherical particles when examined electron microscopically (Fig.1.).

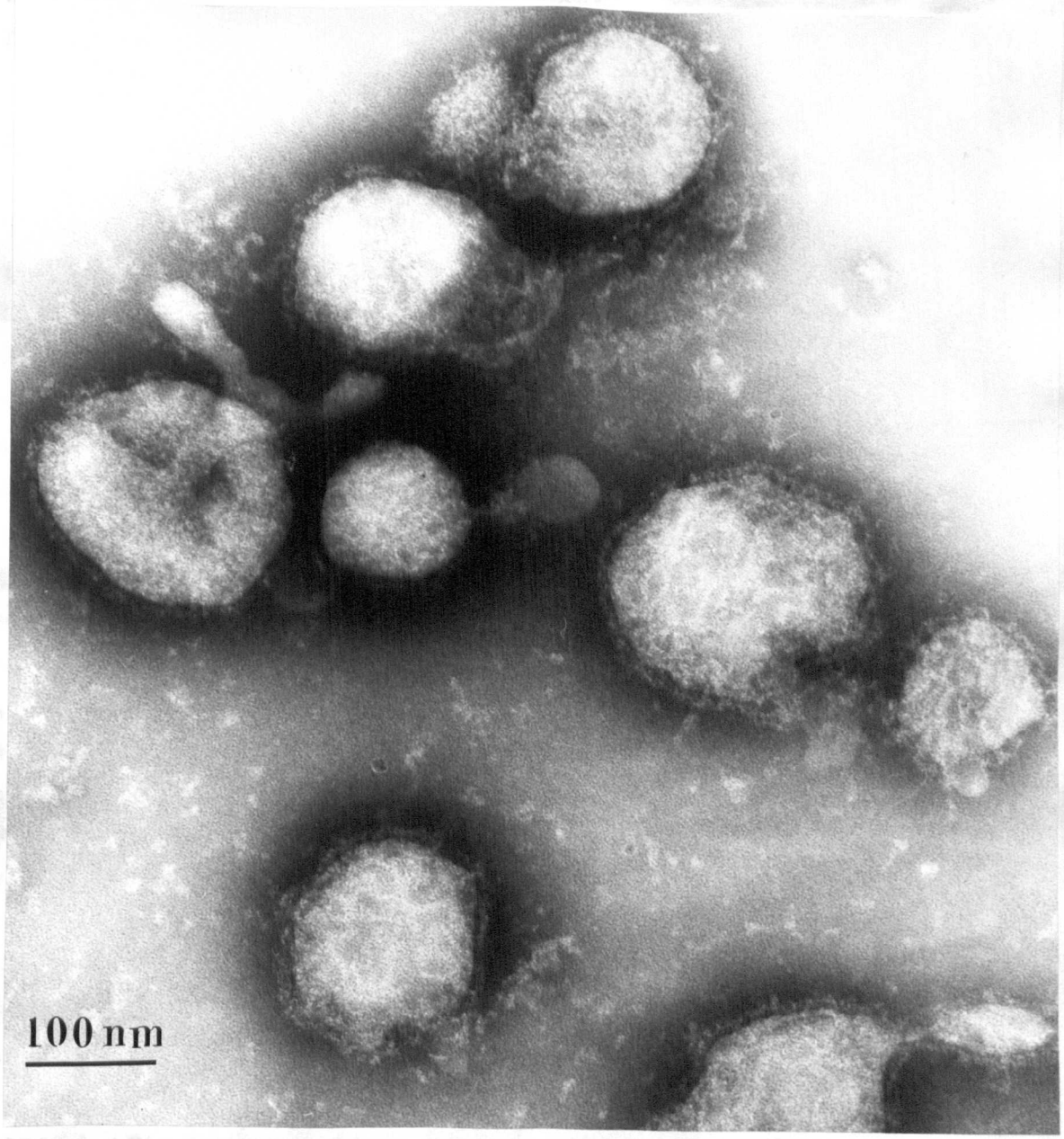
Table 1. Classification of Myxoviruses and some of their biophysical and biochemical properties*

	Subgroup 1	Subgroup 2
CLASSIFICATION	Influenza A Human influenza (man) Swine influenza (swine) Horse influenza (horse) Duck influenza (duck) Fowl plague (chicken)	Newcastle disease (NDV) (chicken) Mumps virus (man) Measles virus (man) Rinderpest virus (cattle) Distemper virus (dog) Respiratory syncytial virus (man)
	Influenza B (man)	Parainfluenza 1
	Influenza C (man)	Haemadsorption virus 2 (man) Sendai virus (mouse)
		Parainfluenza 2 Croup associated virus (man)
		Parainfluenza 3 Haemadsorption virus (man)
		Parainfluenza 4 SV5 (monkey)
Particle size	Small (80 - 120nm)	Large (150 - 250nm)
(a) Spontaneous disruption on spraying for electron microscopy	rare	common
(b) Approximate diameter of internal RNP	9nm	18nm
(c) Genetic recombination	+	-
(d) Multiplicity reactivation	+	-
(e) Formation of von Magnus (incomplete) virus	+	-
(f) Formation of syncytia	-	+
(g) Haemolysis	-	+
(h) Site of appearance of RNP antigen	nucleus	cytoplasm
(i) Early AMD sensitivity	+	-

*Taken from Robinson and Duesberg (1968) and Blair and Duesberg (1970)

Figure 1. Electron micrograph of purified NDV particles.

Fig 1

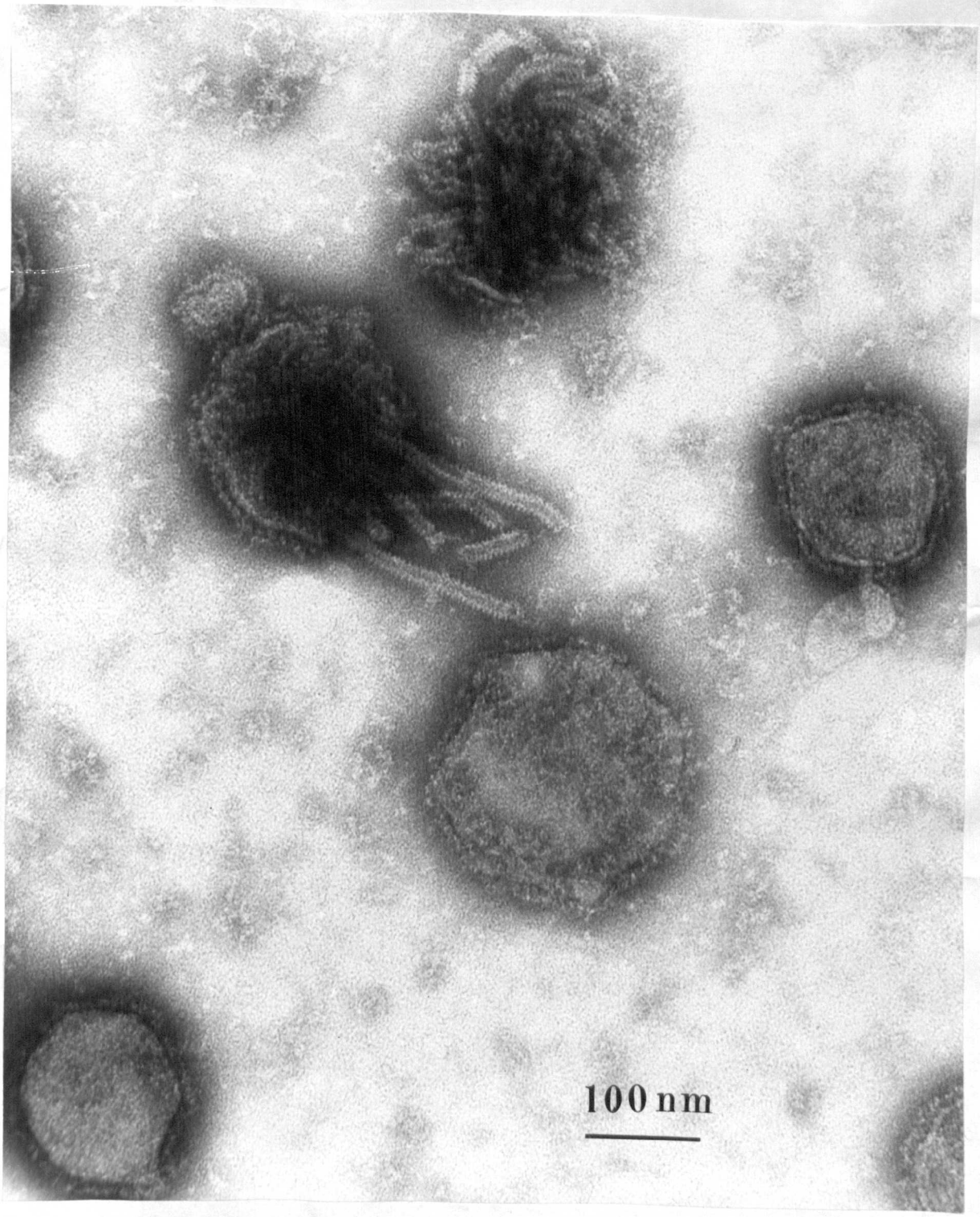


The presence of small amounts of cellular material cannot be ruled out though as, if this is present, it must have a similar buoyant density to the virus particles which has been reported to be between 1.18 and 1.24g/cc (Stenback and Durand, 1963; Duesberg and Robinson, 1965; Reeve and Alexander, 1970a; Huang et al., 1971).

The spherical virus particles have diameters between 90 and 150 nm (Barry et al., 1964) and an average particle weight of 800 x 10^6 daltons (Scholtissek et al., 1969). These particles are often disrupted in the preparation for electron microscopy, and the internal component can be seen spilling out (Fig.2.). Thus the basic virus architecture is readily discernible from electron micrographs. The fundamental structural component, common to all paramyxoviruses, is the ribonucleoprotein helix or nucleocapsid first described by Schafer and Rott (1959) and later, in more structural detail, by Horne and Waterson (1960) and Rott and Schafer (1961). This is rod-shaped, about 1μm long and is comprised of identical structural units packed into a helix 17 to 18nm in diameter with a central channel of 5nm in diameter (Horne and Waterson, 1960; Compans and Choppin, 1967 a, b; Hosaka et al., 1966; Hosaka, 1968; Hosaka and Shimizu, 1968). Further study of NDV nucleocapsids and those of related viruses, e.g. mumps virus, has shown that the structural unit contains one chemical protein subunit (Bikel and Duesberg, 1969; Evans and Kingsbury, 1969; Haslam et al., 1969; Finch and Gibbs, 1970; Mountcastle et al., 1970), and that between 2,400 and 2,800 structural units are arranged into a herringbone-like pattern in each nucleocapsid (Finch and Gibbs, 1970). Their morphology has been likened to tobacco mosaic virus (TMV) (Horne and Waterson, 1960; Choppin and Stoeckenius, 1964). Each complete nucleocapsid contains one copy of the virus' genetic material which is ribonucleic acid (RNA). Nucleocapsids have a buoyant density of 1.30-1.31g/cc in caesium chloride (Compans and Choppin, 1967 b), 1.24-1.26g/cc in tartrate

Figure 2. Electron micrograph of NDV particles showing two disrupted particles with the internal nucleocapsid clearly visible.

Fig 2



solution (de The and O'Connor, 1966) and 1.275g/cc in sucrose solution (Blair, 1968; Robinson, 1971 b). They have sedimentation coefficients of 200-300S (Blair, 1968; Compans and Choppin, 1967 b, 1968; Hosaka, 1968; Kingsbury and Darlington, 1968).

Within the intact virus particle the nucleocapsid is folded and surrounded by a membrane-like structure known as the viral envelope. This is studded with projections or spikes about 8nm long and spaced 8-10nm apart (Waterson, 1964). As mentioned earlier, the viral envelope is easily deformable during preparation of virus particles for electron microscopy, and this probably accounts for the observed pleomorphism. The viral envelope of all myxoviruses is made up of protein and lipid, the latter being derived from the plasma membranes of cells in which the virus is grown (Kates et al., 1961, 1962; Klenk and Choppin, 1969b, 1970a,b; Quigley et al., 1971). Associated with the exterior of the viral envelope are the haemagglutinating and neuraminidase activities which are characteristic of myxoviruses in general. NDV possesses a third functional property, the haemolysin, which is also present on the surface of the virus particle.

(iii) chemical composition

The chemical composition of NDV, and myxoviruses in general, has been difficult to determine accurately because of the limitations involved in the assessment of purity of virus preparations. Stenback and Durand (1963) have shown that NDV grown in different avian and mammalian cells have different densities which probably reflects slight variation in host-cell derived material incorporated into virus and hence variable chemical composition. The chemical analyses of preparations of paramyxoviruses that have been made demonstrate the presence of large amounts of protein and lipid, and smaller amounts of carbohydrate and RNA. Modern reports of the chemical composition of

NDV are non-existent. However, Klenk and Choppin (1969a) have determined the chemical composition of SV5, a paramyxovirus, and this contains 0.91% RNA, 73% protein, 20% lipid and 6.1% non-nucleic acid carbohydrate. Blough and Lawson (1968) have reported values of 24% and 28% for the lipid content of the Blacksburg (Bl) strain of NDV and Sendai virus, respectively. An early report by Cunha et al. (1947) on the chemical composition of NDV gives values which resemble those determined for SV5 (3.5% RNA, 65% protein, 27% lipid and 7% carbohydrate) although these were estimated using partially purified virus. Chemical analyses of paramyxovirus nucleocapsids have shown that the nucleocapsid of NDV contains about 5.7% RNA (Schäfer and Rott, 1959), that of SV5 about 4.1% RNA (Compans and Choppin, 1967b) and that of Sendai virus about 3.7% RNA (Hosaka, 1968). These values are similar to the RNA content of tobacco mosaic virus (TMV) which is 5.1% (Knight and Woody, 1958).

Chemical components of NDV (i) ribonucleic acid

The ribonucleic acid (RNA) of NDV was first isolated by Duesberg and Robinson (1965) using a detergent phenol method of extracting RNA from purified virus. They found that the RNA could be fractionated into two components by sucrose density gradient centrifugation. The faster sedimenting component had a molecular weight of 7.5×10^6 as calculated from its sedimentation coefficient in 0.1M salt ($S_{20,w} = 57$) using the empirical relation:

$$M = 1550\ (S_{20,w})^{2.1}$$

($M \equiv$ molecular weight) determined by Spirin (1963) for TMV RNA under similar conditions. One such RNA molecule per virus particle would account for all of the RNA found in the purified virus preparations, and following on from this work, RNAs of similar S value have been isolated from all other paramyxoviruses studied. These include other strains of NDV (Adams, 1965; Kingsbury, 1966a; Nakajima

and Obara, 1967; Sokol et al., 1966), Sendai virus (HVJ) (Barry and Bukrinskaya, 1968; Blair and Robinson, 1968; Iwai et al., 1966), SV5 (Compans and Choppin, 1968) and mumps virus (East and Kingsbury, 1971). The other component, also found in Sendai virus RNA, which sediments at approximately 3-4S and is heterogeneous, is thought to consist of degraded viral RNA and/or cellular RNA (Duesberg and Robinson, 1965; Kingsbury, 1966a). More careful preparation of virus and RNA has led to some reduction of this low molecular weight RNA (Blair and Robinson, 1968), and it was absent or present in small amount in virus RNA isolated from purified NDV as reported in this thesis. Kingsbury and Darlington (1968) have also shown that NDV nucleocapsids contain only the high molecular weight species of RNA. Therefore, it is almost certain that the high molecular weight RNA carries the virus genetic information, although this RNA has never been shown to be infectious.

The base compositions of high molecular weight RNAs from some purified paramyxoviruses are given in Table 2. These all show a high molar proportion of uracil, and the non-equality of U and A indicates that they are all single-stranded. Sensitivity to degradation by ribonuclease (Duesberg and Robinson, 1965; Kingsbury, 1966a; Compans and Choppin, 1968) and the dependence of sedimentation velocities on ionic strength (Duesberg and Robinson, 1965; Kingsbury, 1966a; Barry and Bukrinskaya, 1968) are further evidence of single-strandedness. Because of the dependence of sedimentation velocities on ionic strength, it has been difficult for workers to determine the molecular weight of virus RNA. Duesberg and Robinson (1965) and Blair and Robinson (1968) report a value of 57S in 0.1M NaCl corresponding to a molecular weight of 7.5×10^6 for NDV and Sendai virus RNAs. However, the sedimentation coefficient falls as the salt concentration is reduced. For instance,

TABLE 2. BASE COMPOSITIONS OF PARAMYXOVIRUS RNAs

Virus	C	A	G	U	Ref
NDV (L-Kansas)	23.0	23.8	23.8	29.4	1
NDV (C)	23.2	20.1	25.4	31.2	2
Sendai (HVJ)	24.3	24.2	21.5	30.0	3
Sendai (Harris)	23.4	24.1	22.9	29.6	4
SV5	20.1	27.8	22.1	30.8	5

References: (1) Duesberg and Robinson, 1965; (2) Kingsbury, 1966a; (3) Iwai et al., 1966; (4) Blair and Robinson, 1968; (5) Compans and Choppin, 1968.

Kingsbury (1966) and Compans and Choppin (1968) reported a value of 50S (mol. wt. 5.7×10^6) for NDV and SV5 RNAs in 0.05M NaCl. If the salt was removed completely, the sedimentation coefficient fell to 40S (mol. wt. 3.6×10^6) as shown by Compans and Choppin (1968) for SV5 RNA, and Barry and Bukrinskaya (1968) for Sendai virus RNA. The similar sedimentation velocities of NDV RNA, SV5 RNA and Sendai virus RNAs under identical salt concentrations, indicated that the size of these RNAs is virtually the same. However, estimates of the molecular weight based on sedimentation coefficient alone are highly uncertain as the frictional coefficients of these RNA molecules are unknown, (Gesteland and Boedtker, 1964). Strauss and Sinsheimer (1963) have also demonstrated that the Spirin formula does not apply accurately to all single-stranded RNAs.

If RNA is sedimented in 99% dimethyl sulphoxide, then a molecular weight estimate can be obtained that is independent of conformation of RNA in aqueous solvents (Duesberg, 1968). Strauss et al. (1968) found that NDV RNA had a sedimentation coefficient of 6.7S in 97% dimethylsulphoxide, and this corresponds to a mol. wt. of 6.3×10^6. This value is in fair agreement with those determined by sedimentation velocity in aqueous solvents.

Compans and Choppin (1967b, 1968) used the length and RNA content of nucleocapsids to estimate the molecular weight of SV5 RNA, and calculated a value of 6.8×10^6. The nucleocapsids of NDV, Sendai virus and SV5 have all been estimated by electron microscopic means to be about 1μm long (Compans and Choppin, 1967 a & b; Hosaka et al., 1966; Hosaka and Shimizu, 1968). Since TMV particles, which are similar morphologically to paramyxovirus nucleocapsids, are 0.3μm long and contain a single-stranded RNA molecule of mol. wt. 2.0×10^6 (Gierer, 1957; Knight and Woody, 1958), it has been suggested by Compans and Choppin (1967b) that paramyxovirus RNA would have a mol.

wt. of 6.7×10^6, i.e. 3.34 times that of TMV RNA. Nakajima and Obara (1967) reported a mol. wt. of 5.8×10^6 for NDV RNA derived from the RNA content of NDV particles.

In summary, it can be said that although paramyxovirus RNAs have similar base compositions (Table 2) and sediment at the same rate under uniform conditions (Blair and Robinson, 1968), suggesting they are closely similar as regards conformation and size, the methods available for estimating the mol. wt. of RNA are not sufficient for a reliable estimate of the mol. wt. of virus RNA. The introduction of analytical RNA polyacrylamide gel electrophoresis by Loening (1967), and the establishment of linear relationships between the relative electrophoretic mobility of a series of RNA molecules in polyacrylamide gels and the logarithm of the mol. wt. (Bishop et al., 1967; Weinberg and Penman, 1970) has provided another method by which the mol. wt. of virus RNA could be estimated. A relationship of this type was used to determine the mol. wt. of NDV RNA and is reported in this thesis.

It has been established that the base compositions of paramyxovirus RNAs are similar (Table 2). However, the failure of one virus RNA to hybridise to an RNA complementary in base sequence (i.e. the majority of RNA found in paramyxovirus infected cells - see later) to a large portion of another has shown that there is little sequence homology among paramyxovirus RNAs. For example, Sendai virus RNA will hybridise to Sendai virus specific intracellular RNA (vsi - RNA) but not to ND vsi - RNA, or mumps vsi - RNA, (Blair and Robinson, 1968; East and Kingsbury, 1971). This is not surprising in view of the likelihood that the avian pathogen, NDV, the human pathogen, mumps virus, and Sendai virus, apparently of murine origin, diverged long ago in evolutionary history.

(ii) proteins

A great deal more work has been done on the proteins of the subgroup I myxoviruses (i.e. orthomyxoviruses) than on those of the subgroup II myxoviruses (i.e. paramyxoviruses). This is partly due to the greater interest in the study of orthomyxoviruses in recent years, and partly due to certain characteristics of myxoviruses in general which have made virus protein studies difficult. The main difficulty is the assessment of purity of myxovirus preparations, for as has been stated previously, these viruses have physical and chemical properties not unlike cellular membranes and it is often not known whether all non-viral material is removed on purification. Since myxoviruses also assemble in and bud through cellular membranes, it is not inconceivable that incorporation of some cellular proteins may result.

NDV and myxoviruses in general possess at least four functional properties: the haemagglutinin, neuraminidase, haemolysin and RNA-dependent RNA polymerase activities. A fifth property which NDV may inherently possess is the capacity to induce interferon. The haemagglutinin, neuraminidase and haemolysin are activities associated with intact virus particles which indicates that the proteins involved are probably on the virus surface, and thus in the viral envelope. RNA polymerase activity can only be revealed by treating the virus particles with a nonionic detergent which probably removes a large part of the viral envelope (Huang et al., 1971; Robinson, 1971; Stone et al., 1971; Hutchinson and Mahy, 1972; Sheaff et al., 1972; Meager and Burke, in preparation; Chow and Simpson, 1971; Bishop et al., 1971). This indicates that the RNA polymerase protein(s) is associated with inner core structures or the nucleocapsids of virus particles.

Further enzyme activities have been demonstrated in purified preparations of NDV and other myxoviruses such as adenosine diphosphatase (ADPase), adenosine triphosphatase (ATPase) (Neurath and Sokol, 1963) and an endonuclease (Rosenbergova et al., 1971), but these are probably contaminating host-cell proteins. It has also been suggested that the haemolysin of NDV and Sendai virus is a host cell protein (Neurath, 1965; Matsumoto and Maeno, 1962; Homma, 1961).

In addition to virus functional proteins, there must also be virus structural proteins which give the virus particles their overall morphology, and serve to protect the fragile viral RNA genome. It is not unlikely that some virus proteins are both structural and functional. Until the advent of analytical protein polyacrylamide gel electrophoresis separation of virus proteins and correlation of protein molecules with known virus functions was virtually impossible. However, it was known that most myxoviruses could be disrupted with ether or a mixture of the detergent Tween 80 and ether, and that two antigenically distinct components were left in the aqueous phase (Hoyle, 1952; Schafer, 1963; Rott, 1964). These two components were separated by sedimentation, and could be identified with the haemagglutinin and the nucleocapsid. The haemagglutinin of NDV appears as rosette-shaped structures with diameters of 35-65nm, and has a sedimentation coefficient of about 80S (Rott, 1964). The haemagglutinin component also contained neuraminidase activity. Nucleocapsids have been previously described on page 3.

Evans and Kingsbury (1969) were the first workers to use the analytical protein polyacrylamide gel electrophoretic technique developed by Maizel (1966) to characterise the proteins of NDV. This technique requires the use of sodium dodecyl sulphate (SDS) to extract the virus proteins. Unfortunately, the detergent often destroys completely the

biological properties of proteins, although it has the useful advantage of eliminating protein charge so that proteins are separated according to their size alone on polyacrylamide gels. Therefore, correlation of individual protein species with the various virus biological functions is often not possible. The only way this can be done is by disruption of the virus particles with ether and/or detergents, separation of the released components by physical methods, analysis of the components for biological activity, and then extraction of component proteins with SDS followed by protein polyacrylamide gel electrophoresis. This approach has been used with limited success using both non-radioactive virus and radioactive virus in which all the virus proteins have been labelled by growth of the virus in the presence of radioactive amino acids.

Evans and Kingsbury (1969) found that if NDV proteins were extracted with SDS and electrophoresed on polyacrylamide gels that three major and five minor proteins could be identified. The three major protein species have since been confirmed for NDV by Haslam, Cheyne and White (1969), Bikel and Duesberg (1969), Mountcastle et al., (1971) and Iinuma et al. (1971). Comparison with the protein electrophoretic patterns of two other paramyxoviruses, SV5 and Sendai, showed that they all contain at least six proteins with some degree of similarity among the patterns (Mountcastle et al., 1971).

The protein of the nucleocapsid is the only paramyxovirus structural protein to have been identified for certain. Nucleocapsid protein or ribonucleoprotein (RNP) was quite simply identified as nucleocapsids could be isolated from paramyxovirus preparations after detergent (e.g. sodium deoxycholate) treatment. For instance, if the protein(s) extracted from NDV', Sendai virus' and SV5' nucleocapsids, labelled with [^{14}C]-amino acids were co-electrophoresed on a polyacrylamide gel with the proteins

extracted from their respective virus particles, labelled with [^{3}H]-amino acids, then the coincidence of radioactive peaks in the gel electrophoretogram showed the protein(s) common to both nucleocapsids and virus particles (Evans and Kingsbury, 1969; Haslam et al., 1969; Bikel and Duesberg, 1969; Mountcastle et al., 1970). The molecular weight of RNP has been estimated by calibrating polyacrylamide with proteins of known molecular weight (estimated by other means) and found to be about 60,000. The latest reported values are 56000 for NDV RNP, 61,000 for SV5 RNP and 60,000 for Sendai virus RNP (Mountcastle et al., 1971). It has also been shown that RNP can be converted to a smaller protein form (mol. wt. ~46,000) which migrates faster on polyacrylamide gels if it is treated with pronase (Bikel and Duesberg, 1969) or if nucleocapsids are isolated from infected cells treated with trypsin (Caliguiri et al., 1969; Mountcastle et al., 1970; Bikel and Duesberg, 1969).

Haslam et al. (1969) reported that when NDV was treated with deoxycholate (DOC) or Tween 80 and ether, the haemagglutinin and neuraminidase subunits could be separated from the virus in biologically active form. Isolation of the haemagglutinin fraction followed by extraction of the proteins and fractionation on polyacrylamide gels showed that it was composed of two major proteins. These have molecular weights of 74,000 and 41,000, and correspond to two of the major proteins of virus particles, the third being the nucleocapsid protein (Haslam et al., 1969; Mountcastle et al., 1971). By absorption of the haemagglutinin fraction to chick erythrocytes it was deduced that haemagglutinin activity is probably associated with the larger of the two proteins (Evans and Kingsbury, 1969; Iinuma et al., 1971). The smaller of the two proteins has thus been implicated as the neuraminidase (Iinuma et al., 1971), although neuraminidase activity has also been associated with higher molecular weight proteins by other workers (Evans and Kingsbury, 1969; Haslam et al., 1969). The NDV protein associated with haemagglutinating

Table 3. Proteins of Paramyxoviruses

Below is recorded recent data taken from Mountcastle et al. (1971) on the present state of knowledge concerning paramyxovirus proteins.

SV5				NDV				Sendai virus			
Protein number	Function	Glyco-protein	mol.wt.	Protein number	Function	Glyco-protein	mol.wt.	Protein number	Function	Glyco-protein	mol.wt.
1(?)	unknown	no	76,000	1	protein associated with HA activity	yes	74,000	1	unknown	no?	69,000
2	protein of viral envelope associated with haemag-glutinating activity	yes	67,000	2	unknown	yes	56,000	2	protein associated with HA activity	yes	65,000
3	ribonucleo-protein	no	61,000	3	ribonucleo-protein	no	56,000	3	ribonucleo-protein	no	60,000
4	protein of viral envelope	yes	56,000	4	unknown	no	53,000	4	unknown	no	58,000
5	unknown	no	50,000	5	unknown	no	46,000	5	unknown	yes	46,000
6	unknown	no	41,000	6	unknown	no	41,000	6	unknown	no	38,000

activity has recently been shown to be a glycoprotein by labelling virus carbohydrate with [^{3}H]-glucosamine (Mountcastle et al., 1971). In addition, they discovered a second glycoprotein which since it migrates to the same position on polyacrylamide gels as RNP was not distinguishable from it by radioactive amino acid labelling of proteins. This glycoprotein is not the RNP, however, as they showed that NDV nucleocapsids contained no (non-nucleic acid) carbohydrate. Therefore, it would appear that there are now at least seven NDV proteins detectable by polyacrylamide gel electrophoresis. Klenk et al. (1970a) and Mountcastle et al. (1971) have also shown two other paramyxoviruses, SV5 and Sendai virus, to each contain two glycoproteins. A preliminary study by Chen et al., mentioned in the Mountcastle et al. (1971), indicates that treatment of SV5 with a proteolytic enzyme removes the virus projections, and the resulting particles have lost their haemagglutinating and neuraminidase activities. Since loss of the virus projections corresponds to loss of the two virus glycoproteins, they suggested haemagglutinating and neuraminidase activities were associated with these glycoproteins.

(iii) lipid and carbohydrate

All myxoviruses contain between 20 and 30% lipid and this is found predominately in the viral envelope. As with paramyxovirus proteins, research into paramyxovirus lipids has lagged behind that into influenza lipids. However, it has been shown with NDV, in common with myxoviruses, that virus particles are disrupted by Tween 80 - ether to release the internal nucleocapsid thus indicating a structural role for the lipid in the viral envelope (Schafer and Rott, 1959). Similarly, phospholipase C has been shown to degrade virus lipid and inactivate infectivity of NDV (Franklin et al., 1957) indicating that lipid is an essential component for virus infectivity.

Myxoviruses acquire their viral envelopes from the host cell surface by a process analogous to that of budding (Bang, 1953; Berkaloff, 1963; Compans et al., 1966). It is then perhaps not surprising that the majority of virus lipid is derived from the plasma membrane. [^{32}P]-labelling experiments have shown that virus phospholipid is derived from pre-formed host-cell phosphatides (Wecker, 1957). Kates et al. (1961, 1962) demonstrated using a single strain of influenza that its lipid composition qualitatively reflected the host lipid composition when grown on different host cells. This has also been demonstrated indirectly for NDV by Stenback and Durand (1963) who showed that virus grown on different host cells had different densities. More recently, Klenk and Choppin (1969b, 1970a & b) have demonstrated that the composition of virus lipids in the paramyxovirus, SV5, reflected the lipid composition of the plasma cell membranes of the cells in which it is grown. A comparative study of the lipids of different RNA viruses (Rous sarcoma virus, NDV, Sendai virus and Sindbis virus) grown in chick embryo fibroblasts showed the lipid composition in the virus particles to be similar (Quigley et al., 1971). However, Blough and co-workers (Blough et al., 1967; Blough and Lawson, 1968; Tiffany and Blough, 1969a) reported that there was some difference between the lipids of myxoviruses and paramyxoviruses, and also between the lipids of NDV and Sendai virus grown in chick embryos. This has led them to postulate that the lipid composition of the viral envelope is determined by the structural proteins of the envelope. This concept appears to be at variance with the majority of evidence so far reported. Klenk and Choppin (1970b) using SV5 grown in different host cells have found only one instance in which there was convincing evidence for selection of the lipids in the viral envelope. This they suggest to be the exception rather than the rule, and point

out that the multi-cycle conditions of virus grown in chick embryos used by Blough and co-workers may account for the differences in lipid composition they observed in different myxoviruses. These multi-cycle growth conditions may also account for the fatty-acid differences between NDV and Sendai virus (Blough and Lawson, 1968) and among NDV strains (Tiffany and Blough, 1969b). Therefore, it is concluded that although there is a possibility that virus structural proteins can determine the lipid composition of the viral envelope, this is thought unlikely in view of the finding that the lipid composition of the viral envelope is qualitatively the same as that in the plasma membranes of infected cells for the enveloped viruses. However, the virus structural proteins almost certainly influence the arrangement and packing of lipids in the viral envelope, and this may have an important role in determining the biological properties of enveloped viruses.

Phospholipids, glycolipids, fatty acids and cholesterol are among the more important lipids which have been isolated from purified myxoviruses. Phospholipids, in particular sphingomyelin, phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine, make up more than half the total lipid content of paramyxoviruses (Blough and Lawson, 1968; Klenk and Choppin, 1969b). There are also significant amounts of cholesterol.

The carbohydrate of paramyxoviruses exists in two bound forms, (i) glycoproteins (Klenk et al., 1970a; Mountcastle et al., 1971), (ii) glycolipids (Blough and Lawson, 1968; Klenk and Choppin, 1970a). Klenk and Choppin (1970a) reported that one-third of the carbohydrate content of SV5 particles is present in glycolipids, and the remaining two-thirds in the two virus glycoproteins. Similar analyses on the carbohydrates of NDV have not been reported. However, the carbohydrate contents of SV5 and NDV are likely to be fairly similar. The mono-saccharides found in the viral glycoproteins of SV5 are galactose, mannose, glucosamine and fucose. Remarkably, neuraminic acid which is present in plasma membranes is absent

from viral glycoproteins and glycolipids (Klenk et al., 1970a). The absence of neuraminic acid is common to viruses possessing a neuraminidase, e.g. myxoviruses. Other viruses which have no neuraminidase such as Sindbis virus and vesicular stomatitis virus (VSV) do contain neuraminic acid in their glycoproteins (Strauss et al., 1970; Burge and Strauss, 1970; Burge and Huang, 1970). Klenk et al., (1970b) have shown in an electron microscopic study that neuraminidase activity is concentrated in areas of the plasma membrane where virus maturation is taking place. They suggested that removal of neuraminic acid from plasma membrane glycolipids may play some role in virus assembly, such as facilitation of the incorporation of other viral proteins or the stimulation of budding.

The glycoproteins are on the surface of the viral envelope and constitute the virus projections or spikes (Mountcastle et al., 1971). They are probably responsible for the virus surface antigenicity, and play a major role in (i) increasing virus dispersion in aqueous media because of their hydrophilic moieties, (ii) adsorption of virus to specific host-cell receptors. It is also very likely that the glycoproteins are associated with haemagglutinating and neuraminidase activities (Mountcastle et al., 1971). The carbohydrate portion of viral glycoproteins appear to be at least in part specified by the host-cell (i.e. it is the host-cell enzymes which attach carbohydrate to virus proteins) for all enveloped viruses studied so far (Klenk et al., 1970a; Strauss et al., 1970; Burge and Strauss, 1970; Burge and Huang, 1970; Compans et al., 1970).

The function of glycolipids in viruses is unknown, but Blough and Lawson (1968) suggested carbohydrate containing lipids may be involved in viral envelope-mediated phenomena such as haemolysis. Klenk and Choppin (1970a) suggested the elimination of gangliosides where the virus buds through the plasma membrane may play an important role in virus yield, cell fusion and cell death.

Replication

Progress in the understanding of biochemical events involved in virus replication depends largely on chemical characterisation of virus structural and functional components. This has been far from easy for myxoviruses, because they are structurally complex and may contain a proportion of non-viral material. Biologically, the events of the paramyxovirus' replicative cycle are fairly well known, but biochemically very little is understood about these replicative events. Briefly, the paramyxovirus' replicative cycle can be divided into three phases: - (i) adsorption to and penetration into cells; (ii) the lag phase in which vsi-RNA and protein synthesis occur; (iii) maturation in and release by budding of virus particles in the plasma membranes. These events are described more fully elsewhere (Robinson and Duesberg, 1968). Some progress has been made in the understanding of vsi-RNA synthesis as described later in this introduction. However, vsi-protein synthesis has been left relatively unexplored. Fluorescent antibody studies indicate that all paramyxovirus antigens are synthesized and accumulate in the cytoplasm (Reda et al., 1964). Incorporation of radioactive amino acids into vsi-proteins can be detected by double labelling procedures such as that of Hay et al. (1968) in which the proteins from infected cells labelled with [^{3}H]-valine and uninfected cells labelled with [^{14}C]-valine are extracted, mixed and fractionated on polyacrylamide gels. The [^{3}H] : [^{14}C] ratio removes peaks due to cellular proteins. Using this technique the synthesis of the three major proteins described by Evans and Kingsbury (1969) and Haslam et al. (1969) in purified NDV preparations has been detected in cells infected with six different strains of NDV from 4 - 6 h p.i. (Lomniczi et al., 1971). It was also shown that cells infected with avirulent NDV strains were still synthesizing virus proteins 24 h after infection, whereas no virus or cellular proteins were synthesized in cells infected with virulent NDV strains 14 h after infection. The biochemical basis of virulence is not understood at all.

Virus specific intracellular RNA synthesis

In some virus infected cells (e.g. polio virus infected HeLa cells) virus specific intracellular RNA synthesis can be detected as the infection almost totally depresses or "cuts-off" host-cell RNA synthesis. This is not so with NDV infected cells as, although host-cell RNA synthesis is progressively "cut-off" after infection, this is never totally depressed even at late stages in the replicative cycle (Wilson, 1968; Moore et al., 1972). However, it has been shown that in common with a number of other RNA viruses (excluding influenza) the growth of paramyxoviruses is only moderately inhibited by the drug actinomycin D (AMD) which blocks host cell DNA-dependent RNA synthesis (Bratt, 1969b; Bratt and Robinson, 1967; Kingsbury, 1962; Rott and Scholtissek, 1964). It has also been reported that AMD stimulates vsi-RNA synthesis (Kingsbury, 1967; Bratt, 1969b) and reduces the time required for the production of infectious progeny virus (Bukrinskaya and Zhdanov, 1963; White and Cheyne, 1965, 1966). This latter phenomenon may be caused by breakdown of host cell polyribosomes in the presence of AMD, thus making available a greater pool of ribosomes which could take part in vsi-protein synthesis (Cheyne and White, 1966). Therefore, NDV or paramyxovirus specific intracellular RNA synthesis is normally detected by the addition of radioactive RNA precursors such as [^{3}H]-uridine after host cell RNA synthesis has been inhibited with AMD. ND vsi-RNA synthesis is usually first detectable about 3 h p.i., and then increases up to a maximum about 7 h p.i. at a rate paralleling the production of infectious virus (Wheelock, 1963; Kingsbury, 1962; Granoff and Kingsbury, 1964; Kingsbury, 1966b; Bratt and Robinson, 1967; Bratt, 1969b). However, using high concentrations of AMD (20μg/ml) detection of vsi-RNA synthesis can be made within 90 minutes p.i. (Robinson, 1971a; Clavell and Bratt, 1971) indicating that vsi-RNA synthesis occurs at a very early stage in infection. This work might be criticised because of the high level of actinomycin present which is extremely cytotoxic to chick embryo cells in which these studies were made.

Isolation of NDV specific intracellular RNA from infected cells has general ly been effected by SDS-phenol methods (Kingsbury, 1966b; Bratt and Robinson, 1967; Blair and Robinson, 1968). Examination of the base composition of this RNA has shown it to contain a higher amount of A than U (~20% U and 30% A). Thus it was readily recognised that RNA molecules made in infected cells in the presence of AMD were, on the whole, not replicas of virus RNA (which contains ~30% U and 20% A), but probably complements of viral genomes (Duesberg and Robinson, 1965; Kingsbury, 1966a,b; Bratt and Robinson, 1967). In vitro RNA-RNA hybridisation tests by Kingsbury (1966b) and Bratt and Robinson (1967) have confirmed that vsi-RNA was predominately complementary to virus RNA. However, some doubts were raised as to the exact proportion of (+) (as in virus RNA) and (-) (as in vsi-RNA) RNA strands in Sendai' and ND vsi-RNA when Robinson (1970) demonstrated self-bybridisation in Sendai virus RNA and NDV RNA. Later reports have failed to confirm self-hybridisation in NDV RNA (Portner and Kingsbury, 1970; Kaverin and Varich, 1971), but not in Sendai virus RNA. Kingsbury (1966b) showed that vsi-RNA from cells infected with four different strains of NDV was complementary to the virus RNA isolated from the C strain which he used indicating a high degree of sequence homology among the RNA molecules of different NDV strains. However, Sendai vsi-RNA did not anneal to NDV RNA and vice versa suggesting that the base sequences of the two paramyxovirus RNAs must be quite different (Blair and Robinson, 1968). Kingsbury (1966b) has also shown that the complementary RNA synthesis was unaffected in the presence or absence of AMD, i.e. AMD does not elicit production of complementary RNA.

Sedimentation of ND vsi-RNA through a sucrose gradient showed it to be comprised of four main components which were assigned S values of 57S, 35S, 22S and 18S by comparison with the sedimentation of cell ribosomal RNAs (18S and 28S) (Bratt and Robinson, 1967). Similar RNA species were found in Sendai vsi-RNA (Blair and Robinson, 1968). The 18S and 22S

RNAs were shown to be single-stranded by their sensitivity to degradation by ribonuclease (Bratt and Robinson, 1967; Blair and Robinson, 1968), whilst 35S RNA was shown to contain some base-paired RNA, and was thus implicated as a replicative intermediate (Bratt and Robinson, 1971). The complementary RNA species make up to 95% of the total vsi-RNA, the 18S RNA being always present in largest amount (Bratt and Robinson, 1967; Blair and Robinson, 1968), and hybridise 90-100% to virus RNA (Kingsbury, 1966b; Bratt and Robinson, 1967). Generally, only small amounts of 57S RNA are present intracellularly, but the amount of this and the complementary RNA species do vary from one NDV strain to another (Bratt, 1969a; Lomniczi et al., 1971). The fraction of intracellular 57S RNA annealing to NDV RNA is 30% indicating the presence of 57S (-) RNA strands besides 57S (+) RNA strands presumably destined for new progeny virus particles (Bratt and Robinson, 1967). Larger amounts of 57S (+) RNA strands are usually found in Sendai virus infected cells suggesting that virus particle maturation (i.e. encapsidation of 57S (+) RNA) may be taking place more slowly in this case (Blair and Robinson, 1968, 1970).

A mixture of all three of the smaller unlabelled intracellular RNA species annealed to no more than 70% of [^{32}P] labelled 57S NDV RNA (Bratt and Robinson, 1967). Thus it was concluded that the 35S, 22S and 18S species did not collectively contain the entire complement of 57S RNA. However, because neither the absolute concentration and purity of the intracellular RNA species (possible contamination with fragments of 57S (+) RNA strands) nor the specific radioactivity of the virus RNA were known in these hybridisation experiments, the above conclusion must be considered an estimate. An unusual finding was that the 18S RNA fraction with a molecular weight of ~0.7 x 10^6 daltons could account for 50% of the base sequences in NDV RNA with a molecular weight of 4.8 - 7.5 x 10^6 daltons (Bratt and Robinson, 1967). Kingsbury (1970) suggested that either 18S RNA is heterogeneous

with respect to nucleotide sequences or that the 18S molecules are identical with half the virus genome redundent for these sequences. The increased resolving power of polyacrylamide gel electrophoresis has shown that 18S RNA is heterogeneous, there being at least three different RNA molecules, making the first suggestion the more likely (Lomniczi et al., 1971). It has also been made possible by the use of this technique to estimate the molecular weights of ND vsi-RNA species, and determine whether the sub-genomic complementary RNAs were related by size to the virus RNA. These results have been published (Lomniczi et al., 1971) and are also reported later.

Recent work in which the drug cycloheximide has been used to block protein synthesis indicates that synthesis of complementary RNA continued unchanged or was stimulated, whilst production of 57S (+) RNA strands was suppressed (Robinson, 1971a; Clavell and Bratt, 1971; Kaverin and Varich, 1971). This suggests the presence in infected cells of two RNA polymerases; one to synthesize complementary RNA, and the other to synthesize new virus RNA (Robinson, 1971a). The insensitivity of vsi-RNA synthesis to cycloheximide was one of the reasons for the postulation of a virus particle RNA polymerase (see later). Kaverin and Varich (1971) offered an alternative hypothesis to the "two RNA polymerases" hypothesis. This second hypothesis assumed a competition for 57S (+) RNA strands between the ribonucleoprotein (RNP) and the RNA polymerase. When protein synthesis was inhibited the pool of RNP was rapidly exhausted, and the protein could not compete effectively with the RNA polymerase. The latter preferred (+) strands as templates when they were available; therefore under this condition (-) strands were synthesized while the synthesis of (+) strands was suppressed.

The site of NDV specific RNA synthesis in infected cells is almost certainly in the cytoplasm. Autoradiographic studies (Wheelock, 1963) and cell fractionation (Bratt and Robinson, 1967) have shown ND vsi-RNA synthesis to be confined to the cytoplasm of infected cells. However, reports of nuclear sites (Azadova et al., 1965; Bukrinskaya et al., 1966) cannot be ruled out until conclusive evidence using short

pulses of labelled precursor is presented to eliminate these.

Kingsbury (1966b) was the first to suggest that the large amount of complementary RNA of sub-genomic sizes present in paramyxovirus infected cells was functioning as messenger RNA for viral protein synthesis. The association of NDV complementary RNA, especially 18S and 35S, with polyribosomes of infected cells demonstrated by Bratt and Robinson (1967) and Kingsbury (1970) makes this likely. A similar situation is found for vesicular stomatitis virus (VSV), a rhabdovirus, which also synthesizes sub-genomic complementary RNA (Huang et al., 1970; Mudd and Summers, 1970). The lack of infectivity of NDV RNA or any paramyxovirus RNA (Kingsbury, 1966a; Robinson, 1971b) is also consistent with the hypothesis that the complementary RNAs are the viral messenger RNAs. However, the direct test of this hypothesis, involving cell-free synthesis of identifiable viral gene products programmed by pure vsi-RNAs, has not been made.

If the NDV complementary RNAs are indeed the viral messenger RNAs, then this pattern of RNA replication and virus replication will distinguish it from the "orthodox" model of replication of the small RNA viruses (e.g. picornaviruses). These viruses contain infectious RNA (i.e. the virus RNA is itself the messenger RNA from which new virus proteins are produced) (Montagnier, 1968; Hofschneider and Hanson, 1968).

NDV and paramyxovirus RNA-dependent RNA polymerase

An RNA-dependent RNA polymerase or RNA transcriptase was first demonstrated in the virions of the rhabdovirus, VSV, which like the paramyxoviruses produces RNA complementary to the virus RNA in infected cells (Baltimore et al., 1970; Huang et al., 1970). A comparison of NDV particles with VSV particles showed NDV to possess less than 3% of the RNA transcriptase activity of VSV, and this was initially thought to be negligible (Baltimore et al., 1970). However, a later study by Huang et al. (1971) showed that the enzyme

activity in NDV particles, although very low, synthesised complementary RNA. They found that the RNA transcriptase activity in NDV particles, in common with VSV particles, was only revealed after treatment of the particles with a non-ionic detergent, specifically Triton N101 or Non-idet P40. Maximal enzyme activity was obtained at pH 7.3 and incubation at 32°C, and the reaction was dependent on the presence of (i) divalent cations, e.g. Mg^{2+}, (ii) a sulphydryl reagent, e.g. dithiothreitol, (iii) all four nucleoside triphosphates, ATP, CTP, GTP and UTP, (iv) a non-ionic detergent, e.g. Triton N101. The reaction was inhibited by ribonuclease (but not deoxyribonuclease) and trypsin. Actinomycin D which blocks DNA-dependent RNA synthesis had no effect on the reaction. The majority of these findings have been confirmed, and these are reported in this thesis and elsewhere (Sheaff et al., 1972). A similar RNA transcriptase has since been demonstrated in Sendai virus (Robinson, 1971b; Stone et al., 1971; Hutchinson and Mahy, 1972). The activity of Sendai virion RNA transcriptase was found to be even lower than that of NDV, and there appeared to be some variation in the pH optimum for the enzyme from one group of workers to another. However, both NDV and Sendai virus RNA transcriptases synthesized RNA which has been demonstrated by hybridisation studies to be complementary to the parent virus RNA (Huang et al., 1971; Robinson, 1971b; Stone et al., 1971; Clavell and Bratt, 1971). This newly synthesized RNA is found initially bound to the virus RNA in a partially double-stranded structure. The presence of double-strandedness in the virus RNA is indicated by a reduction in the sedimentation coefficient (50S → 35S) and resistance to ribonuclease (Huang et al., 1971; Robinson, 1971b; Stone et al., 1971; Clavell and Bratt, 1971). On longer incubation in vitro single-stranded complementary RNA has been found. This RNA sediments at about 16S (Stone et al., 1971; Clavell and

Bratt, 1971), only slightly slower than the 18S RNA found in vsi-RNA extracted from paramyxovirus infected cells. Robinson (1971b) and Hutchinson and Mahy (1972) found smaller single-stranded RNAs sedimenting between 4 and 6S. This could be due to endonuclease activity in purified paramyxovirus preparations (Rosenbergova et al., 1971) which degrades larger pieces of single-stranded RNA or the synthesis of only small pieces of RNA by the enzyme in vitro. The reason(s) why the RNA transcriptase does not make the 35S, 22S and 18S RNA species found in vsi-RNA is not known. However, the in vitro assay conditions, whilst allowing maximal enzyme activity, may be far from the conditions found in chick cells. The transcriptase may depend on host-cell factors (Huang and Baltimore, 1971), and have to be associated with some host-cell membrane or component for full activity and the synthesis of those complementary RNA species normally found in infected cells.

Huang, Baltimore and Bratt (1971) examined four NDV strains and found them all to contain RNA transcriptase activity indicating the enzyme activity not to be due to contamination of any one strain with another virus. NDV strain HP had a polymerase activity of specific activity approximately 200 p moles [^{3}H]-GMP per mg protein per hour at 32^{o}C while the three others, NDV-IM, NDV-RO, NDV-N, had lower enzyme specific activities of about 50 p moles [^{3}H]-GMP per mg protein per hour at 32^{o}C.

The role of NDV RNA polymerase or transcriptase in interferon induction

Interferon is a name applied to a heterogeneous group of antiviral proteins whose production can be induced by a wide variety of agents. The biological and chemical properties of interferon have been fully reviewed elsewhere (Finter, 1966). Mechanisms of interferon induction, production and mode of action, are still not understood. The writer does not intend to review the vast amount of

literature on interferon, much of which is irrelevant to the work reported herein, and will confine himself to the production of interferon by inactivated myxoviruses, in particular NDV, and draw analogies to the production of interferon by certain other agents, in particular double-stranded RNA.

Myxoviruses, irradiated with ultra-violet light, have long been known to be inducers of interferon formation (Burke and Isaacs, 1958; Ho and Breinig, 1965; Cantell and Paucker, 1963; Wagner, 1964). Unirradiated virus does not induce interferon formation in chick cells (Burke and Isaacs, 1958; Ho and Breinig, 1965; Cantell and Paucker, 1963), but does so in mouse L cells (Wagner, 1964). Three questions therefore may be asked: (1) why do infective NDV and influenza fail to provoke interferon formation in chick cells; (2) by what mechanism does irradiated, non-infective virus produce interferon; and (3) why is this capacity lost on prolonged irradiation?

It has been established that NDV contains an RNA polymerase (Huang et al., 1971) and that this probably functions to produce complementary RNA of sub-genomic sizes which act as viral mRNA (Bratt and Robinson, 1967) on infection. It is also thought that the newly synthesised RNA is initially bound to the virus RNA in a double-stranded structure (Huang et al., 1971). This is particularly interesting with regards the induction of interferon since double-stranded RNA is the best exogenous inducer of interferon (Colby, 1971; De Clercq and Merigan, 1970; Hilleman, 1968). Therefore, the answers to the three questions may be resolved by examination of the activity of NDV RNA polymerase before and after irradiation. Prior to the present work reported herein and that of Clavell and Bratt (1971) there had been conflicting results reported on the RNA-synthesising capacity of UV-irradiated NDV (Gandhi and Burke, 1970; Gandhi et al., 1970; Huppert et al., 1969).

At the heart of this controversy is the small amount of base-paired, virus specific RNA detectable in cells infected with infective virus (Bratt and Robinson, 1971; Zhdanov and Kingsbury, 1969) and the small amount of any type of virus-specific RNA (single-stranded or base-paired) in cells infected by UV-irradiated virus (Gandhi and Burke, 1970; Gandhi, Burke and Scholtissek, 1970; Huppert et al., 1969). This work can also be criticised because RNA was extracted from infected cells late in infection, and it might be predicted that UV-irradiated virus would only make RNA early after infection. Both Meager and Burke (1972) and Clavell and Bratt (1971) have studied the activity of NDV RNA polymerase with increasing doses of UV-irradiation and their results are similar. Correlation with loss of infectivity and increase in interferon inducing capacity has also been attempted and is reported in this thesis.

The aims of this work were as follows:-

(i) to isolate NDV RNA and ND vsi-RNA and fractionate these on sucrose density gradients and polyacrylamide gels in order to characterise their component RNA species with respect to sedimentation coefficients and molecular weights, and confirm, and subsequently extend, the observations of others.

(ii) to determine whether the presence of certain of the component RNA species, e.g. 18S RNA, of ND vsi-RNA was related to virulence.

(iii) to isolate polyribosomes from NDV-infected chick cells, and isolate and identify, if possible, the virus specific RNA species associated with polyribosomes.

(iv) to examine the properties of NDV particle RNA-dependent RNA polymerase.

(v) to investigate the possible role which NDV particle RNA-dependent RNA polymerase may have in interferon induction.

(vi) to attempt to elucidate the structural basis of the RNA polymerase activity of NDV particles.

MATERIALS AND METHODS

MATERIALS

(a) Biological materials

Medium 199 and Eagles minimal essential medium were purchased as tenfold concentrates from Wellcome Reagents Limited, Beckenham, England. Calf serum was obtained from Bio-cult Laboratories, Glasgow, Scotland.

Crystamycin was purchased from Glaxo Laboratories Limited, Greenford, England, and Chloramphenicol from Parke Davis Limited, London.

Trypsin, for preparation of chick embryo fibroblast cultures, was obtained from Difco Laboratories, Detroit, U.S.A. Deoxyribonuclease (free of ribonuclease activity) was purchased from Sigma Chemical Company Limited, London, and pancreatic ribonuclease from C. F. Boehringer V. Soehne, Mannheim, West Germany.

Bovine serum albumin (BSA), fraction V, was obtained from Armour Pharmaceutical Company Limited, Eastbourne, Sussex, and yeast RNA from B.D.H. Chemicals Limited, Poole, Dorset.

ATP, UTP, GTP, and CTP (synthetic or from equine muscle) were purchased from Sigma Chemical Company Limited, London.

Actinomycin D (AMD) was given by Merck, Sharp and Dohme Limited, Rahway, New Jersey, U.S.A.

Purified [^{3}H] SFV and [^{3}H] TMV, both containing RNA labelled with [^{3}H]-UMP, were generous gifts from Dr. S. I. T. Kennedy (Department of Biological Sciences, Warwick University, Coventry.) and Dr. A. J. Gibbs (Rothamstead Research Station, nr. Harpenden, Herts.) respectively.

(b) Chemical materials

Arcton 113 was obtained from Imperial Chemical Industries, Mond Division, Runcorn. 2-mercaptoethanol, Triton N-101 and β-propiolactone (BPL) were purchased from Sigma Limited, London. Water d_2 puriss (D_2O) was purchased from Koch Light Laboratories, Ltd., Colnbrook, Bucks. Sodium deoxycholate

was purchased from Hopkin and Williams Limited, Chadwell Heath, Essex. Tween 80, Triton X-100, Sodium lauryl (or dodecyl) sulphate (SDS), specially pure, and Schiffs reagent were all purchased from B.D.H. Chemicals Limited, Poole, Dorset. Poly-L-lysine hydrochloride (average mol. wt. 100,000) was purchased from Schwarz/Mann, Orangeburg, New York, U.S.A.

NNN^1N^1-Tetramethylethylenediamine (TEMED) was purchased either from Koch Light Laboratories Limited, Colnbrook, Bucks., or fromEastman Kodak Company, Rochester, New York, U.S.A. Acrylamide and NN^1-methylenebisacrylamide were purchased from Eastman Kodak Company. Cleland's reagent (A grade) was purchased from Calbiochem, London.

Coomassie brilliant blue R and Fuchsin Basic were obtained from G. T. Gurr Limited, London.

(c) Radioactive materials

[5-^{3}H]-uridine (30.7 Ci/mmole), [2-^{14}C]-uridine (53 mCi/mole), [^{14}C]-L-valine (225 mCi/mmole), [^{35}S]-L-methionine (17 Ci/mmole), [8-^{3}H]-GTP (8.0 or 10.0 Ci/mmole) and [^{32}P] (orthophosphate in dilute hydrochloric acid pH 2-3 at 50-100 Ci/mg P) were purchased from the Radiochemical Centre, Amersham, Bucks.

2-5-Diphenyloxazole (PPO), 1,4-di (2-(5-phenyloxazolyl))-benzene (POPOP) and naphthalene (scintillation grade) were obtained from Nuclear Enterprises (G.B.) Limited, Edinburgh. Aerosil standard silica was obtained from Bush Beach Segner Bayley Limited, London.

(d) Various

Plastic petri dishes were purchased from Sterilin Limited, Richmond, Surrey. Plastic scintillation vials were obtained from Packard Instruments Limited, Wembley, Middlesex.

(e) Centrifugation equipment

All centrifugation procedures were carried out exclusively in MSE centrifuges, rotors and tubes (Measuring and Scientific Equipment, Ltd., London, England.). Unless otherwise specified, all the centrifuge rotors used were made of aluminium.

PREPARATIVE METHODS

Media

1. Growth medium

Growth medium consisted of medium 199 containing 7% (v/v) calf serum, 0.19% (w/v) sodium bicarbonate, and crystamycin (100 units/ml of penicillin and 0.1mg/ml of streptomycin).

2. Maintenance medium

Maintenance medium was identical to growth medium except that it contained 2% (v/v) calf serum instead of 7% (v/v).

3. Earle's balanced salt solution

Earle's balanced salt solution, as defined by Earle (1943), was made from a prepared salt mixture, which was purchased from Oxo Limited, Oxoid Division, London. The salt mixture was dissolved in distilled water, sterilised by autoclaving, and adjusted to pH 7.2 - 7.5 with 5% (w/v) sodium bicarbonate, using phenol red as an indicator of pH.

Preparation of chick embryo fibroblast monolayers

Primary chick embryo cells were prepared using a modification of the procedure of Porterfield (1960). 11-day old chick embryos were used. After removal from the eggs, the embryos were decapitated and rinsed in phosphate-buffered saline, pH 7.3 (PBS) (Dulbecco and Vogt, 1954), minced finely with sharp scissors, and the mince suspended in 0.1% (w/v) trypsin in PBS (4ml/embryo). The suspension was then shaken on an orbital shaker for 20 min at 37°C. The supernatant was decanted into a pre-cooled bottle on ice, and the sedimented pieces of embryo re-trypsinized. Altogether, four trypsinizations were performed. The cell-containing supernatant was then

passed through a 40 mesh stainless steel filter, and the cells pelleted by centrifugation at 1,000 rev/min (300 g_{av}) for 20 min at 4°C. The cells were resuspended at 2ml per embryo in growth medium and filtered through sintered glass funnels (grade 0 first and then grade 1). The yield of cells was 2 to 3 x 10^8 cells/embryo.

Monolayer cultures were prepared as follows:- The cell suspension was diluted to 3 x 10^6 cells/ml, 6 x 10^6 cells/ml, or 10 x 10^6 cells/ml with growth medium. 20ml aliquots of the 3 x 10^6 cells/ml suspension were dispensed onto glass petri dishes of 9cm diameter; 25ml aliquots of the 6 x 10^6 or 10 x 10^6 cells/ml suspension were dispensed onto glass petri dishes of 14cm diameter; and 3ml or 5ml of the 3 x 10^6 cells/ml suspension were dispensed onto plastic petri dishes (5cm) (Sterilin Limited, Richmond, Surrey). Cultures were incubated at 37°C in a humid atmosphere of 95% air/5% carbon dioxide, and in most experiments used the day after setting up.

Growth of Newcastle disease virus

The Herts 33, Texas L, H, F and LaSota strains of Newcastle disease virus were used. The origin, growth and biological properties of these NDV strains has been described previously (Lomniczi, 1970; Lomniczi, Meager and Burke, 1971). NDV strains were stored frozen at -20° or -70°C, or at 4°C if virus purification was to follow. The allantoic cavity of 9-day old embryonated eggs was inoculated with 50µl of a dilution of virus suspension in maintenance medium (about 5 x 10^3 p.f.u./egg for plaque forming strains, and about 8 x 10^4 EID_{50}/egg for the non-plaque forming strains). Allantoic fluid was harvested after 36 - 48 h at 37°C followed by 4 h at 4°C, and contained virus at approximately 1 x 10^9 p.f.u./ml (3 x 10^{10} EID_{50}/ml). The virus containing fluid was clarified by centrifugation at 10,000 rev/min (11,000 g_{av}) for 30 min at 4°C in a 6 x 250 ml angle rotor before storage.

In other cases, virus was grown in chick chorio-allantoic membranes and in monolayer cultures. Growth of NDV in chorio-allantoic membranes was based on the method of Joss et al. (1969). Chorio-allantoic membranes were removed from 11-day old embryonated eggs, and washed twice with Earle's balanced salt solution, buffered to pH 7.5 with 5% (w/v) sodium bicarbonate, containing 100µg/ml chloramphenicol. About 30 washed membranes were transferred to 1 litre Flow bottles and suspended in 60ml Earle's balanced salt solution containing 0.4% (w/v) sodium bicarbonate and 100µg/ml chloramphenicol, and buffered to pH 7.5 by bubbling with 90% O_2/10% CO_2. The membranes were infected with NDV at 2-3 p.f.u./cell, and the preparations shaken on a mechanical device in a 37°C hot-room. The yield of virus obtained after 24 h was about 4 x 10^{10} p.f.u./Flow bottle. For production of virus from cell monolayers, cultures on glass petri dishes (14cm diameter) were infected with NDV (10 p.f.u./cell). The yield of virus obtained was approximately 2.5 x 10^8 p.f.u./culture after 24 h incubation at 37°C.

Preparation of radioactively labelled NDV

NDV labelled with [^{32}P] was prepared by a modification of the method of Barry and Bukrinskaya (1968). 9-day old embryonated eggs were inoculated with 50µl of orthophosphate solution containing 0.25mCi of [^{32}P] (carrier-free) just before infecting the eggs with NDV as described above. Labelling of NDV strain Texas with [^{3}H]-uridine was achieved by growing virus in chick chorio-allantoic membranes using 1mCi[^{3}H]-uridine per bottle of 30 membranes. NDV strain Texas labelled with [^{14}C]-L-valine was prepared by infection of chick cells (2.5 x 10^8 cells/petri dish) with virus at 30 p.f.u./cell for 1 h at 37°C. The excess virus was removed and the cell monolayers washed twice with 10ml of maintenance medium containing 0.1µg/ml actinomycin D (AMD). Then 25ml of maintenance medium containing 0.1µg/ml AMD was added until

2.5 - 3.0 h post infection (p.i.), when the medium was replaced with 15ml of Earle's balanced salt solution (without glucose) containing 2% dialysed calf serum, 0.1μg/ml AMD, crystamycin (100 units/ml of penicillin and 0.1mg/ml of streptomycin), and 10μCi [^{14}C]-L-valine. The virus containing medium was harvested after 24 h at 37^{o}C.

Purification of NDV

In the early part of experimental work, virus purification was based on a method described for Sendai virus by Barry and Bukrinskaya (1968). (METHOD I). However, attempts to improve on the final percentage of infectivity retained after purification led to the development of a second method (METHOD II), which was used exclusively for the preparation of concentrated virus suspensions used in NDV RNA-dependent RNA polymerase assays.

METHOD I

Virus was pelleted from clarified allantoic fluid by centrifugation at 30,000 rev/min (75,000 g_{av}) for 1.5 h at 4^{o}C in an 8 x 50ml angle rotor, and then resuspended in 4% of the original volume with phosphate-buffered saline by Dounce homogenisation (2ml capacity glass Dounce homogeniser). This crude virus suspension was shaken with an equal volume of ice-cold fluorocarbon (Arcton 113, Imperial Chemical Industries.) for 30 s, and the phases separated by centrifugation at 2,000 g_{av} for 5 min in an MSE bench centrifuge. The upper aqueous layer which contained virus was removed leaving a yellow precipitate at the interface. Aliquots of virus suspension (2ml) were layered onto 20ml 15 - 60% w/v linear potassium tartrate gradients containing PBS, and centrifuged to equilibrium at 25,000 rev/min (65,000 g_{av}) for 14 h at 4^{o}C in a 3 x 23ml swing-out rotor. The visible white virus band which contained the majority of infectivity, haemagglutinating activity and radioactive label and had a

buoyant density of 1.19 - 1.21 g/cc, was collected and diluted with ice-cold PBS. Virus was pelleted out of the potassium tartrate solution by centrifugation at 30,000 rev/min (59,000 g_{av}) for 45 min at 4°C in an 8 x 25ml angle rotor, resuspended in a small volume of PBS by Dounce homogenisation, and dialysed against 500 vol. PBS overnight at 4°C. The purified virus preparation was best stored at 4°C, but glycerol (to 20% (v/v)) was added if the virus was to be stored frozen at -70°C.

METHOD II

Virus was pelleted from clarified allantoic fluid by centrifugation at 25,000 rev/min (50,000 g_{av}) for 2 h at 4°C in a 6 x 100ml angle rotor, and resuspended in 4% of the original volume with TN buffer (10mM tris, 30mM NaCl, pH 7.3 at 32°C) by Dounce homogenisation (2ml or 10ml capacity glass Dounce homogeniser). The resulting suspension was layered onto 5ml 20% (w/v) sucrose cushions containing TN buffer and centrifuged at 30,000 rev/min (59,000 g_{av}) for 45 min at 4°C in an 8 x 25ml angle rotor. The virus pellets were again resuspended in TN buffer, the suspension layered onto a 50ml 15 - 60% (w/v) linear sodium potassium tartrate gradient buffered to pH 7.3 with TN buffer and the virus spun to equilibrium (24,000 rev/min (70,000 g_{av}) for 14 - 18 h at 4°C in a 3 x 65ml swing-out rotor). On occasion, virus was first centrifuged to equilibrium on a 50ml 20 - 65% (w/v) linear sucrose density gradient (24,000 rev/min for 14 - 18 h at 4°C) before the sodium potassium tartrate gradient to further improve purity. The virus band was collected, diluted with ice-cold TN buffer, and virus re-pelleted at 30,000 rev/min (59,000 g_{av}) for 45 min at 4°C in an 8 x 25ml angle rotor. The pellet was resuspended as before in a small volume of TN buffer and dialysed against TN buffer overnight at 4°C. Virus was stored under the same conditions described in Method I.

Virus grown in chorio-allantoic membranes or chick cell monolayer cultures was purified by Method II.

Fractionation of linear density gradients containing virus

Linear sucrose or potassium (sodium) tartrate density gradients were fractionated by piercing the bottom of the centrifuge tubes with a stainless steel hollow needle fitted into an MSE (Measuring and Scientific Equipment Limited, London, England.) tube piercer apparatus, and collecting 10 drop (0.5ml) or 20 drop (1.0ml) fractions. Fractions were assayed for infectivity and haemagglutinating activity as described on page 53, and for radio-activity by suspension in gel scintillation cocktail followed by counting in a Packard liquid scintillation spectrometer, Model 3320.

Preparation of the sub-viral particles produced by the action of Triton N101

Triton N101 in distilled water was added to purified NDV suspensions to a final concentration of 0.08% (w/v) and the preparation incubated at 32°C (or 37°C) for 0.5 - 2.0 h. The sub-viral particles so produced were separated from the detergent by layering 6ml of the preparation onto a 3ml 20% (w/v) sucrose cushion containing TN buffer and pelleting the particles by centrifugation at 30,000 rev/min ($56,000g_{av}$) for 45 min at 4°C in a 10 x 10ml angle rotor. The pellet was resuspended in a small volume of TN buffer by shaking on a vortex mixer (Whirlimixer, Fisons Scientific Apparatus Limited, Loughborough, Leicestershire.). The sub-viral particle suspension was either used immediately or stored at 4°C for a period of up to 2 days before use.

Preparation of NDV nucleocapsids

Initially, the method of Mountcastle et al. (1970) was used for preparing NDV nucleocapsids. However, modifications were made when it was found that the nucleocapsid preparation was inhomogeneous. The following method was adopted.

Nucleocapsids were prepared by adding 0.5ml of 5% (v/v) Triton X-100 and 0.2ml of 10% (w/v) sodium deoxycholate (DOC) to 2ml of concentrated virus suspension (5-10mg virus protein/ml) and incubating at 41°C for 2 h. The nucleocapsids were purified by centrifugation to equilibrium on a 15ml 20 - 65% (w/v) linear sucrose gradient containing D_2O buffered with 10mM tris, 30mM NaCl, pH 7.3 at 32°C (TN buffer) at 25,000 rev/min (78,000 g_{av}) for 14 h at 4°C in a 6 x 15ml swing-out rotor. The particulate band sedimenting to a buoyant density of 1.27g/cc was collected, diluted 3-4 times with TN buffer, and incubated for 30 min at 41°C with DOC at the same concentration as before. Nucleocapsids were then re-banded on a 20 - 65% sucrose-D_2O gradient as described, and after dilution with TN buffer, pelleted by centrifugation at 30,000 rev/min (56,000 g_{av}) for 45 min at 4°C in a 10 x 10ml angle rotor. The pellet was dispersed in a small volume of ice-cold TN buffer by gentle shaking and stored at 4°C prior to use.

Ultraviolet irradiation of NDV

Purified NDV suspensions were diluted to approximately 0.2mg/ml with TN buffer, pH 7.3 at 32°C. 3ml aliquots of the diluted suspension were cooled to 4°C and then ultraviolet irradiated in glass petri dishes (6cm diameter) with continuous stirring at a fixed distance under a Hanovia germicidal lamp. Initially, the lamp emitted 6.1 x 10^3 ergs/cm^2/s, but later this reduced to 2.35 x 10^3 ergs/cm^2/s with aging of the lamp. The method of Hatchard and Parker (1956) was used to calibrate the lamp.

β-propiolactone inactivation of NDV

The method of inactivation was a modification of that described for Sendai virus by Neff and Enders (1968). A 10% (v/v) solution of β-propiolactone (BPL) in distilled water was prepared immediately before use. The 10% solution was then diluted to the required percentage in a saline bicarbonate solution (1.68g of $NaHCO_3$ in 100ml of 0.9% (w/v) NaCl),

to form a series of dilutions from 0.005 - 0.300% (v/v) BPL. The dilute BPL solutions were added in the proportion of 1 part to 9 parts of concentrated NDV. All materials used were pre-cooled in ice water prior to use. The dilutions of BPL were prepared as quickly as possible at 0-4°C. After addition of BPL to the concentrated NDV the preparation was shaken for 10 min at 4°C to mix, and then kept at 37°C for 2 h to allow the reaction to go to completion. (β-propiolactone was rapidly hydrolysed to β-hydroxypropionic acid under these conditions). The preparation was maintained at 4°C overnight.

Extraction of NDV RNA from NDV infected chick embryo cell monolayers

Labelling of virus nucleic acids with [^{3}H]-uridine was based on the method of Cartwright and Burke (1970), and extraction of total cell RNA was based on the method of Bratt and Robinson (1967).

Chick embryo cell monolayer cultures in glass petri dishes (9cm diameter; 6 x 10^7 cells/dish) were infected with NDV (10 p.f.u./cell) for 1 h at 37°C, the cultures washed twice and overlaid with 10ml of maintenance medium. At 4½ h post infection AMD (100μg/ml) was added to a final concentration of 2μg/ml. [^{3}H]-uridine (50μCi/culture) was added at 5 h p.i. and incubation continued for 2 h at 37°C. The medium was then removed, and the cells washed three times with buffer (100mM NaCl, 10mM tris, 1mM EDTA, pH 8.5 at 20°C). This was followed by addition of 2ml of 1% (w/v) sodium dodecyl sulphate (SDS) in buffer, pH 8.5, containing 0.5% (v/v) 2-mercaptoethanol. The cells were removed by swirling the SDS solution over the culture, transferred, using a Pasteur pipette, to 5ml of phenol (redistilled) saturated with buffer, pH 8.5, and shaken vigorously for 10 min on a Griffin flask shaker (Griffin and George Limited, London, England.). The aqueous phase was separated out by centrifugation at 4000g for 10 min in an MSE bench centrifuge and re-extracted with phenol. This process was repeated until the interface between aqueous and phenolic

phases was clear. Then the aqueous phase was removed and residual phenol extracted with ether (3 x 5ml redistilled ether). Excess ether was removed in a stream of nitrogen. Ribonucleic acids were precipitated by addition of 2 vol. of redistilled ethanol and storage at -20°C overnight.

Extraction of RNA from purified NDV

RNA was extracted from purified NDV suspensions by treating with 1% (w/v) SDS in T buffer (100mM NaCl, 10mM Tris, 1mM EDTA, pH 7.2 at 20°C) at room temperature, and then shaking with 5ml buffer saturated phenol as described previously. In later experiments, purified NDV was heated briefly to 50°C with 1% SDS before addition of phenol. The RNAs of purified Semliki Forest virus and purified tobacco mosaic virus were extracted under the conditions described above at room temperature.

Preparation of radioactive chick cell ribosomal RNA

Chick cell ribosomal RNA contains RNA species with known sedimentation coefficients and molecular weights, and was frequently used to calibrate linear sucrose density gradients and analytical RNA polyacrylamide gels. It was prepared radioactive by the addition of 5μCi [^{3}H]-uridine, or 1μCi [^{14}C]-uridine, or 100μCi [^{32}P] (as orthophosphate) to chick cell monolayer cultures (1.5 x 10^{8} cells/petri dish). The cultures were incubated for 24 h at 37°C and radioactive ribosomal RNA was extracted in the same way as NDV RNA as described on page 38.

Preparation of [^{32}P] HeLa cell cytoplasmic and nucleolar RNA

He La cell nucleoli were prepared by a method based on that described by Penman, Vesco and Penman (1968), and nucleolar RNA extracted as described by Penman (1966) and Wagner, Katz and Penman (1967).

HeLa cells were grown to confluence (72 h at 37°C) in 1 litre Flow bottles in Eagle's minimal essential medium containing 7% calf serum and 0.5mCi [^{32}P] (as orthophosphate) per bottle.

The cells were removed from the bottles by brief incubation at 37°C with 10ml of 0.1% trypsin in PBS and then pelleted by centrifugation at 500g for 5 min in an MSE bench centrifuge. Then the cells were washed with ice-cold Earles balanced salt solution, buffered to pH 7.5 with 5% (w/v) sodium bicarbonate, re-pelleted by centrifugation, and resuspended in 2ml of RSB hypotonic buffer (10mM Tris, 10mM NaCl, 1.5mM Mg $Cl_2.6H_2O$, pH 7.4 at 20°C). After allowing the cells to swell for 10 min at 4°C, they were broken by 25-30 strokes in a tight-fitting glass Dounce homogeniser (2ml capacity). The damaged cells and released nuclei were pelleted by centrifugation at $3000g_{av}$ for 5 min at 4°C in an MSE bench centrifuge, the supernatant (CYTOPLASMIC EXTRACT) was removed and kept for extraction of cytoplasmic RNA, and the pellet resuspended in 2ml of RSB buffer. To the resuspended cells and nuclei was added 0.3ml of a mixture comprised of 10% (w/w) sodium deoxycholate (DOC) and 10% (w/w) Tween 80 in the proportions 1 : 2 by volume, and the preparation mixed briefly on a Vortex shaker (Whirlimixer). This treatment solubilised all cell membranes except the nuclear membrane, and the nuclei released were observed to be intact on microscopic examination. Nuclei were pelleted by centrifugation at $3000g_{av}$ for 5 min at 4°C in an MSE bench centrifuge, the supernatant removed and combined with the CYTOPLASMIC EXTRACT. Nuclei were digested by resuspension in 2ml of HS (high-salt) buffer (10mM Tris, 500mM NaCl, 50mM $MgCl_2.6H_2O$, pH 7.4 at 20°C) containing 100µg deoxyribonuclease (DNA'ase) and incubation of the preparation at 30°C for 10 min. After this time the preparation had become markedly less viscous and was layered onto a 20ml 15-30% (w/w) linear sucrose density gradient and centrifuged at 17,000 rev/min ($32,000g_{av}$) for 15 min at 4°C in a 3 x 23ml swing-out rotor. Under these conditions the nucleoli, which remain intact, sediment to the bottom of the centrifuge tube. The nucleolar pellet was immediately resuspended in 2ml of SDS buffer (0.5% (w/v) SDS, 10mM Tris, 100mM NaCl, 1mM EDTA, pH 7.4 at 20°C). The CYTOPLASMIC EXTRACT was made to 0.5% (w/v) SDS. Both cytoplasmic and nucleolar RNA were extracted using the following

hot-phenol method. 2ml of water saturated phenol (re-distilled) was added to the SDS-aqueous RNA solution, shaken and heated to 60°C. The two phases were again shaken and 2ml of chloroform containing 1% (v/v) isoamyl alcohol (an anti-foaming agent) added. After re-heating to 60°C and shaking, the phases were separated by centrifugation at $4000g_{av}$ for 10 min in an MSE bench centrifuge. The phenol-chloroform phase was turbid at this stage, but cleared on heating to 60°C. This lower phase was then removed using a long Pasteur pipette and discarded. A further 2ml of the chloroform/isoamyl alcohol solution was added, and the procedure repeated. Chloroform was used until the interface between aqueous and organic phases contained near transparent protein residues. The aqueous phase was then removed, and RNA precipitated by addition of 2 vol of re-distilled ethanol and storage at -20°C for at least 1 h.

Extraction of virus structural proteins

The structural proteins of NDV and NDV subviral particles were extracted by treatment of purified suspensions with 1% (w/v) SDS, 0.5M urea and 1% (v/v) 2-mercaptoethanol, incubating at 37°C for 3 h, and dialysing overnight at room temperature against 1000 vol of 0.005M Tris + 0.038M-glycine, pH 8.4, containing 0.1% (w/v) SDS and 0.01%(w/v) Cleland's reagent.

Preparation of polyribosomes from (i) chick embryo fibroblasts in monolayer cultures

The isolation of polyribosomes from chick embryo fibroblasts in monolayer cultures was based on the method of Morse *et al.* (1971). All of the glassware and the solutions (except detergents) used in the isolation procedure were autoclaved to destroy any ribonuclease activity. All glassware, centrifuge rotors and solutions were pre-cooled to 0-4°C before use. Rapid cooling of the cells was essential to preserve large polyribosomes because at lower temperatures chain initiation

was more strongly inhibited than chain extension. Maintenance of low temperatures throughout the procedure also minimised activity of ribonuclease, since the detergent lysis of cells used in the method described below was known to release ribonuclease from lysosomes.

Chick embryo cells were grown in monolayer cultures (2.5×10^8 cells/culture) on glass petri dishes (14cm diameter). The medium was removed and the cells washed with 10ml of ice-cold TKM buffer (10mM Tris, 150mM KCl, 10mM $MgCl_2.6H_2O$, pH 7.4 at 20°C). Then a further 10ml of TKM buffer was added, and the cells scraped off the glass dishes with sterile rubber policemen. The cell suspension was removed with a large Pasteur pipette and put into a chilled 25ml universal bottle. The cells were sedimented by centrifugation at $500g_{av}$ for 5 min at 4°C in an MSE bench centrifuge, the supernatant discarded, and the cells resuspended in ice-cold TKM buffer by gently taking up and down in a large Pasteur pipette (1ml TKM buffer per monolayer culture). 5% (v/v) Triton X-100 was added to a final concentration on 0.5% (v/v) and the cells lysed by slow taking up and down (about 20 times) in a large Pasteur pipette. Nuclei, unbroken cells and cell debris were removed by centrifugation at 11,000 rev/min ($7,000g_{av}$) at 4°C for 10 min in an 8 x 25ml angle rotor. The supernatant or cytoplasmic extract (CE) was removed, and 2.0M sucrose in TKM buffer added to a final concentration of 0.25M. Aliquots of CE (6.5ml) were layered onto 10ml discontinuous sucrose gradients comprised of 5ml of 2M sucrose in TKM buffer and 5ml of 0.5M sucrose in TKM buffer, and the gradients centrifuged at 40,000 rev/min ($104,000g_{av}$) for 2 h at 4°C in an 8 x 25ml angle rotor. After centrifugation, the sucrose layers were carefully removed with a Pasteur pipette and the centrifuge tube wiped dry above the polysome pellet. The translucent polysome pellet was washed twice with 5ml ice-cold TKM buffer, and then allowed to swell in a small volume (< 1ml) of TKM buffer for 5-10 min at 4°C. The pellet was finally dispersed by swirling the TKM buffer gently over it. The opalescent polyribosome suspension (5-10 OD_{260} units) was then layered onto a 15ml or 21ml 8 - 45% linear sucrose density gradient containing TKM buffer, and the

polysomes separated by centrifugation in MSE swing-out rotors at 25,000 rev/min ($78,000g_{av}$) for 100 min at 4°C or 30,000 rev/min ($93,000g_{av}$) for 75 min at 4°C for 15ml and 21ml gradients, respectively. The polysome profile was obtained by unloading the gradient by upwards displacement of sucrose through an LKB UVICORD II ultraviolet absorptiometer (L.K.B. Instruments Limited, Croydon, Surrey.) reading at 254nm linked to a SERVOSCRIBE RE 511.20 potentiometric recorder (Smiths Industries Limited, Wembley, Middlesex.). Fractions of the gradient were collected in an LKB ULTRORAC fraction collector.

(ii) Chorio-allantoic membranes

Chorio-allantoic membranes were prepared from 11-day old embryonated eggs as described previously. These were isolated from medium by centrifugation at 10,000 rev/min ($11,000g_{av}$) for 15 min at 4°C in a 6 x 250 ml angle rotor, washed once with ice-cold PBS and once with ice-cold Medium A (50mM Tris, 25mM KCl, 5mM$MgCl_2.6H_2O$, pH 7.6 at 20°C, 20mM 2-mercaptoethanol, 1 drop of baycovin (diethyl pyrocarbonate) per 400ml solution), and then minced finely with sharp scissors. 1ml of Medium A was added per membrane and the preparation homogenised with a Teflon (polytetrafluoroethylene) pestle in a glass tube. The Teflon pestle was tight-fitting (clearance 0.25mm) in the glass tube in order to break the tough membranes. The preparation was given 12 strokes (30 s) at speed setting 8 on a Tri R Stir R (Model S63C) overhead-drive homogeniser (Tri-R Instruments Inc., Rockville Centre, New York.). Then the homogenate was made 0.25M with respect to sucrose by addition of 2.0M sucrose in Medium A, and centrifuged at 11,000 rev/min ($7,000g_{av}$) for 10 min at 4°C in an 8 x 25ml angle rotor to remove mitochondria, nuclei and cell debris. The post mitochondrial supernatant (PMS) was removed from the centrifuge tube with a Pasteur pipette, taking care to avoid the white lipid matter which floated on the top. 10% (w/v) DOC was added to a final concentration of 0.5% (w/v) and the preparation

stirred with a glass rod until it became clear. Aliquots (6.5ml) were layered onto 10ml discontinuous sucrose gradients and centrifuged as described in (i). The procedure was exactly the same as (i) except that the TKM buffer used in polysome re-suspension and the linear sucrose density was 10mM Tris, 10mM KCl and 1.5mM $MgCl_2.6H_2O$, pH 7.6 at 20°C.

Ribonuclease treatment of (i) RNA samples (ii) sucrose gradient fractions containing RNA (iii) chick polyribosomes

(i) RNA samples

The RNA sample was made 0.2M with respect to NaCl and incubated for 1 h at 37°C with pancreatic ribonuclease at 1μg/ml final concentration.

(ii) Sucrose gradient fractions containing RNA

Sucrose gradient fractions containing RNA were treated in the same manner with pancreatic ribonuclease as described in (i) before TCA precipitation as described on page 46.

(iii) Chick polyribosomes

Suspensions of chick polyribosomes in TKM buffer were incubated with 1μg of pancreatic ribonuclease made up in TKM buffer for 15 min at 37°C before linear sucrose density gradient centrifugation.

Purification of acrylamide and N,N^1-methylenebisacrylamide

Acrylamide and N,N^1-methylenebisacrylamide were re-crystallised as described by Loening (1967). 70g acrylamide were dissolved in 1 litre of chloroform at 50°C, the solution filtered hot, and crystals obtained by cooling first to room temperature and then to -20°C. The crystals were collected by filtration and washed with ice-cold chloroform. 10g N,N^1-methylenebisacrylamide were dissolved in 1 litre of acetone at 40-50°C and re-crystallised in a similar way to acrylamide except that ice-cold acetone was used to wash the crystals. Solutions of acrylamide and N,N^1-methylenebisacrylamide were stored in brown bottles at 4°C for up to 1 month.

Scintillation cocktails

1. Toluene - Triton X-100 scintillation cocktail

This consisted of a solution of 0.4% (w/v) 2,5-diphenyloxazole (PPO) and 0.005% (w/v) 1,4-Di-[2-(phenyloxazolyl)]-benzene (POPOP) in toluene containing 33% (v/v) Triton X-100.

2. Gel scintillation cocktail

This was essentially the Medium of Geegeebuoy (quoted by Sargent and Campbell, 1965). It was a mixture of naphthalene (50g), PPO (7g), POPOP (150mg), ethanol (30ml), toluene (A.R.) (200ml), made up to 1 litre with dioxane (A.R.). To the solution 35g of finely divided silica were added and the silica suspended by mixing in a blendor.

ANALYTICAL METHODS

Measurement of NDV specific RNA synthesized in infected chick embryo cell monolayers

Monolayer cultures of chick embryo cells in plastic petri dishes (1.5×10^7 cells/petri) were infected with NDV (10 - 30 p.f.u./cell) for 1 h at 37°C. Excess virus was then removed, the monolayer cultures washed twice with maintenance medium (2 x 2ml) and 2ml of maintenance medium left on the cultures. The infected cultures were incubated at 37°C, and at suitable times after infection host-cell RNA synthesis was inhibited by the addition of AMD (2μg/ml final concentration) for 30 min. [^{3}H]-uridine (5μCi/petri) was added and the cultures incubated 1 or 2 h at 37°C. The cultures were then washed with ice-cold PBS (3 x 3ml), ice-cold 10% TCA (3 x 3ml) and ethanol (2 x 3ml). After drying in air at 37°C, the precipitated cells were dissolved in 0.5ml of 0.5N sodium hydroxide. Aliquots (0.1ml) were taken for protein and radioactivity determinations. Gel scintillation cocktail was used to suspend alkaline samples for radioactive counting.

Sucrose density gradient analysis of NDV RNA

Alcohol precipitated NDV RNA was re-dissolved in a small volume of S buffer (100mM NaCl, 100mM Tris, 1mM EDTA, pH 7.4 at 20°C) and layered onto a 5-25% (w/v) linear sucrose gradient containing S buffer formed in a 15ml polycarbonate tube. Centrifugation was for 14-16 h at 17,500 rev/min ($40,000g_{av}$) at 4°C in a 6 place swing-out rotor. Gradient unloading was achieved by upward displacement of sucrose as described on page 43.

Determination of acid-insoluble radioactivity in fractions containing radioactive RNA

To the whole or part of each fraction of sucrose gradients, an equal volume of ice-cold 10% (w/v) trichloroacetic acid (TCA)

containing 0.04M sodium pyrophosphate was added, followed by 500μg of bovine serum albumin. After precipitation at 4^{o}C for at least 1 h, the precipitates were collected on Whatman GF/C glass fibre discs using a Millipore 3025 sampling manifold (Millipore (U.K) Limited, Wembley, Middlesex.). The discs were washed 3-4 times with 2ml of 10% (w/v) TCA containing 0.04M sodium pyrophosphate, twice with 2ml of ethanol, and twice with 2ml of ether. After drying in air for 15 min the discs were placed in glass or plastic scintillation vials. Toluene - Triton X-100 scintillation cocktail (10ml) was added to each vial and radioactivity determined using a Packard Tri-Carb liquid scintillation spectrometer, Model 3320, as described below.

Determination of radioactivity

Samples were suspended in either toluene-Triton X-100 scintillation cocktail or gel scintillation cocktail. A Packard Tri-Carb liquid scintillation spectrometer, Model 3320, incorporating a Model 543 tape perforator or a Teletype tape perforator (Teletype Corporation, Skokie, Illinois) was used to monitor radioactivity. When both [^{3}H] and [^{14}C] were present the radioactivity due to each isotope was determined using the channels-ratio method of Hendler (1964) and data processed by an ICL computer. [^{3}H] and [^{32}P] were counted simultaneously by sufficient separation of the Packard's gain settings for the two isotopes without correction - there was less than 5% spillage of radioactivity due to [^{32}P] registered in the channel registering radioactivity due to [^{3}H].

Analytical RNA polyacrylamide gel electrophoresis

In the early part of experimental work, RNA was fractionated on 2.2% and 2.5% polyacrylamide gels as described by Peacock and Dingman (1967). (METHOD I). However, it was quickly established that the gel system described by Loening (1967) gave superior resolution of RNA species, and this became the method of choice. (METHOD II).

METHOD I

The buffer system used consisted of 900mM boric acid, 900mM Tris, 25mM EDTA, pH 8.3 at 20°C. This buffer was used undiluted in the preparation of the gels, but was diluted 1 : 9 with glass-distilled water for use in the buffer reservoir of the Shandon disc electrophoresis apparatus (Shandon Scientific Company Limited, Willesdon, London, NW10).

The appropriate volumes of 20% (w/v) acrylamide, 1% (w/v) N,N^1-methylenebisacrylamide, borate buffer and distilled water were mixed, the solution de-aerated, and ammonium persulphate (freshly made up) and NNN^{1}N^1-tetramethylethylene diamine (TEMED) added to 0.07% (w/v) and 0.075% (v/v) final concentrations, respectively. After brief mixing 7.5-8.0cm gels were cast in perspex tubes (0.4cm diameter), carefully overlaid with 0.2ml distilled water and allowed to polymerise for at least 30 min at room temperature. The gels were inserted vertically in the electrophoresis apparatus and pre-electrophoresed for 30 min at 150 V and 2mA per gel at 4°C to remove catalysts. After electrophoresis, the buffer was removed and samples (50µl) of RNA in 100mM sodium acetate buffer, pH 5.0, containing 10-15% (w/v) sucrose layered onto the gels. Electrophoresis buffer was replaced carefully to fill the perspex tubes, the apparatus reassembled, and electrophoresis continued at 150 V and 3mA per gel at 4°C for 1 h for 2.2% polyacrylamide gels and 45 min for 2.5% polyacrylamide gels.

METHOD II

The buffer system used consisted of 180mM sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$), 150mM Tris, 5mM EDTA, pH 7.7-7.8. This buffer was used undiluted in the preparation of the gels, but was diluted 1 : 4 with glass distilled water and made 0.2% (w/v) with respect to SDS for use as electrophoresis buffer.

The appropriate volumes of an acrylamide stock solution (15% (w/v) acrylamide and 0.75% (w/v) NN^1-methylene bisacrylamide), phosphate buffer and distilled water were mixed, the solution de-aerated, and ammonium persulphate (freshly made up) and NNN^1N^1-tetramethylethylenediamine (TEMED) added to 0.07% (w/v) and 0.075% (v/v) final concentrations, respectively. After brief mixing, 8.0cm gels were cast in perspex tubes (0.4cm diameter), carefully overlaid with 0.2ml distilled water and allowed to polymerise for 30 min at room temperature. On occasion, longer gels (13-15cm) were made when a larger molecular weight range of RNA species was to be fractionated. The polymerised gels were stored at 4°C for 1-2 h before insertion into a box-type electrophoresis apparatus specially constructed so that the entire length of the perspex tubes were immersed in electrophoresis buffer. This ensured uniform cooling of the gels. 8cm gels were pre-electrophesed at 60 V and 4mA per gel, and 13-15cm gels at 100 V and 3mA per gel, both for 30 min at room temperature. After electrophoresis, the buffer was removed and samples (50µl) of RNA in 100mM NaCl, 50mM Tris, 1mM EDTA, pH 7.4 at 20°C, containing 10-15% (w/v) sucrose layered onto the gels. Electrophoresis buffer was replaced carefully to fill perspex tubes, the apparatus reassembled, and electrophoresis continued at 60 V and 4mA per gel or 100 V and 3mA per gel for 2.5 h at room temperature.

Fractionation of polyacrylamide gels

On completion of electrophoresis, the gels were gently extruded from the tubes and placed between two fine-toothed nylon combs, bolted to the sides of a piece of perspex (0.6cm thick). The gels were frozen at -20°C for 30 min and sections (~ 2mm thick) were cut with thin stainless-steel razor blades placed at right angles to the length of the combs, using the teeth for guidance. This method was abandoned when a mechanical precision gel slicer (Mickle Laboratory Engineering Company, Gomshall, Surrey.) was purchased. Gels

were then gently extruded from the tubes onto parafilm strips mounted on brass plates and frozen at -20°C for 30 min. The brass plates were fitted onto a specially constructed hollow brass block through which methanol at -20°C was circulated to ensure the gels remained frozen during slicing. 1mm sections were cut from the gel by vertical movement, at right angles to the longitudinal axis, of the slicer's blade.

Determination of the radioactivity present in the polyacrylamide gel sections

Gel sections (1 or 2mm thick) were transferred to scintillation vials and 0.2ml of 100mM NaCl, 10mM Tris, 1mM EDTA, pH 8.5 at 20°C, added to each vial. The vials were capped and stored at 37°C overnight. Then 10ml of gel scintillation cocktail was added per vial and mixed thoroughly on a Vortex mixer (Whirlimixer). After 1 h in the scintillation cocktail, the gel sections had disintegrated and were dispersed by mixing again on a Whirlimixer. The vials were cooled to 4°C and then the radioactivity determined using a Packard Tri-Carb liquid scintillation spectrometer, Model 3320, as described on page 47.

Analytical Protein polyacrylamide gel electrophoresis

The method of protein polyacrylamide gel electrophoresis adopted was that described by Morser, Kennedy and Burke (in preparation). The buffer system used throughout the electrophoresis was 0.05 M-tris + 0.384 M-glycine containing 0.1% SDS and 0.1% (v/v) 2-mercaptoethanol. Polyacrylamide gels were cast to a height of 9cm in perspex tubes (0.6cm diameter) and contained 10% (w/v) acrylamide, 0.266% (w/v) NN^1-methylenebisacrylamide, 0.1% (w/v) SDS, 0.5M urea, 0.376 M tris, 0.06N HCl, 0.058% (v/v) NNN^1N^1-tetramethylethylenediamine and 0.07% (w/v) ammonium persulphate (freshly

made up). The gel solution in each tube was overlaid with a small volume of water to ensure a flat top surface as polymerisation took place. After 30 min at room temperature, the gels were fully polymerised and were inserted vertically into a box-type electrophoresis kit. 100µl of 0.1M reduced glutathione were layered onto each gel, and the gels pre-electrophoresed for 1.5 h at constant voltage (100 V). The final current was approximately 2.5mA/gel. Protein samples were made 5% (w/v) with respect to sucrose and 0.01% (w/v) with respect to bromophenol blue, and layered onto the tops of the gels. Electrophoresis was for 5 h at 100 V (2.5mA/gel).

Staining of proteins on SDS-polyacrylamide gels with Coomassie blue

After electrophoresis, the 10% polyacrylamide gels were withdrawn from the perspex tubes and immersed in 12.5% (w/v) TCA overnight at 4°C to fix the protein bands. Gels were stained with Coomassie blue, as described by Maizel et al. (1970). A 0.1% (w/v) solution of Coomassie blue containing 50% (v/v) methanol and 7.5% (v/v) glacial acetic acid was freshly made up, and gels immersed in it for 4 h at room temperature. The gels were destained with several changes of 5% (v/v) methanol in 7.5% (v/v) glacial acetic acid and scanned at 570nm using in a Chromoscan densitometer (Joyce, Loebl and Company Limited, Gateshead, Durham.).

Staining of glycoproteins on SDS-polyacrylamide gels

After electrophoresis, the 10% polyacrylamide gels were withdrawn from the perspex tubes and washed by stirring in 40% (v/v) methanol containg 7% (v/v) glacial acetic acid (250ml/gel) overnight. This procedure removed SDS from the gels and was reported to be essential by Kobylka et al. (1972)

for the periodic acid - Schiff reagent (P.A.S.) staining technique for glycoproteins described by them and carried out as follows:- the gels were further washed with 7% (v/v) glacial acetic acid for 1 hour before immersing in 1% (w/v) periodic acid - 7% (v/v) acetic acid (freshly made up) for 1 h in the dark. Excess periodic acid was removed by extensive washing with 7% (v/v) acetic acid - at least 3 changes of 500ml acetic acid at hourly intervals per gel. Then the acetic acid was rinsed off with distilled water, and the gels immersed in Schiff's reagent (0.5% Fuchsin-sulphite) for 1 h in the dark. By the end of this time glycoproteins were faintly stained with magenta colour and the rest of the gel was colourless. The gels were washed at 10 minute intervals 3-4 times with 1% (w/v) sodium metabisulphite in 0.1 NHCl, and as the magenta bands fade quickly, they were scanned immediately using a Gilford 2000 densitometer at 550nm.

BIOLOGICAL, BIOCHEMICAL, AND BIOPHYSICAL ASSAYS

Assay of infectious virus

The infectious titre of NDV was estimated by plaque assay on monolayer cultures of chick embryo cells (Dulbecco, 1952). 0.5ml aliquots of 10-fold serial dilutions of the virus in maintenance medium were added to monolayer cultures (1.5×10^7 cells/culture) on plastic petri dishes. After adsorption at 37°C for 60 min, the virus inoculum was replaced by 5ml of overlay medium, consisting of growth medium containing 0.9% NOBLE agar. The cultures were incubated at 37°C and plaques were counted after 36-48 h. To facilitate plaque counting, the cells were stained by the addition of 3ml of 0.01% (w/v) of neutral red in Earle's solution to the plates for 1 h at 37°C.

Assay of haemagglutinating activity

Titrations were carried out on lucite plates as described by Lindenmann, Burke and Isaacs (1957). One-day old chicken erythrocytes were obtained from Tissue Culture Services Limited, Slough, Buckinghamshire, and diluted to 1% (v/v) with 0.9% (w/v) saline before use. Titre of haemagglutinating activity was defined as the reciprocal of that dilution giving partial agglutination.

Interferon production and assay

Interferon was produced by infection of monolayer cultures of chick cells (1.5×10^7 cells/plastic petri dish) with 0.5ml of virus (10-50 p.f.u./cell) for 1 h at 37°C. After washing, 2ml of maintenance medium was added and cultures were incubated at 37°C for 21 h before collecting the extra-cellular fluid. Residual virus was inactivated by heating at 65°C for 1 h, and samples stored at

-20°C until assayed. Chick interferon was assayed by a plaque reduction method using Semliki Forest virus to challenge. All chick interferon titres were expressed in international units by using the standard chick interferon preparation obtained from the National Institute for Medical Research, Mill Hill, London.

NDV RNA-dependent RNA polymerase assay

The assay method used was a slight modification of that described by Huang, Baltimore and Bratt (1971). All polymerase assays were carried out in thin walled glass vials (vol. 2ml) and the standard reaction mixture contained per 230µl : 10µmoles of tris, pH 7.3 at 32°C; 0.8µmole of magnesium acetate; 0.6µmole of sodium dithiothreitol (Cleland's reagent); 20µmoles of NaCl; 0.14µmole each of ATP, UTP and CTP; 0.002µmole of [^{3}H]-GTP (1,340 c.p.m. per pmole); 160µg of Triton N-101; and 50-200µg of viral protein in TN buffer (10mM tris, 30mM NaCl, pH 7.3 at 32°C). In particular experiments the constituents were varied as indicated. The reaction mixture was incubated at 32°C for 2 h, and terminated by cooling to 4°C in ice, adding 0.4ml of 0.08M sodium pyrophosphate, 200-500µg bovine serum albumin and 0.6ml of 25% (w/v) trichloroacetic acid (TCA). After precipitation for 1 h at 4°C, the vial contents were transferred to 15ml polypropylene centrifuge tubes. About 8ml of 10% (w/v) TCA containing 0.04M sodium pyrophosphate was added and acid-precipitable material spun down by centrifugation at 6,000 rev/min (5,000g_{av}) for 15 min at 4°C in a 16 x 15ml rotor. The supernatant was discarded and the pellet washed three times with 2ml of TNE buffer (50mM tris, 146mM NaCl, 1mM EDTA, pH 7.6 at 20°C) plus 8ml of 10% TCA by centrifugation. Finally, the pellet was resuspended in 0.2ml of TNE buffer, and 10ml of toluene - Triton X-100 scintillation fluid was added. After thorough mixing, each sample was assayed for acid precipitable [^{3}H]-GMP using a Packard liquid scintillation spectrometer, series 3320. An incubated sample

without virions contained less than 50 c.p.m. using the above procedure.

Protein determination

Virus protein was determined by a modification of the method of Lowry et al. (1951), described by Oyama and Eagle (1956) with bovine serum albumin (SA) as standard.

RNA determination

RNA was determined by the equivalence 1mg RNA/ml = 20 OD_{260} units established using yeast RNA.

Density determination

For sucrose solutions, density was deduced from the refractive index measured in an Abbe 60 refractometer (Bellingham and Stanley Limited, S. Tottenham, London.). Densities of all other solutions were estimated by gravimetric means.

Electron microscopy

Purified virus and sub-viral particles produced by treatment of NDV with Triton N101 were diluted with distilled water, and a small amount of each mixed with an equal volume of 3% phosphotungstic acid adjusted to pH 6.0. A drop of the mixture was then placed on a 400 mesh carbon-formwar-coated grid and the excess fluid withdrawn. After drying, the grid was examined immediately in a Phillips EM 300 at a plate magnification of 60,000 (Hoyle and Almeida, 1971).

RESULTS

RESULTS

Purification of NDV

Using METHOD I (page 34), the infectivity : HA ratio of NDV after Arcton 113 treatment was about 40% of that of the infected allantoic fluid. After potassium tartrate density centrifugation, the infectivity : HA ratio fell to about 10% of its original value. Using METHOD II (page 35), the infectivity : HA ratio of NDV after purification was 20 - 40% of the original infectivity : HA ratio of the allantoic fluid.

Figure 3 shows the result obtained when purified [^{32}P]-labelled NDV prepared by METHOD II was centrifuged to equilibrium on a 50ml 15 - 60% (w/v) sodium potassium tartrate density gradient. One single, white band which had a buoyant density of approximately 1.20g/cc, and contained the majority of infectivity, haemagglutinin and [^{32}P]-radioactivity, was apparent. Examination of purified NDV (METHOD II) by electron microscopy showed it to be comprised of a homogeneous population of roughly spherical particles covered with small projections (Figure 1). No other material, e.g. cell debris, was observed in such preparations. Purified NDV (METHOD II) had infectivity of approximately 5 x 10^{10} p.f.u./mg virus protein, which is in agreement with the values obtained by Huang et al. (1971).

The ribonucleic acid of NDV

(i) Analysis by sucrose density gradient centrifugation

Before investigation of ND vsi-RNA synthesis in infected cells by the use of sucrose density gradient centrifugation and polyacrylamide gel electrophoresis, it was necessary to characterise NDV RNA. The radioactive sedimentation profile of [^{32}P]-NDV RNA, extracted from [^{32}P]-labelled NDV (strain Texas), in a 5 - 25% (w/v) linear sucrose density gradient is shown in Figure 4. [^{32}P]-NDV RNA sedimented as a single high molecular weight RNA species. A similar result was obtained

Figure 3. Centrifugation of [^{32}P]-labelled NDV in a 20-65% (w/v) sucrose density gradient buffered to pH 7.3 with TN buffer.
Radioactivity o——o, infectivity o——o,
haemagglutinin 

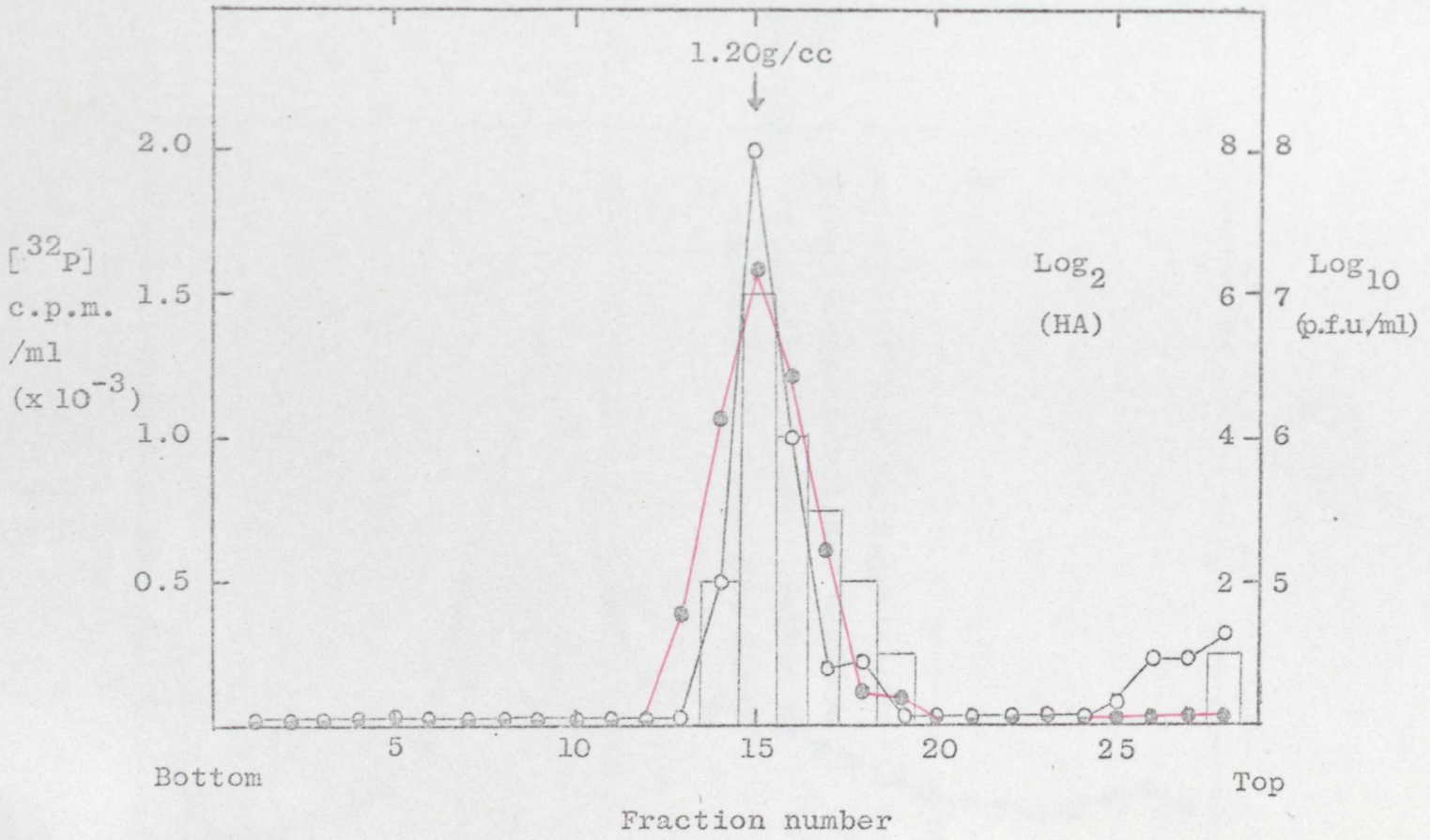
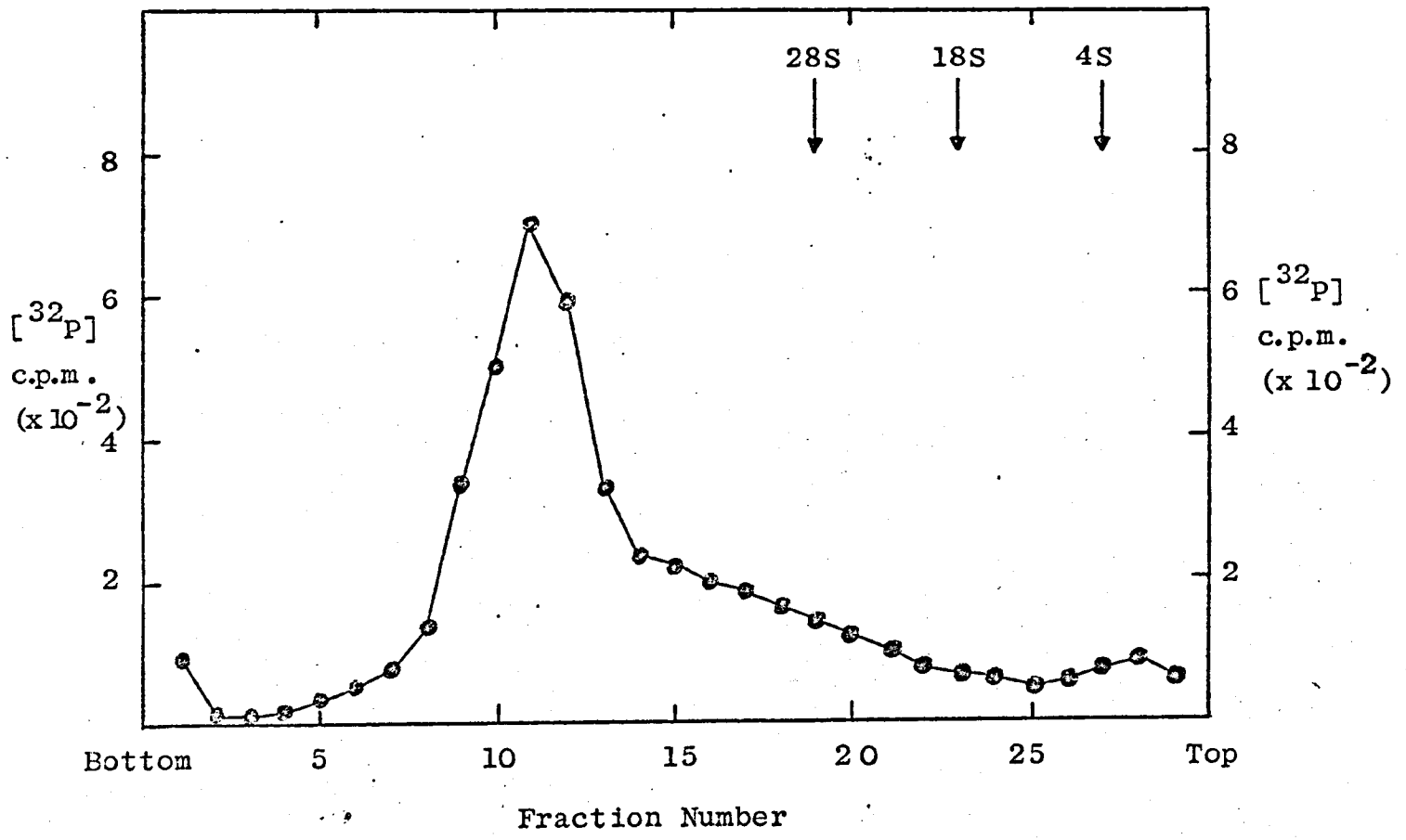

1.20g/cc
2.0
1.5
1.0
0.5
[32P]
c.p.m.
/ml
(x 10-3)
8
6
4
2
Log2
(HA)
8
7
6
5
Log10
(p.f.u./ml)
5
10
15
20
25
Bottom
Top
Fraction number

Figure 4. Centrifugation of [^{32}P]-NDV RNA in a 5 - 25% (w/v) sucrose density gradient buffered to pH 7.4 with S buffer. The arrows show the position of the chick ribosomal RNA markers.

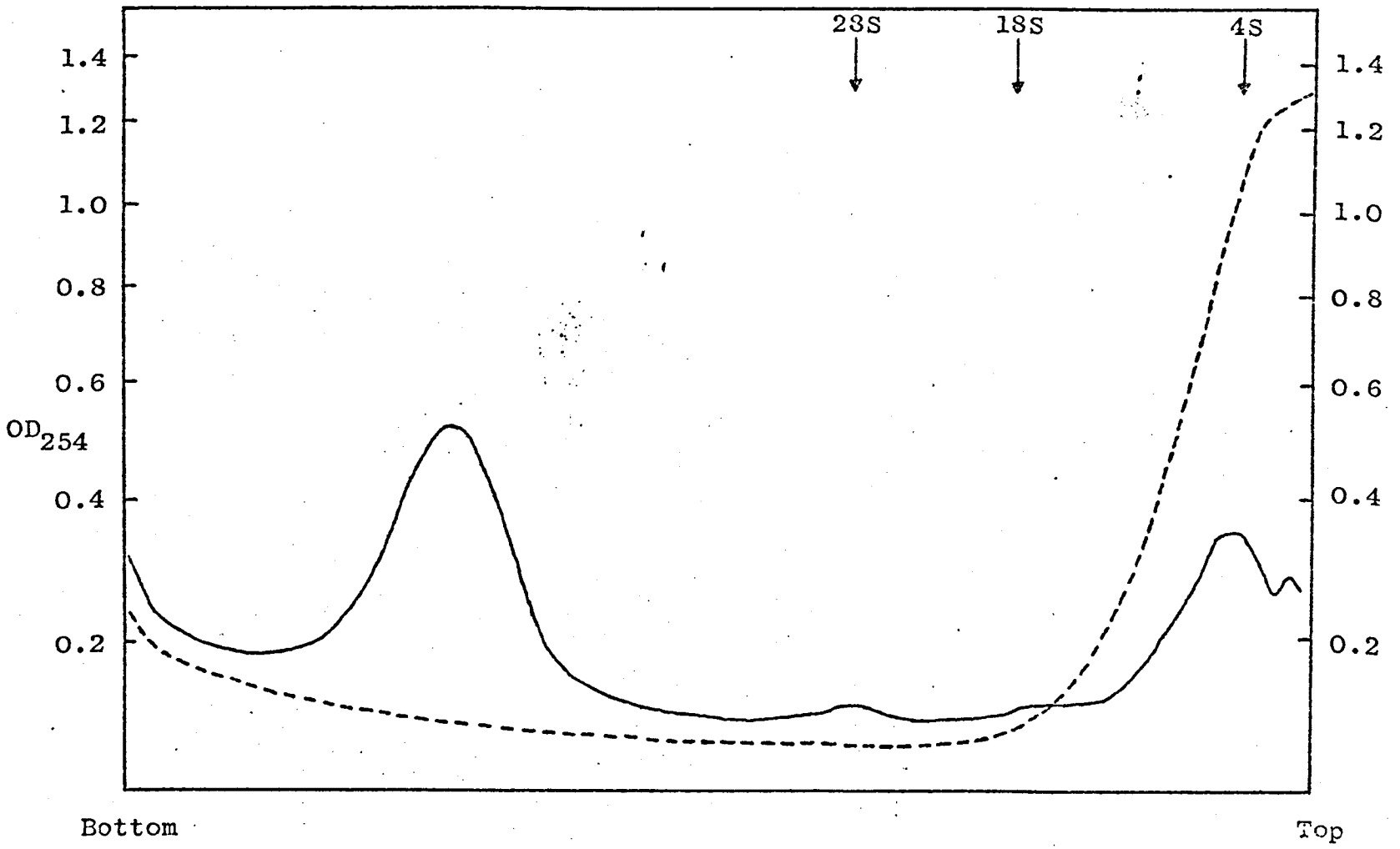

28S
18S
4S
8
6
4
2
[32P]
c.p.m.
(x 10-2)
Bottom
5
10
15
20
25
Top
Fraction Number

when unlabelled NDV RNA was sedimented through a sucrose density gradient (Figure 5). Digestion of NDV RNA with ribonuclease before sucrose density centrifugation degraded it to low molecular weight polynucleotides. These sedimented very slowly, and were found at the top of the sucrose gradient (Figure 5). The sedimentation coefficient (S_{20},W) of NDV RNA, as calculated with reference to the 28S and 18S chick ribosomal markers (Martin and Ames, 1961) was approximately 50S. This corresponded to a molecular weight of 5.7×10^6 using the Spirin formula (Spirin, 1963). The low molecular weight RNA found in NDV RNA preparations by Duesberg and Robinson (1965), which was probably a degradation product and/or cellular RNA, was present in variable amount. This RNA sedimented at 4S on sucrose density gradients, and was always present in smaller amount than 50S NDV RNA (Figure 5). In most preparations of [^{32}P]-NDV RNA, 4S RNA was present only to the extent of 10% of 50S RNA (Figure 4).

(ii) Analysis by polyacrylamide gel electrophoresis

Since NDV RNA was of high molecular weight, low percentage polyacrylamide gels were used to ensure its entry into the gels on electrophoresis. 2.2% polyacrylamide gels were found to be ideal for this purpose, since NDV RNA migrated up to 2.0 cm into these gels. NDV RNA did not enter 2.5% polyacrylamide gels under the conditions used, but 2.5% gels were useful for examination of lower molecular weight material. Electrophoresis of [^{32}P]-NDV RNA extracted from the Texas (Figure 6a) and F (Figure 6b) strains of NDV gave similar radioactive electrophoretograms; a single peak of radioactivity due to high molecular weight RNA was obtained at fraction 8 in both cases (Figure 6a,b). The radioactive peaks were much sharper than those observed on linear sucrose density gradients (compare Figure 6 with Figure 4) indicating the superior resolving power of polyacrylamide gel electrophoresis. Extraction of [^{32}P]-NDV RNA and

Figure 5. Centrifugation of unlabelled NDV RNA in a 5 - 25% (w/v) sucrose gradient buffered to pH 7.4 with S buffer. Aliquots of NDV RNA (~75µg) in S buffer were either (a) untreated, ———, or (b) treated with ribonuclease - - - - - -, and centrifuged in parallel sucrose gradients. The arrows show the approximate position of chick ribosomal RNA markers estimated from another sucrose gradient run in parallel.


23S
18S
4S
1.4
1.2
1.0
0.8
0.6
OD254
0.4
0.2
Bottom
Top

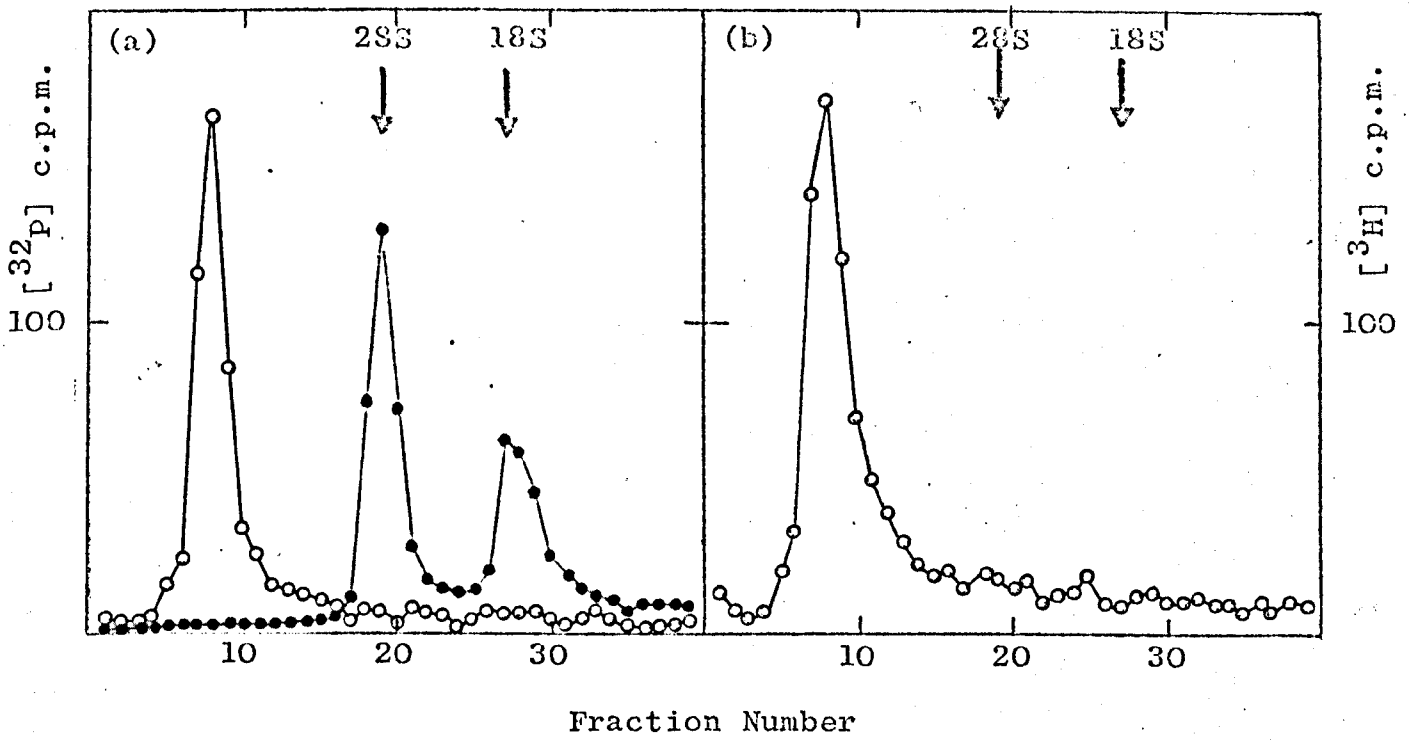


Figure 6. Polyacrylamide gel electrophoresis on 2.2% gels of the RNA (o——o) extracted from (a) the TEXAS and (b) the F strain of NDV. Electrophoresis was at 150V and 2mA per gel for 1 h (Peacock and Dingman, 1967). The arrows indicate the position the ribosomal markers (●——● in a).

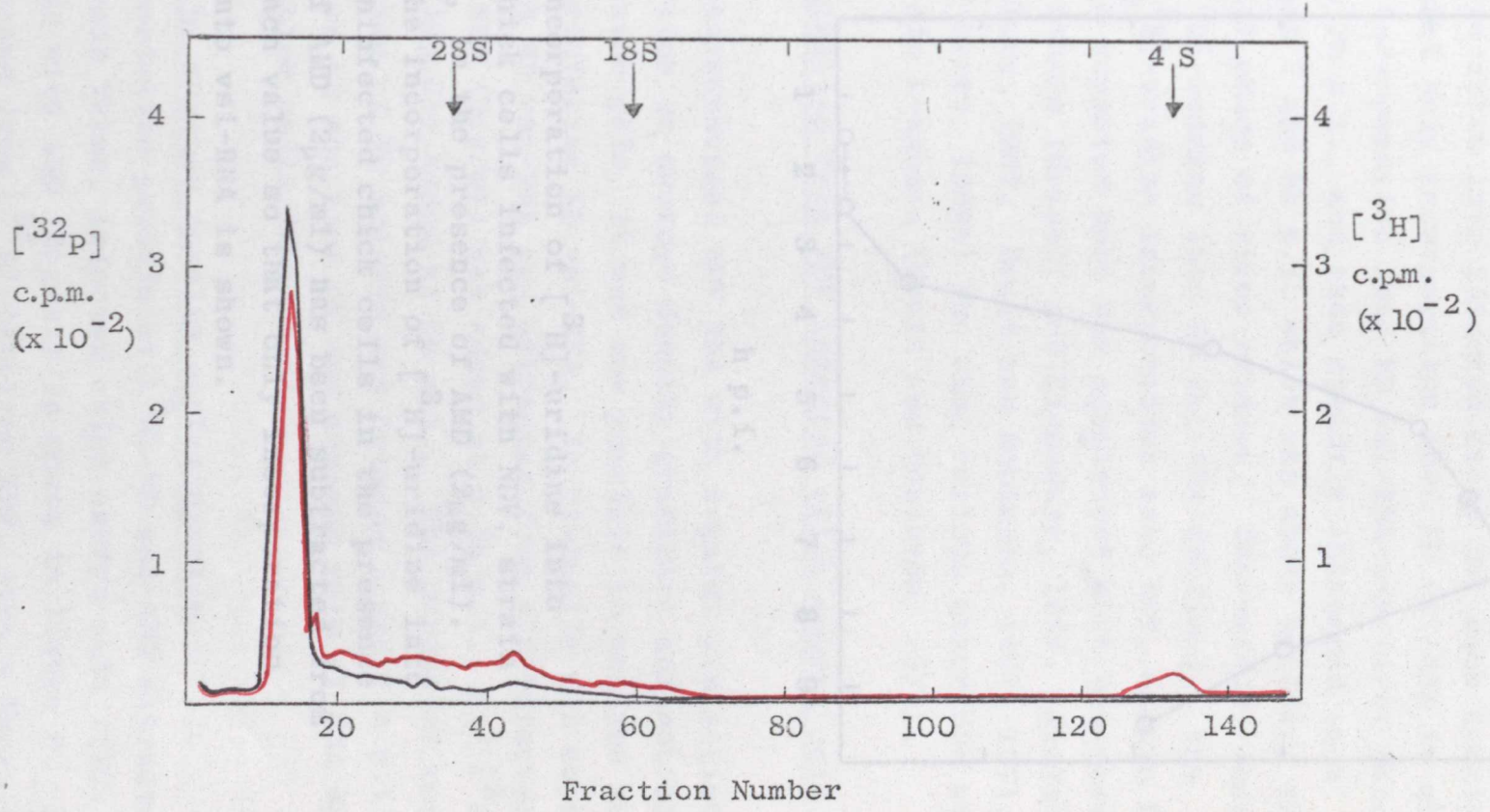
[^{3}H]-NDV RNA from NDV, strain Texas, grown in embryonated eggs and chorio-allantoic membranes respectively, followed by fractionation on a long (15 cm) 2.2% polyacrylamide gel (Figure 7) demonstrated the presence of the high molecular weight RNA species (fraction 13) and also the 4S RNA component of NDV RNA (fraction 133). The latter was present to the extent of less than 10% of the high molecular weight RNA species (Figure 7).

The rate of [^{3}H]-uridine incorporation into NDV infected chick embryo cells in the presence of actinomycin D

To detect ND vsi-RNA synthesis in NDV infected chick embryo cells, it was necessary to completely inhibit cell RNA synthesis with actinomycin D (AMD). This was because cell RNA synthesis decreases only very gradually after infection by NDV (Granoff and Kingsbury, 1964; Moore et al., 1972; Wilson, 1968). A concentration of 2μg AMD/ml in the medium overlaying the chick cells was chosen as this almost completely inhibited cell RNA synthesis, and the cytoxicity of the drug at this level was sufficiently low to allow chick cells to survive up to 8 hours (Bratt and Robinson, 1967). This concentration of AMD (2μg/ml) has also been shown to only moderately decrease the production of infectious progeny virus (Bratt and Robinson, 1967). In the experiments to be described cells were only exposed to AMD for 1.5 - 3.0 hours.

After an initial lag of 0 - 4 hours NDV strains have been shown to grow exponentially in chick embryo cells for up to 16 hours after infection (Lomniczi et al., 1971). The kinetics of growth for all strains examined were similar, although the yield of virus released/cell varied among the strains (Lomniczi et al., 1971). The kinetics of [^{3}H]-uridine incorporation in chick cells infected with NDV, strain L, in the presence of AMD (2μg/ml) is shown in Fig.8.

Figure 7. Co-electrophoresis on a 15cm 2.2% polyacrylamide gel of the RNA extracted from (a) the TEXAS strain of NDV grown in embryonated eggs, [^{32}P] ———— , (b) the TEXAS strain of NDV grown in chorioallantoic membranes, [^{3}H] ———— . Electrophoresis was at 100V and 3mA per gel for 2.5 h (Loening, 1967). The arrows show the approximate position of ribosomal RNA markers estimated from a 2.2% gel run in parallel.


28S
18S
4 S
[32P]
c.p.m.
(x 10-2)
[3H]
c.p.m.
(x 10-2)
1
2
3
4
20
40
60
80
100
120
140
Fraction Number

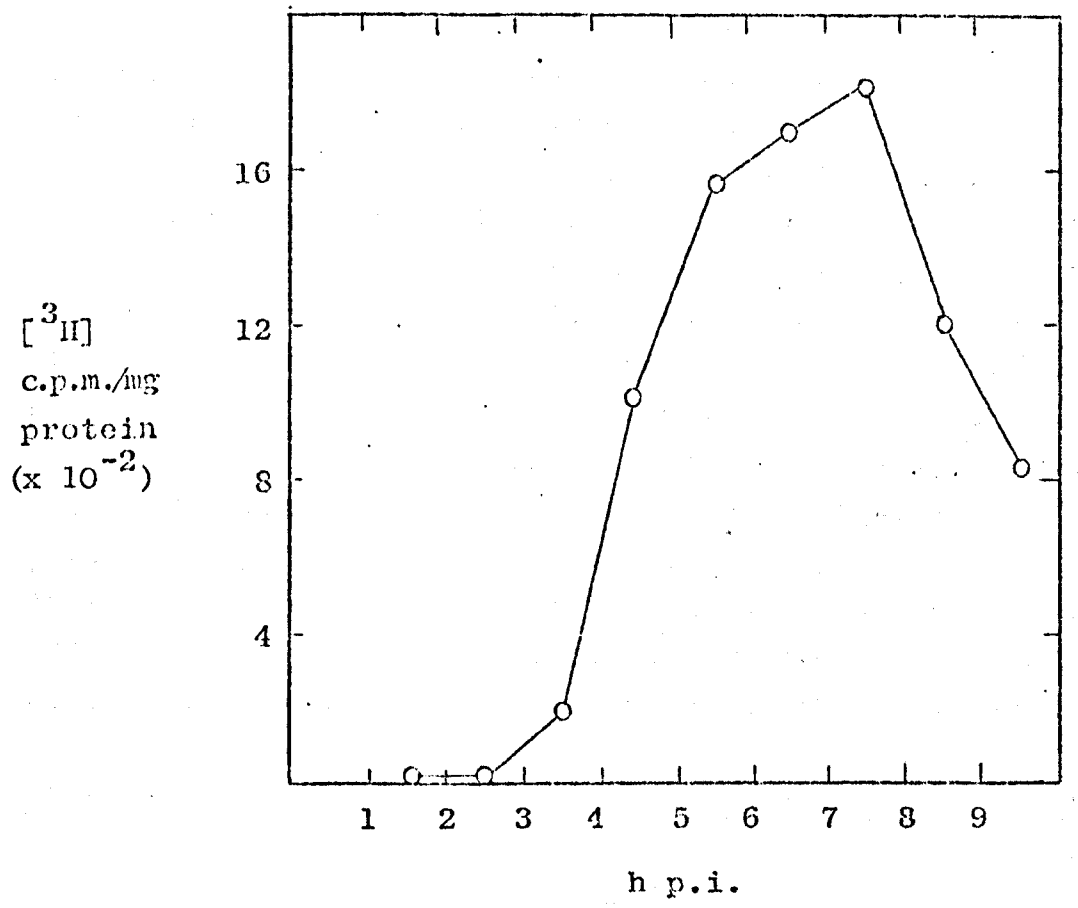


Figure 8. Incorporation of [^{3}H]-uridine into chick cells infected with NDV, strain L, in the presence of AMD (2μg/ml). The incorporation of [^{3}H]-uridine into uninfected chick cells in the presence of AMD (2μg/ml) has been subtracted from each value so that only incorporation into vsi-RNA is shown.

The incorporation of [^{3}H]-uridine into uninfected chick cells under the same conditions has been subtracted from [^{3}H]-uridine incorporation into infected cells for each hourly interval so that only incorporation into ND vsi-RNA is shown. [^{3}H]-uridine incorporation into ND vsi-RNA was first detectable at about 3h p.i., and then steadily increased to a maximum between 7 and 8h p.i. which was about half-way through the exponential phase of virus release. Thereafter, incorporation of [^{3}H]-uridine into ND vsi-RNA declined. The kinetics of [^{3}H]-uridine incorporation into NDV, strain L, infected cells reported here was consistent with the results published by others (Granoff and Kingsbury, 1964; Kingsbury, 1966b; Kingsbury, 1967; Bratt and Robinson, 1967, 1971; Wilson, 1968; Bratt, 1969b) who used various other NDV strains, e.g. NDV, Strain L-Kansas (Bratt and Robinson, 1971).

ND vsi-RNA extracted from NDV infected chick embryo cell monolayers

Having characterised NDV RNA with regards its sedimentation properties on sucrose density gradients and mobility in polyacrylamide gels, it was now possible to analyse and similarly characterise ND vsi-RNA. In virtually all experiments in which ND vsi-RNA was extracted from NDV infected chick cells, AMD (2μg/ml) was added at 4.5h p.i., and [^{3}H]-uridine (50 - 100μCi) added at 5h p.i.. The period of radioactive labelling of ND vsi-RNA was between 5 and 7 h p.i., which was close to the time of the maximum rate of RNA synthesis in infected chick cells (Figure 8).

(i) Analysis by sucrose density centrifugation

The sedimentation profile of [^{3}H]-ND vsi-RNA extracted from NDV, strain Texas, infected chick embryo cell (CEC) monolayers treated with AMD (2μg/ml) is shown in Figure 9. [^{32}P]-NDV RNA extracted from [^{32}P]-labelled NDV, strain Texas, and [^{32}P]-chick cell ribosomal RNA were added to serve as RNA species of known sedimentation coefficients. The [^{3}H]-ND vsi-RNA sedimentation profile (Figure 9) was similar to those

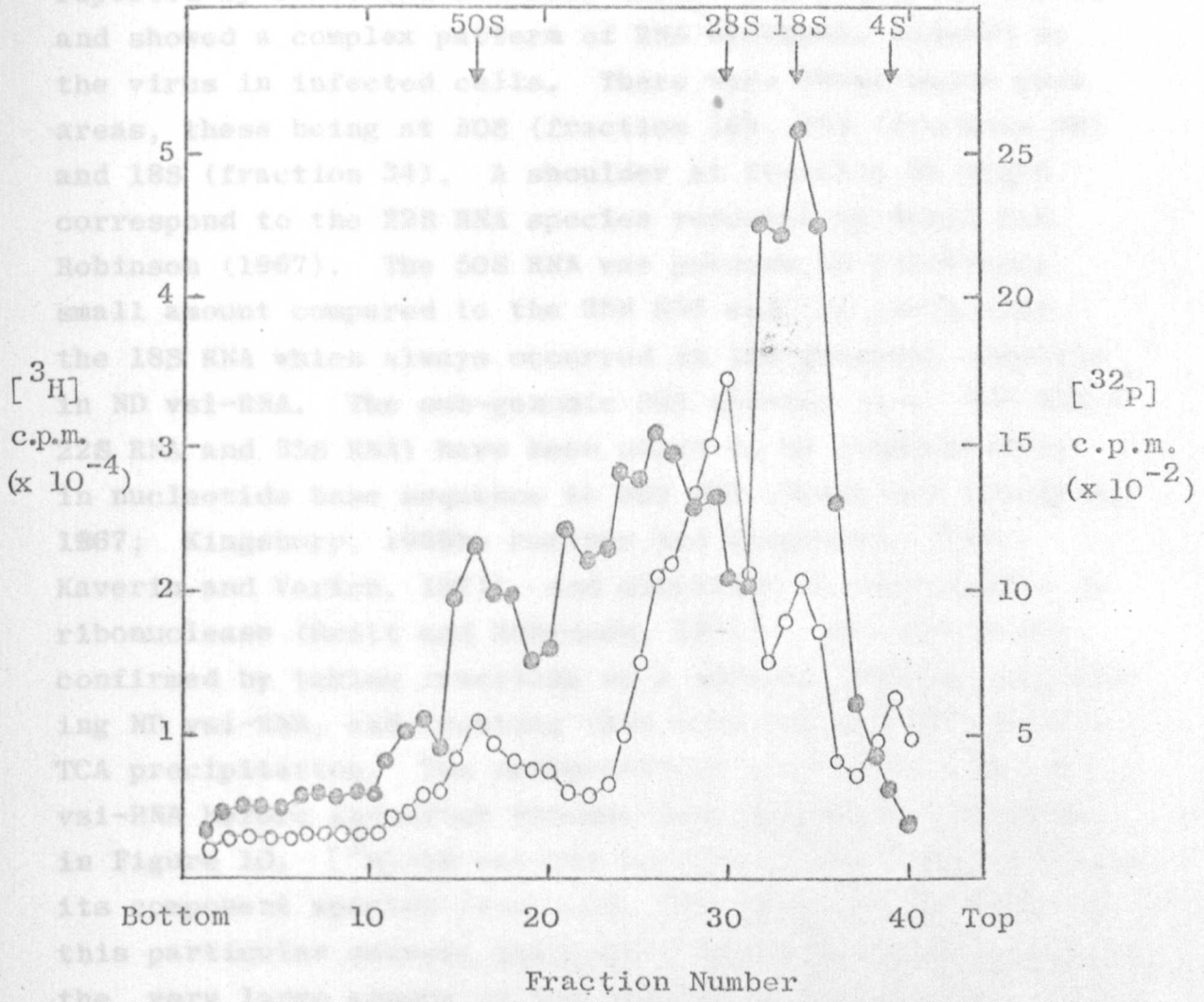


Figure 9. Co-sedimentation in a 5 - 25% (w/v) sucrose density gradient buffered to pH 7.4 with S buffer of [^{3}H]-ND (strain TEXAS) vsi-RNA (●——●), [^{32}P]-NDV (strain TEXAS) RNA and [^{32}P]-chick ribosomal RNA (o——o). The arrows show the position of NDV RNA (at 50S) and ribosomal RNA (at 28S, 18S and 4S).

reported by Bratt and Robinson (1967) and Bratt (1969a,b), and showed a complex pattern of RNA synthesis induced by the virus in infected cells. There were three major peak areas, these being at 50S (fraction 16), 35S (fraction 26) and 18S (fraction 34). A shoulder at fraction 32 might correspond to the 22S RNA species reported by Bratt and Robinson (1967). The 50S RNA was present in relatively small amount compared to the 35S RNA, and, in particular, the 18S RNA which always occurred in the greatest quantity in ND vsi-RNA. The sub-genomic RNA species (i.e. 18S RNA, 22S RNA and 35S RNA) have been shown to be complementary in nucleotide base sequence to NDV RNA (Bratt and Robinson, 1967; Kingsbury, 1966b; Portner and Kingsbury, 1970; Kaverin and Varich, 1971), and sensitive to degradation by ribonuclease (Bratt and Robinson, 1971). The latter was confirmed by taking fractions of a sucrose gradient containing ND vsi-RNA, and treating them with ribonuclease before TCA precipitation. The sedimentation profilesof [^{3}H]-ND vsi-RNA before and after ribonuclease digestion are shown in Figure 10. [^{3}H]-ND vsi-RNA was not as well resolved into its component species (i.e. 50S, 35S, 22S and 18S RNAs) on this particular sucrose gradient. This was probably due to the very large amount of the 18S RNA species in this sample of [^{3}H]-ND vsi-RNA. However, all the RNA contained in fractions taken from the sucrose gradient was apparently completely sensitive to degradation by ribonuclease (Figure 10).

(ii) Analysis by polyacrylamide gel electrophoresis

The poor resolution of ND vsi-RNA into its component RNA species by sucrose density gradient centrifugation (e.g. Figure 10) emphasised the need for a technique with superior resolving power for RNA molecules. Analytical RNA polyacrylamide gel electrophoresis developed by Peacock and Dingman (1967) and Loening (1967) has considerably improved separation of high molecular weight RNAs in complex mixtures of RNA molecules. Therefore, systems for analytical RNA polyacrylamide

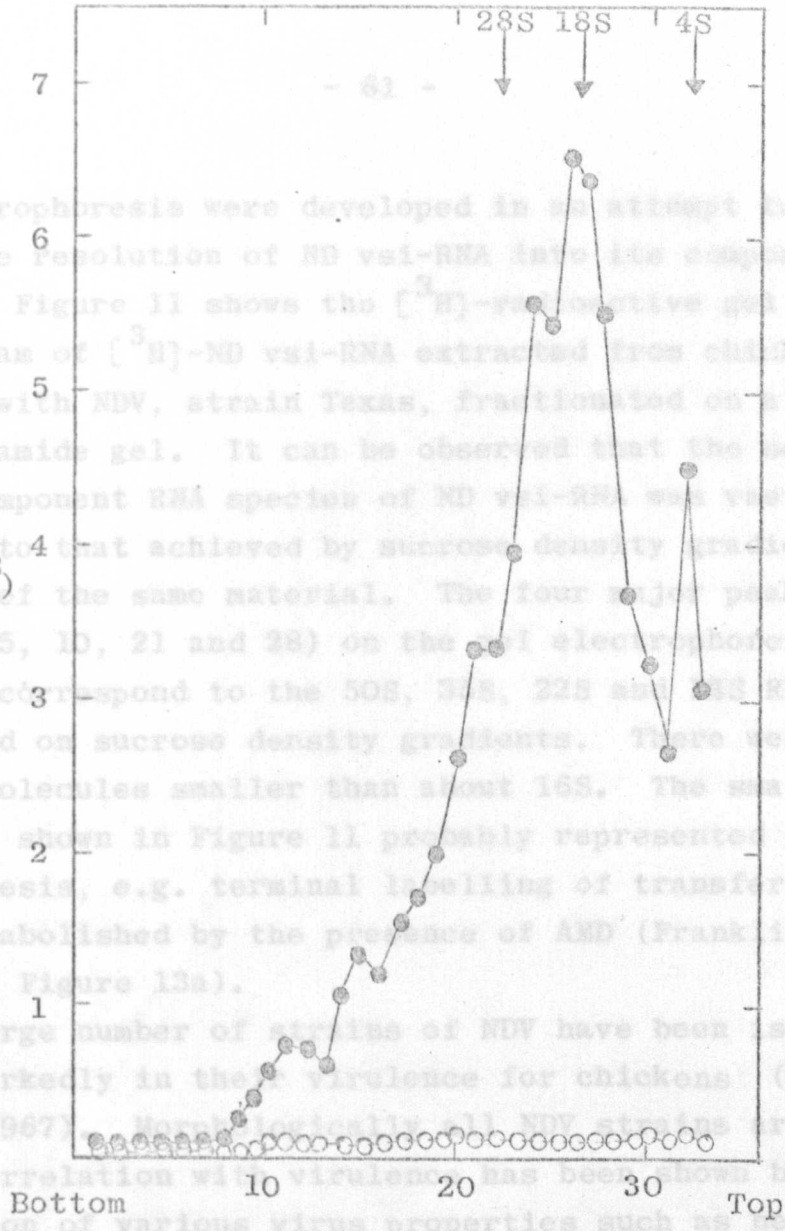


Figure 10. The sensitivity to degradation by ribonuclease of [^{3}H]-ND (strain TEXAS) vsi-RNA. Equal amounts of [^{3}H]-ND vsi-RNA were centrifuged in 5 - 25% (w/v) sucrose gradients containing S buffer, and the fractions collected from the gradients either (a) treated immediately with TCA ●——●, or (b) made 0.2M with respect to NaCl and incubated for 1 h at 37°C with pancreatic ribonuclease (1µg/ml) before TCA precipitation o——o. The arrows show the position of the chick ribosomal RNA markers.

gel electrophoresis were developed in an attempt to better effect the resolution of ND vsi-RNA into its component RNA species. Figure 11 shows the [^{3}H]-radioactive gel electrophoretogram of [^{3}H]-ND vsi-RNA extracted from chick cells infected with NDV, strain Texas, fractionated on a 15cm 2.2% polyacrylamide gel. It can be observed that the separation of the component RNA species of ND vsi-RNA was vastly improved compared to that achieved by sucrose density gradient centrifugation of the same material. The four major peaks (at fraction 5, 10, 21 and 28) on the gel electrophoretogram probably correspond to the 50S, 35S, 22S and 18S RNA species identified on sucrose density gradients. There were no ND vsi-RNA molecules smaller than about 16S. The small amount of 4S RNA shown in Figure 11 probably represented cellular RNA synthesis, e.g. terminal labelling of transfer RNA, not entirely abolished by the presence of AMD (Franklin, 1963) (see also Figure 13a).

A large number of strains of NDV have been isolated which differ markedly in their virulence for chickens (Waterson et al., 1967). Morphologically all NDV strains are similar, and no correlation with virulence has been shown by the examination of various virus properties such as heat stability, serology, inhibition of protein and RNA synthesis in infected cells, etc., (Kohn and Fuchs, 1969; Reeve et al., 1970; Reeve and Waterson, 1970; Moore et al., 1972). However, the capacity to produce plaques and syncytia (polykaryocytes) in chick embryo cells was found to be related to virus virulence (Schloer and Hanson, 1968; Kohn and Fuchs, 1969; Reeve and Alexander, 1970b; Reeve and Poste, 1971). It has also been suggested that the large amounts of 18S RNA in ND vsi-RNA might be related to virulence (Blair and Robinson, 1968; Bratt, 1969a). Therefore, an investigation into the production of vsi-RNA by different NDV strains was carried out. Six strains of NDV were used: two were velogenic (Herts 33, Texas); two were mesogenic (H and L); and two were lentogenic (F and LaSota). The origin, growth, assay and biological properties of these NDV strains has been previously described

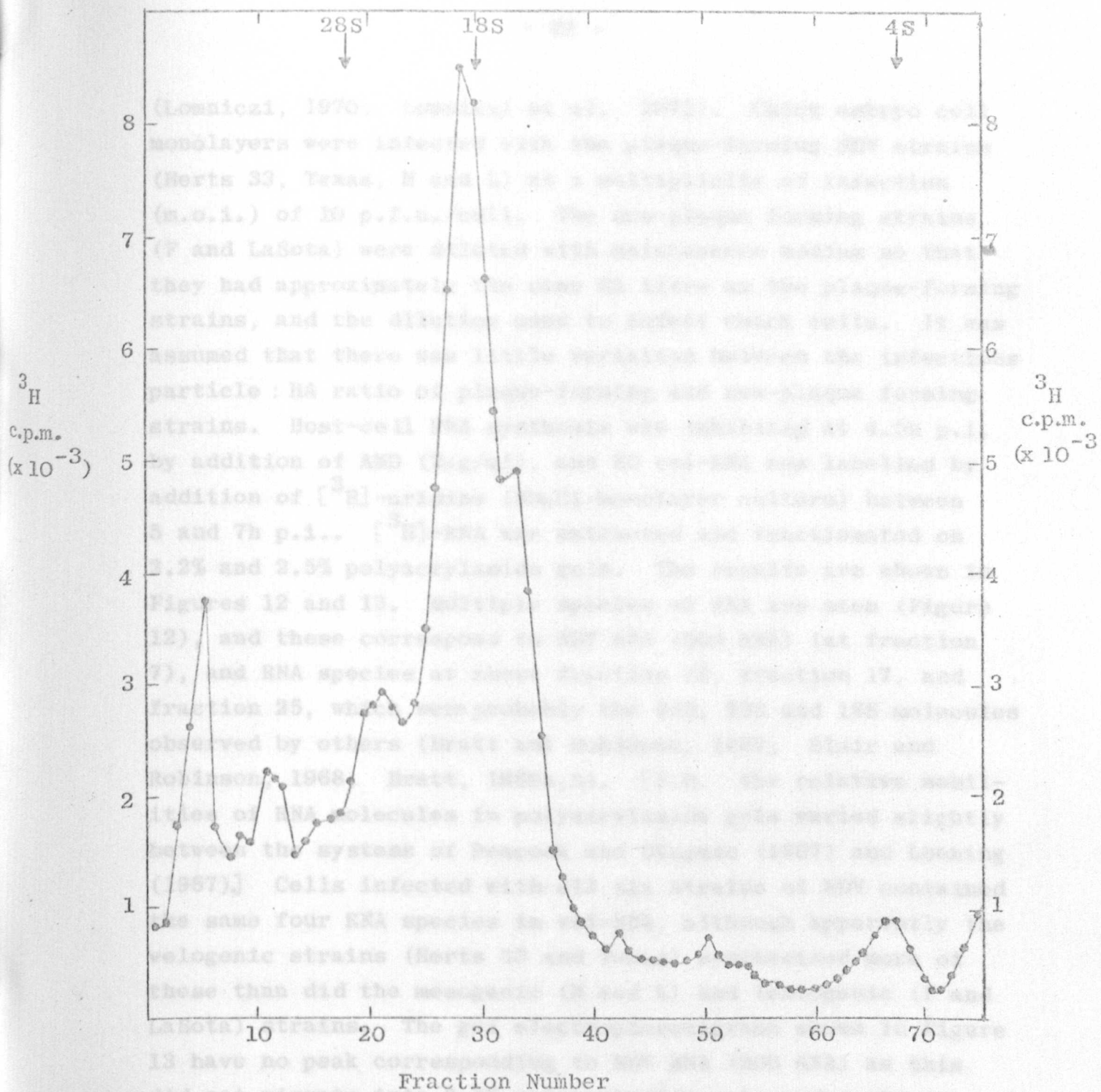


Figure 11. Polyacrylamide gel electrophoresis on a 15cm 2.2% gel of [^{3}H]-ND (strain TEXAS) vsi-RNA. Electrophoresis was at 100V at 3mA per gel for 2.2 h (Loening system). The arrows show the position of [^{32}P]-ribosomal RNA species.

(Lomniczi, 1970; Lomniczi et al., 1971). Chick embryo cell monolayers were infected with the plaque-forming NDV strains (Herts 33, Texas, H and L) at a multiplicity of infection (m.o.i.) of 10 p.f.u./cell. The non-plaque forming strains (F and LaSota) were diluted with maintenance medium so that they had approximately the same HA titre as the plaque-forming strains, and the dilution used to infect chick cells. It was assumed that there was little variation between the infectious particle : HA ratio of plaque-forming and non-plaque forming strains. Host-cell RNA synthesis was inhibited at 4.5h p.i. by addition of AMD (2μg/ml), and ND vsi-RNA was labelled by addition of [^{3}H]-uridine (50μCi/monolayer culture) between 5 and 7h p.i.. [^{3}H]-RNA was extracted and fractionated on 2.2% and 2.5% polyacrylamide gels. The results are shown in Figures 12 and 13. Multiple species of RNA are seen (Figure 12), and these correspond to NDV RNA (50S RNA) (at fraction 7), and RNA species at about fraction 12, fraction 17, and fraction 25, which were probably the 35S, 22S and 18S molecules observed by others (Bratt and Robinson, 1967; Blair and Robinson, 1968; Bratt, 1969a,b). [N.B. the relative mobilities of RNA molecules in polyacrylamide gels varied slightly between the systems of Peacock and Dingman (1967) and Loening (1967)] Cells infected with all six strains of NDV contained the same four RNA species in vsi-RNA, although apparently the velogenic strains (Herts 33 and Texas) synthesised more of these than did the mesogenic (H and L) and lentogenic (F and LaSota) strains. The gel electrophoretograms shown in Figure 13 have no peak corresponding to NDV RNA (50S RNA) as this did not migrate into 2.5% polyacylamide gels under the conditions used. However, the sub-genomic RNAs, 35S, 22S and 18S, are seen at about fraction 5, 9 and 13, respectively. Figure 13(a) also shows a typical gel electrophoretogram of [^{3}H]-RNA extracted from uninfected chick cells treated with AMD (2μg/ml). Most of the chick cell ribosomal RNA synthesis was suppressed, but some 4S RNA was always synthesised under these conditions. Examination of ND vsi-RNA throughout the entire growth cycle of NDV showed that the four RNA species,

Figure 12. Polyacrylamide gel electrophoresis of vsi-RNA extracted from cells infected with various strains of NDV. [^{3}H]-ND vsi-RNA was fractionated on 2.2% gels by electrophoresis at 150V and 2mA per tube for 1 h. The arrows indicate the position of [^{14}C]-ribosomal RNA species. Note the different scales. (Peacock and Dingman system).

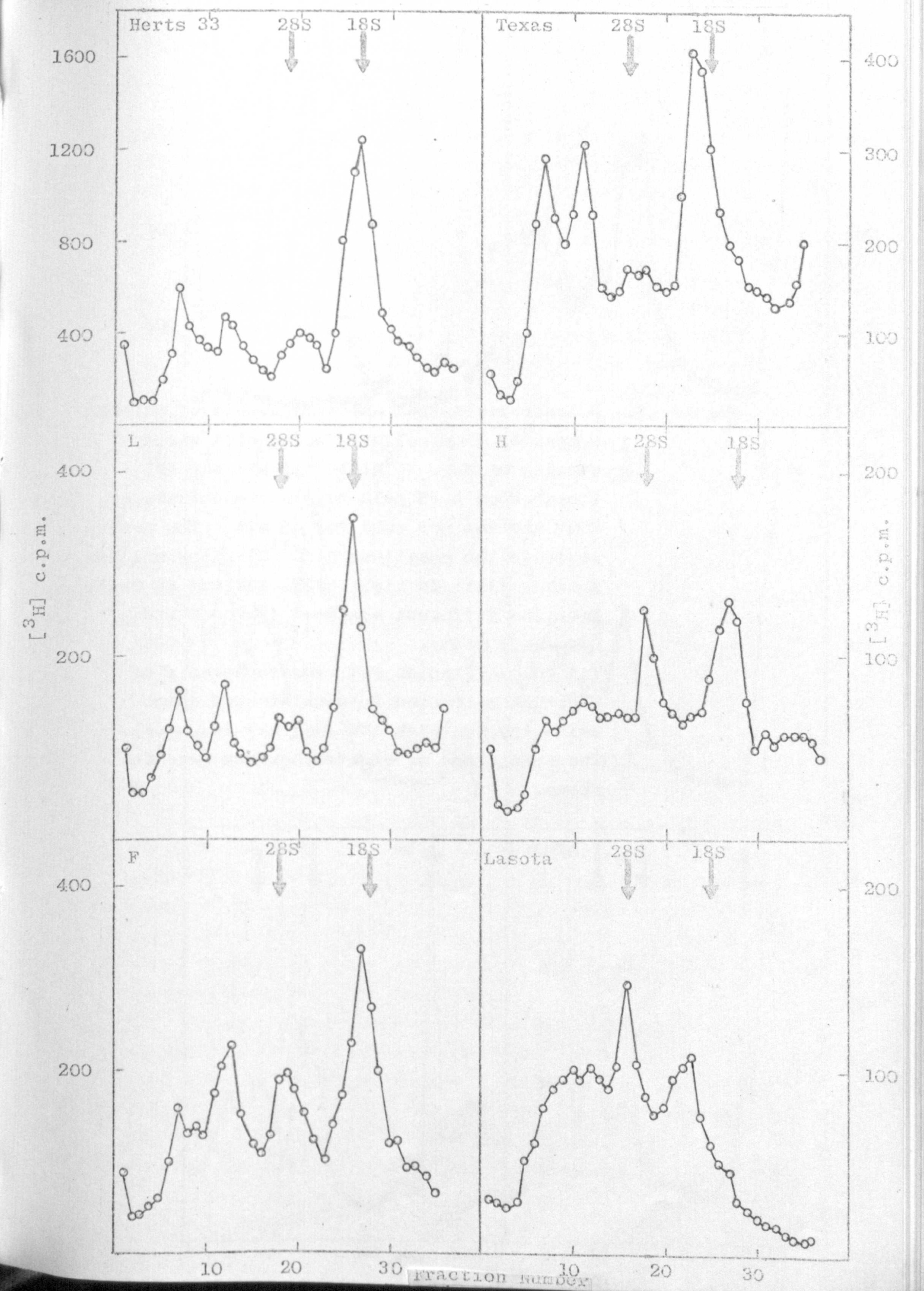
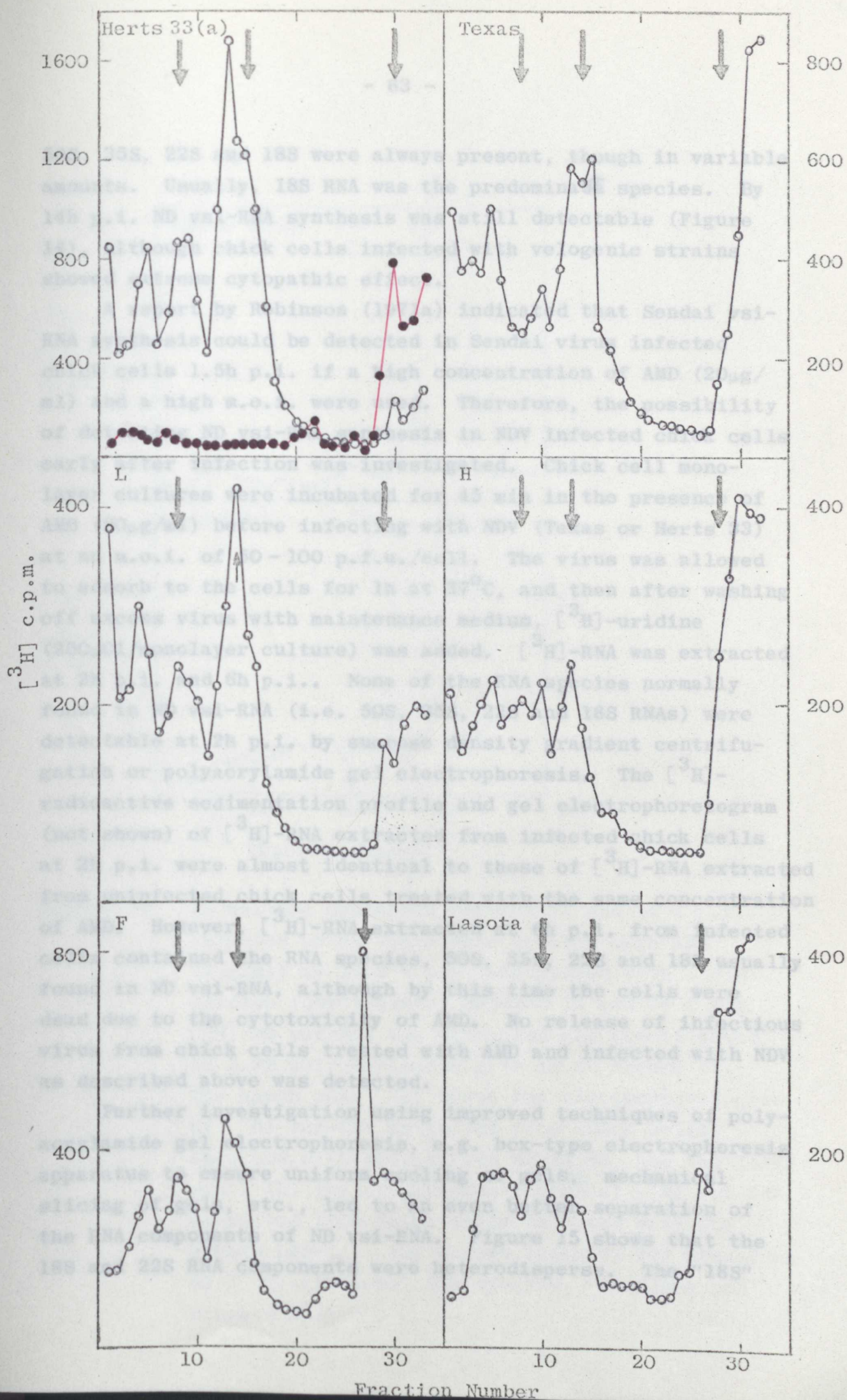

Herts 33
28S
18S
Texas
L
H
F
Lasota
1600
1200
800
400
300
200
100
[3H] c.p.m.
10
20
30
Fraction Number

Figure 13. Polyacrylamide gel electrophoresis of vsi-RNA extracted from cells infected with various strains of NDV. [^{3}H]-ND vsi-RNA was fractionated on 2.5% gels by electrophoresis at 150V and 2mA per tube for 45 min. The arrows indicate the positions of [^{14}C]-ribosomal RNA species (left to right, 28S, 18S and 4S RNA). Note the different scales. (Peacock and Dingman system).

(a) Polyacrylamide gel electrophoresis of [^{3}H]-RNA extracted from uninfected chick cells treated with AMD (2μg/ml) (●———●). The conditions of electrophoresis were as above.


Herts 33(a)
Texas
L
H
F
Lasota
1600
1200
800
400
800
600
400
200
400
200
400
200
800
400
400
200
[3H] c.p.m.
10
20
30
10
20
30
Fraction Number

50S, 35S, 22S and 18S were always present, though in variable amounts. Usually, 18S RNA was the predominant species. By 14h p.i. ND vsi-RNA synthesis was still detectable (Figure 14), although chick cells infected with velogenic strains showed extreme cytopathic effect.

A report by Robinson (1971a) indicated that Sendai vsi-RNA synthesis could be detected in Sendai virus infected chick cells 1.5h p.i. if a high concentration of AMD (20μg/ml) and a high m.o.i. were used. Therefore, the possibility of detecting ND vsi-RNA synthesis in NDV infected chick cells early after infection was investigated. Chick cell monolayer cultures were incubated for 45 min in the presence of AMD (20μg/ml) before infecting with NDV (Texas or Herts 33) at an m.o.i. of 50 - 100 p.f.u./cell. The virus was allowed to adsorb to the cells for 1h at 37°C, and then after washing off excess virus with maintenance medium, [^{3}H]-uridine (250μCi/monolayer culture) was added. [^{3}H]-RNA was extracted at 2h p.i. and 6h p.i.. None of the RNA species normally found in ND vsi-RNA (i.e. 50S, 35S, 22S and 18S RNAs) were detectable at 2h p.i. by sucrose density gradient centrifugation or polyacrylamide gel electrophoresis. The [^{3}H]-radioactive sedimentation profile and gel electrophoretogram (not shown) of [^{3}H]-RNA extracted from infected chick cells at 2h p.i. were almost identical to those of [^{3}H]-RNA extracted from uninfected chick cells treated with the same concentration of AMD. However, [^{3}H]-RNA extracted at 6h p.i. from infected cells contained the RNA species, 50S, 35S, 22S and 18S usually found in ND vsi-RNA, although by this time the cells were dead due to the cytotoxicity of AMD. No release of infectious virus from chick cells treated with AMD and infected with NDV as described above was detected.

Further investigation using improved techniques of polyacrylamide gel electrophoresis, e.g. box-type electrophoresis apparatus to ensure uniform cooling of gels, mechanical slicing of gels, etc., led to an even better separation of the RNA components of ND vsi-RNA. Figure 15 shows that the 18S and 22S RNA components were heterodisperse. The "18S"

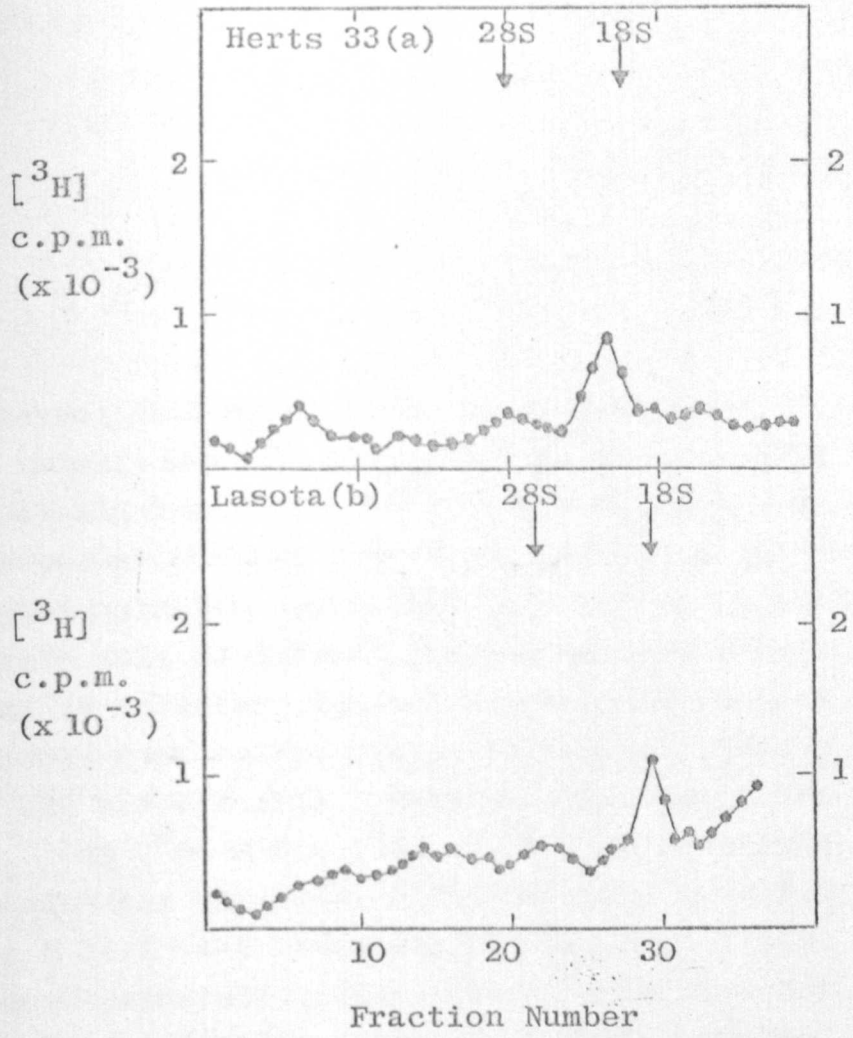
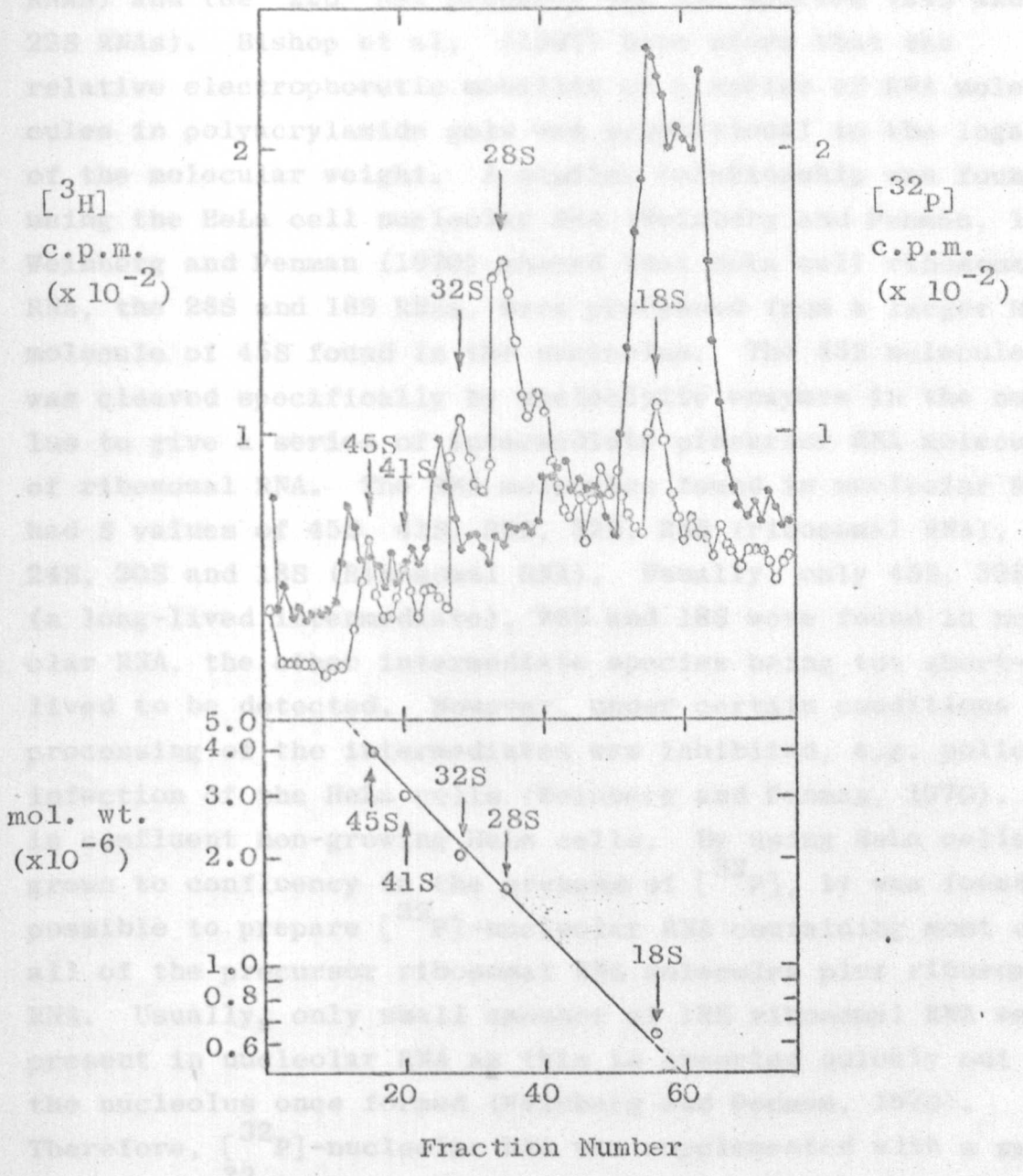


Figure 14. ND vsi-RNA synthesis between 12 and 14 h p.i.. Infected chick cells were treated with AMD (2μg/ml) at 11.5 h p.i. and pulsed with [^{3}H]-uridine (50μCi/plate) between 12 and 14 h p.i.. [^{3}H]-ND vsi-RNA was extracted and fractionated on 2.2% polyacrylamide gels as in Figure 12. The upper panel (a) shows the electrophoretogram of [^{3}H] vsi-RNA extracted from chick cells infected with NDV, strain HERTS 33, and the lower panel (b) the electrophoretogram of [^{3}H] vsi-RNA extracted from chick cells infected with NDV, strain LASOTA. The arrows indicate the positions of [^{14}C]-ribosomal RNA species. (Peacock and Dingman system).

Figure 15. The electrophoretic mobility in 2.2% polyacrylamide gels of the HeLa cell [^{32}P]-nucleolar RNA (o——o), HeLa cell [^{32}P]-ribosomal RNA (o——o) and [^{3}H]-ND (strain TEXAS) vsi-RNA (●——●). All three types of RNA were co-electrophoresed at 60V and 4mA per gel for 2.5 h. The different species of cellular RNA are indicated by the arrows, and the following values were taken for their molecular weights: 45S, 41S and 32S nucleolar RNA, 4.1×10^6, 3.1×10^6, and 2.1×10^6, respectively (Weinberg and Penman, 1970); 28S and 18S ribosomal RNA, 1.65×10^6 and 0.65×10^6, respectively (Petermann and Pavlovec, 1966). (Loening system).


[3H]
c.p.m.
(x 10-2)
[32P]
c.p.m.
(x 10-2)
28S
32S
18S
45S
41S
mol. wt.
(x10-6)
Fraction Number
20
40
60

RNA probably contained three RNA species (19S, 17S and 15S RNAs) and the "22S" RNA probably two RNA species (24S and 22S RNAs). Bishop et al. (1967) have shown that the relative electrophoretic mobility of a series of RNA molecules in polyacrylamide gels was proportional to the logarithm of the molecular weight. A similar relationship was found using the HeLa cell nucleolar RNA (Weinberg and Penman, 1970). Weinberg and Penman (1970) showed that HeLa cell ribosomal RNA, the 28S and 18S RNAs, were processed from a larger RNA molecule of 45S found in the nucleolus. The 45S molecule was cleaved specifically by nucleolytic enzymes in the nucleolus to give a series of intermediate precursor RNA molecules of ribosomal RNA. The RNA molecules found in nucleolar RNA had S values of 45S, 41S, 36S, 32S, 28S (ribosomal RNA), 24S, 20S and 18S (Ribosomal RNA). Usually, only 45S, 32S (a long-lived intermediate), 28S and 18S were found in nucleolar RNA, the other intermediate species being too short-lived to be detected. However, under certain conditions processing of the intermediates was inhibited, e.g. polio infection of the HeLa cells (Weinberg and Penman, 1970), or in confluent non-growing HeLa cells. By using HeLa cells grown to confluency in the presence of [^{32}P], it was found possible to prepare [^{32}P]-nucleolar RNA containing most or all of the precursor ribosomal RNA molecules plus ribosomal RNA. Usually, only small amounts of 18S ribosomal RNA were present in nucleolar RNA as this is exported quickly out of the nucleolus once formed (Weinberg and Penman, 1970). Therefore, [^{32}P]-nucleolar RNA was supplemented with a small amount of [^{32}P]-HeLa cell ribosomal RNA extracted from cell cytoplasm so that all the RNA species could be identified after fractionation by polyacrylamide gel electrophoresis. The molecular weights of five of the nucleolar RNA species had been previously determined:- these were 4.1×10^6, 3.1 and 10^6 and 2.1×10^6 for the 45S, 41S and 32S RNAs, respectively (Weinberg and Penman, 1970), and 1.65×10^6 and 0.65×10^6 for the 28S and 18S RNAs (Petermann and

Pavlovec, 1966). By using these molecular weights, 2.2% polyacrylamide gels were calibrated as in Figure 15. Co-electrophoresis of [^{3}H]-ND vsi-RNA and [^{32}P] HeLa cell nucleolar and ribosomal RNA (Figure 15) allowed molecular weights to be estimated for the component RNA species of ND vsi-RNA. The molecular weights determined from Figure 15, using the logarithm of mol. wt. vs. electrophoretic mobility relationship, were 4.8×10^6 for NDV RNA (50S RNA), 2.5×10^6 for 35S RNA, about 1.2×10^6 and 1.0×10^6 for 24S and 22S RNAs of "22S" RNA, and 0.7×10^6, 0.55×10^6, and 0.45×10^6 for 19S, 17S and 15S RNAs of "18S" RNA. Loening, U.E. (Dept. of Zoology, Edinburgh University, Edinburgh) estimated a mol. wt. of 5.2×10^6 for NDV RNA using a similar system (personal communication).

Polyribosomes isolated from chick chorioallantoic membranes

The following studies involving the isolation of polyribosomes from chick chorioallantoic membranes and chick embryo cells were carried out with the aim of confirming the association of NDV complementary RNA, i.e. the majority of ND vsi-RNA, with polyribosomes described by Bratt and Robinson (1967). Chorioallantoic membranes were used initially for three reasons: (i) growth of NDV in these membranes was not inhibited by AMD (Barry et al., 1962); (ii) the yield of infectious virus per cell of chorioallantoic membranes was greater than that obtained per cell of chick embryo cells (10 - 50 p.f.u./cell for chorioallantoic membranes as compared to 1 - 5 p.f.u./cell for chick embryo cells); (iii) large quantities of membranes were available from which potentially large amounts of polyribosomes could be isolated. The method for isolating polyribosomes from chorio-allantoic membranes was based on that developed by Wettstein et al. (1963) for rat liver (see also Noll, 1969). Certain modifications were made, in particular with regards to breakage of cells. Fairly severe homogenisation was required to break the cells of chorio-allantoic membranes.

Polyribosomes were obtained in good yield from chorio-allantoic membranes (12mg of polyribosomes/30 membranes) using the method described on page 43. The OD_{260}/OD_{280} ratio of the resuspended polyribosomes was 1.7 - 1.8 indicating high purity of the polyribosomes (Noll, 1969). The OD_{254} polyribosomal sedimentation profile obtained by centrifugation of polyribosomes in an 8 - 45% (w/v) sucrose density gradient is shown in Figure 16. There were large amounts of monosomes (one ribosome), di-somes (two ribosomes), and the smaller polyribosomes. The polyribosomes were susceptible to breakdown by ribonuclease to yield predominately monosomes (Figure 16). Addition of [^{35}S]-L-methionine to chorio-allantoic membranes prior to the isolation procedure radioactively labelled the polyribosomes with [^{35}S] (Figure 17). The heterodisperse association of [^{35}S]-methionine with polyribosomes probably represented newly synthesised methionine containing polypeptides.

It was already established that NDV containing its RNA labelled with [^{3}H]-UMP could be harvested from chorio-allantoic membranes after 24 hours growth of the virus at 37°C in the presence of [^{3}H]-uridine (see Figure 7). Therefore, it was surprising to find that ND vsi-RNA could not be detected in the infected cells of chorio-allantoic membranes in the presence of AMD (2μg/ml). Figure 18 a and b shows the sedimentation profile of [^{3}H]-RNA extracted from the post mitochondrial supernatant (PMS) derived from uninfected (Figure 18a) and infected (Figure 18b) chorio-allantoic membranes. The membranes were treated with AMD at 4.5h p.i., and pulsed with [^{3}H]-uridine between 5 and 7h p.i.. Except for a small peak at 15 - 16S in the [^{3}H]-RNA extracted from infected PMS, there was no difference in the sedimentation profiles of [^{3}H]-RNA extracted from infected and uninfected PMS. Relatively large amounts of 4S RNA were present in the [^{3}H]-RNA extracted from both infected and uninfected PMS. However, this was to be expected as 4S RNA synthesis was never entirely suppressed even in chick embryo cells treated with high concentrations of AMD (10 - 20μg/ml)

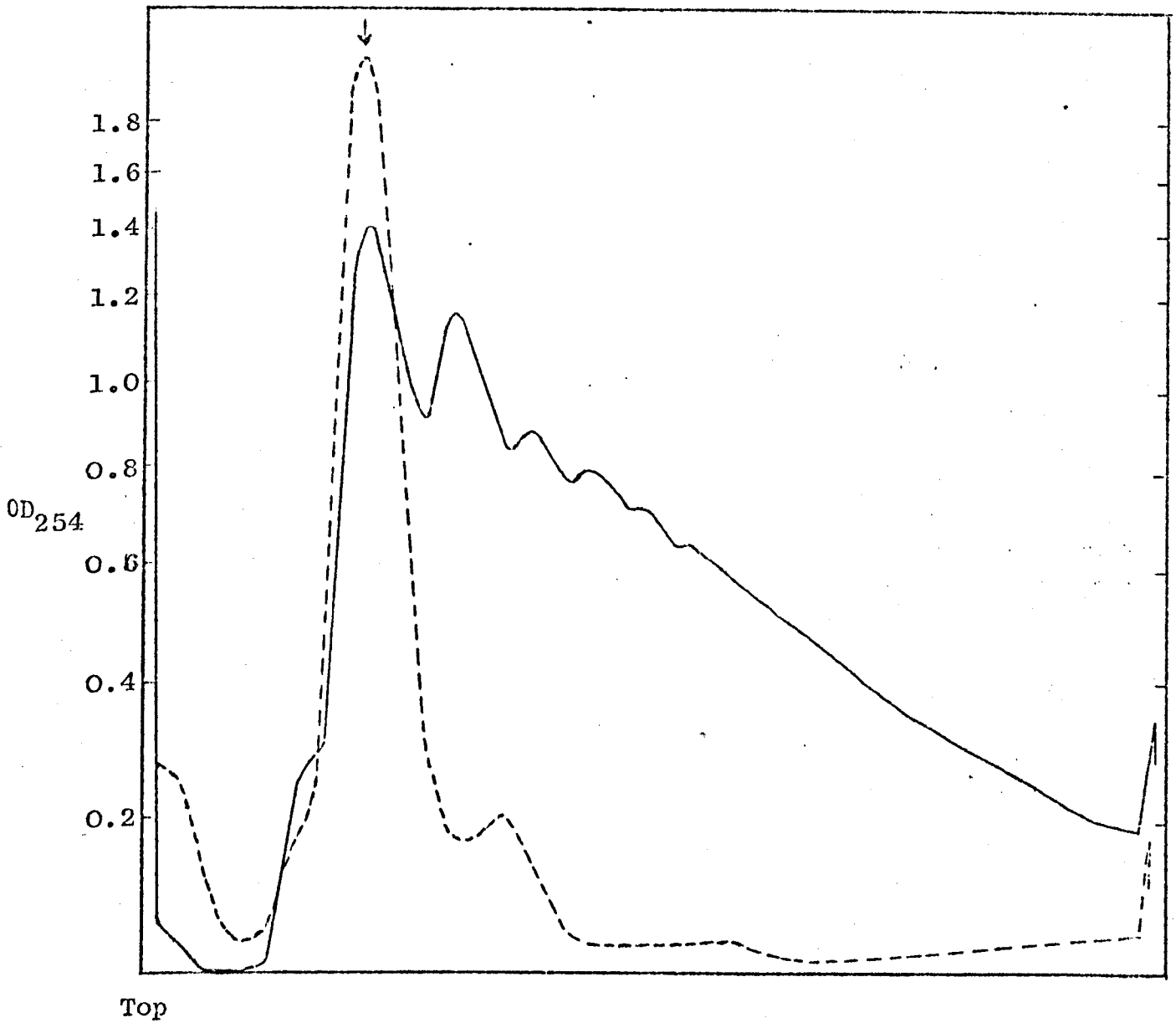


Figure 16. Centrifugation of polyribosomes isolated from chorioallantoic membranes in an 8 - 45% (w/v) sucrose density gradient buffered to pH 7.6 with TKM buffer. Aliquots of polyribosomes in TKM buffer were either (a) untreated (0.75 mg polyribosomes)———, or (b) treated with ribonuclease (0.3 mg polyribosomes)- - - -, and centrifuged in parallel sucrose gradients. The arrow indicates the position of the 80S ribosome.

Figure 17. Centrifugation of [^{35}S]-labelled polyribosomes isolated from chorioallantoic membranes in an 8 - 45% (w/v) sucrose density gradient buffered to pH 7.6 with TKM buffer. Chorioallantoic membranes were incubated with 5μCi of [^{35}S]-L-methionine for 15 min at 37°C before the start of the isolation procedure. OD_{254} ———, [^{35}S]-radioactivity (acid-insoluble) o———o. The arrow indicates the position of the 80S ribosome.

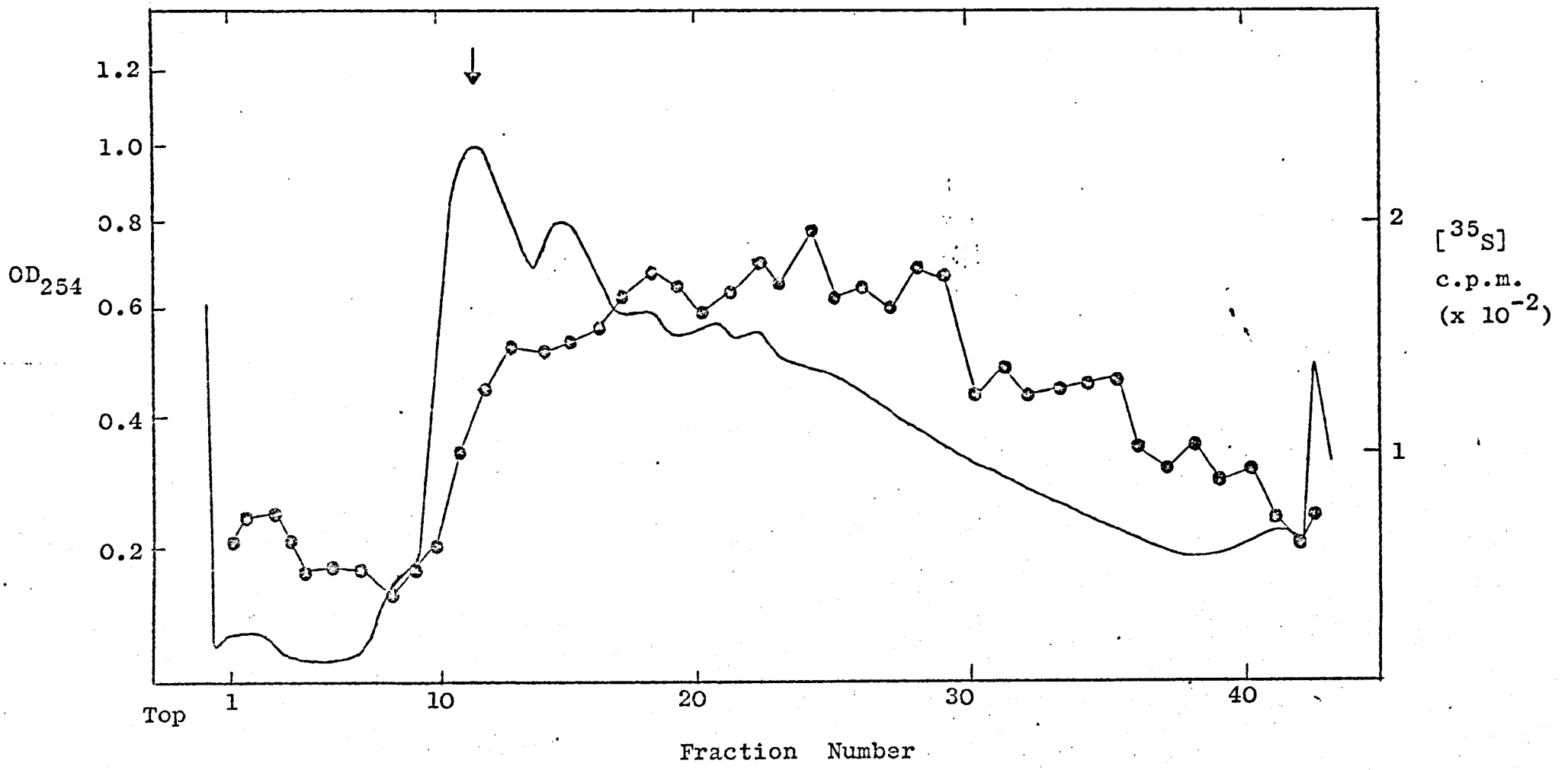
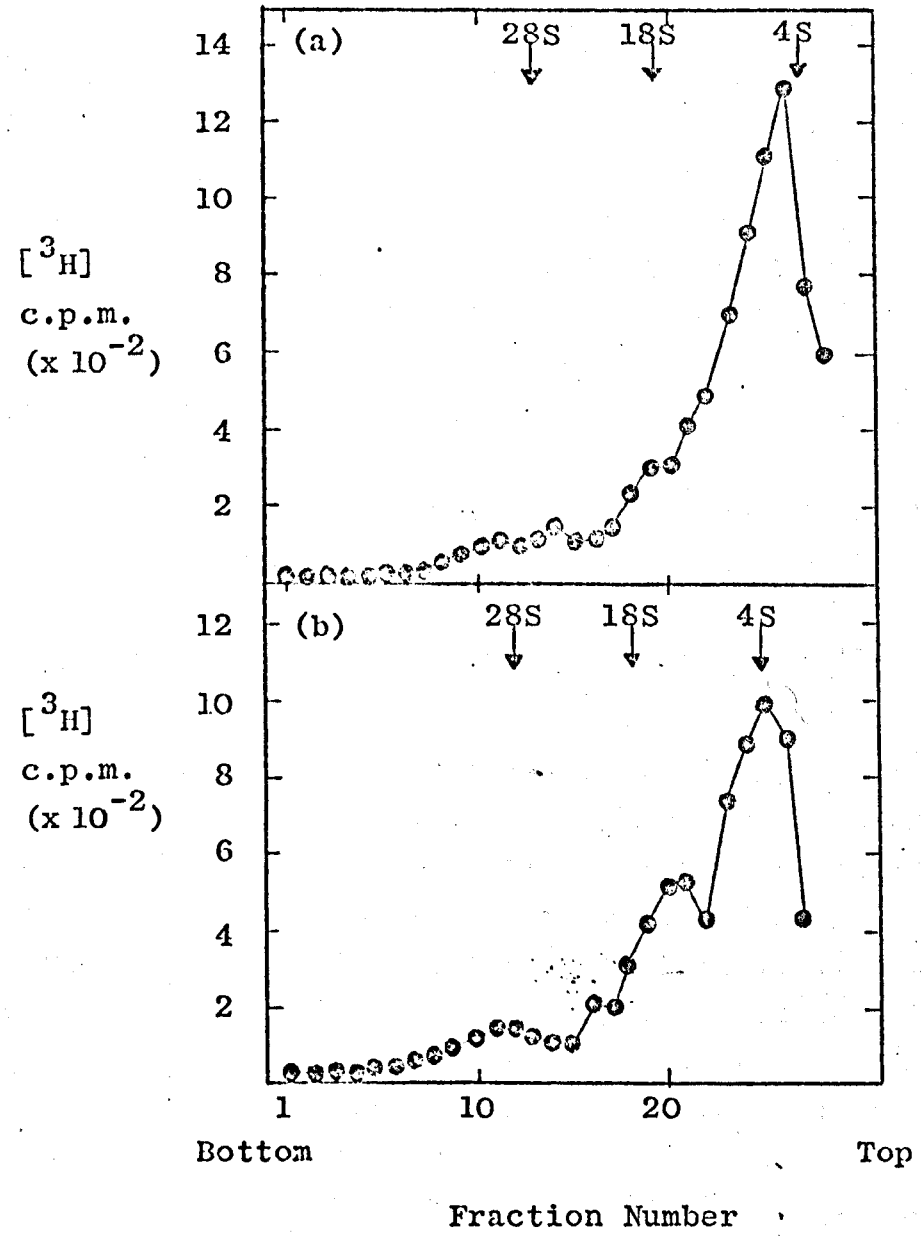

1.2
1.0
0.8
0.6
0.4
0.2
OD_{254}
2
1
$[^{35}S]$
c.p.m.
(x 10^{-2})
Top
1
10
20
30
40
Fraction Number

Figure 18(a). Centrifugation of [^{3}H]-RNA extracted from the PMS of uninfected chorioallantoic membrane cells in a 5 - 25% (w/v) sucrose density gradient buffered to pH 7.4 with S buffer. Chorioallantoic membranes were treated with AMD and pulsed with [^{3}H]-uridine as described below.

Figure 18(b) Centrifugation of [^{3}H]-RNA extracted from the PMS of chorioallantoic membrane cells infected with NDV, strain TEXAS, in a 5 - 25% (w/v) sucrose density gradient buffered to pH 7.4 with S buffer. Chorioallantoic membranes were treated with AMD (2μg/ml) at 4.5 h p.i., and pulsed with [^{3}H]-uridine (1mCi/30 membranes) between 5 and 7 h p.i. before disrupting the cells. Centrifugation in both (a) and (b) was at 50,000g_{av} for 16h at 4°C (not 40,000g_{av} as described in the Methods). The arrows indicate the position of the ribosomal RNA species.


(a)
28S
18S
4S
14
12
10
8
6
4
2
[3H]
c.p.m.
(x 10-2)
(b)
28S
18S
4S
12
10
8
6
4
2
[3H]
c.p.m.
(x 10-2)
1
10
20
Bottom
Top
Fraction Number

and membranes were known to be less sensitive to AMD (Barry et al., 1962). The absence of any identifiable RNA components of ND vsi-RNA in [^{3}H]-RNA isolated from infected chorio-allantoic membranes made it improbable that any further progress could be made using such a system. In fact, it was found that polyribosomes isolated from infected chorio-allantoic membranes labelled with [^{3}H]-uridine in the presence of AMD (2μg/ml) contained very low levels of [^{3}H]-label (Figure 19). The incorporation of [^{3}H]-label into polyribosomes isolated from uninfected membranes was about the same (Figure 19). At this stage the use of chorio-allantoic membranes was abandoned in favour of chick embryo cells grown in monolayers, as it had been established that ND vsi-RNA synthesis was readily detectable in these cells.

Polyribosomes isolated from chick embryo cells grown in monolayers

Polyribosomes were prepared from chick embryo cells in monolayer cultures using the method described on page 41. The optimal salt concentrations of the TKM buffer, and the centrifugation conditions for sucrose density gradients were worked out in collaboration with Dr. S. I. T. Kennedy (Dept. of Biological Sciences, University of Warwick, Coventry.). It was found that polyribosomes could be obtained in good yield (2mg of polyribosomes/6 monolayer cultures), and that the OD_{260}/OD_{280} ratio of the polyribosome suspension was 1.7 - 1.8 indicating high purity (Noll, 1969). Figure 20 shows the polyribosomal OD_{254} sedimentation profile of (i) untreated chick embryo cell polyribosomes, and (ii) ribonuclease treated chick embryo cell polyribosomes on 8 - 45% (w/v) sucrose density gradients. About 95% of the absorption at 254nm was in the polyribosomes (i.e. 2 or more ribosomes), and polyribosomal peaks up to heptamers were discernable. Often the tetramer (4 ribosomes) was a large peak suggesting that it might be a stable degradation produce of larger polyribosomes. The polyribosomes were completely sensitive to RNA'ase (Figure 20) indicating that they were ribosomes linked by messenger RNA, and not ribosome aggregates.

Figure 19. Centrifugation of [^{3}H]-labelled polyribosomes isolated from (a) uninfected chorioallantoic membranes - - - - -, (OD_{254}), o- - -o ([^{3}H]-radioactivity), and (b) chorioallantoic membranes infected with NDV, strain TEXAS, ———— (OD_{254}), o——o ([^{3}H]-radioactivity), in parallel 8 - 45% (w/v) sucrose density gradients buffered to pH 7.6 with TKM buffer. Chorioallantoic membranes were treated with AMD (2μg/ml) at 4.5 h p.i., and pulsed with [^{3}H]-uridine (1mCi/30 membranes) between 5 and 7 h p.i. before disrupting the cells. The arrow indicates the position of the 80S ribosome.

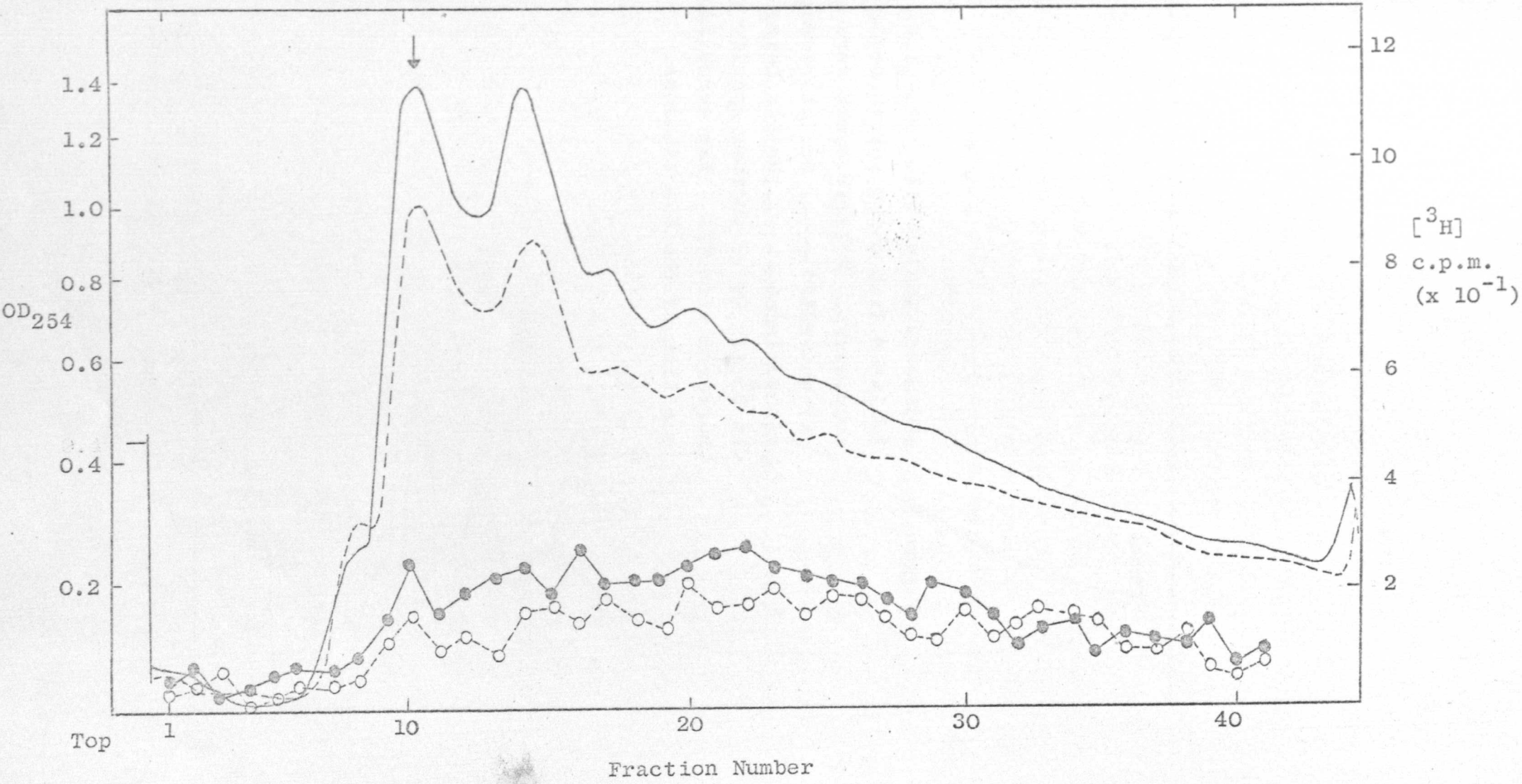
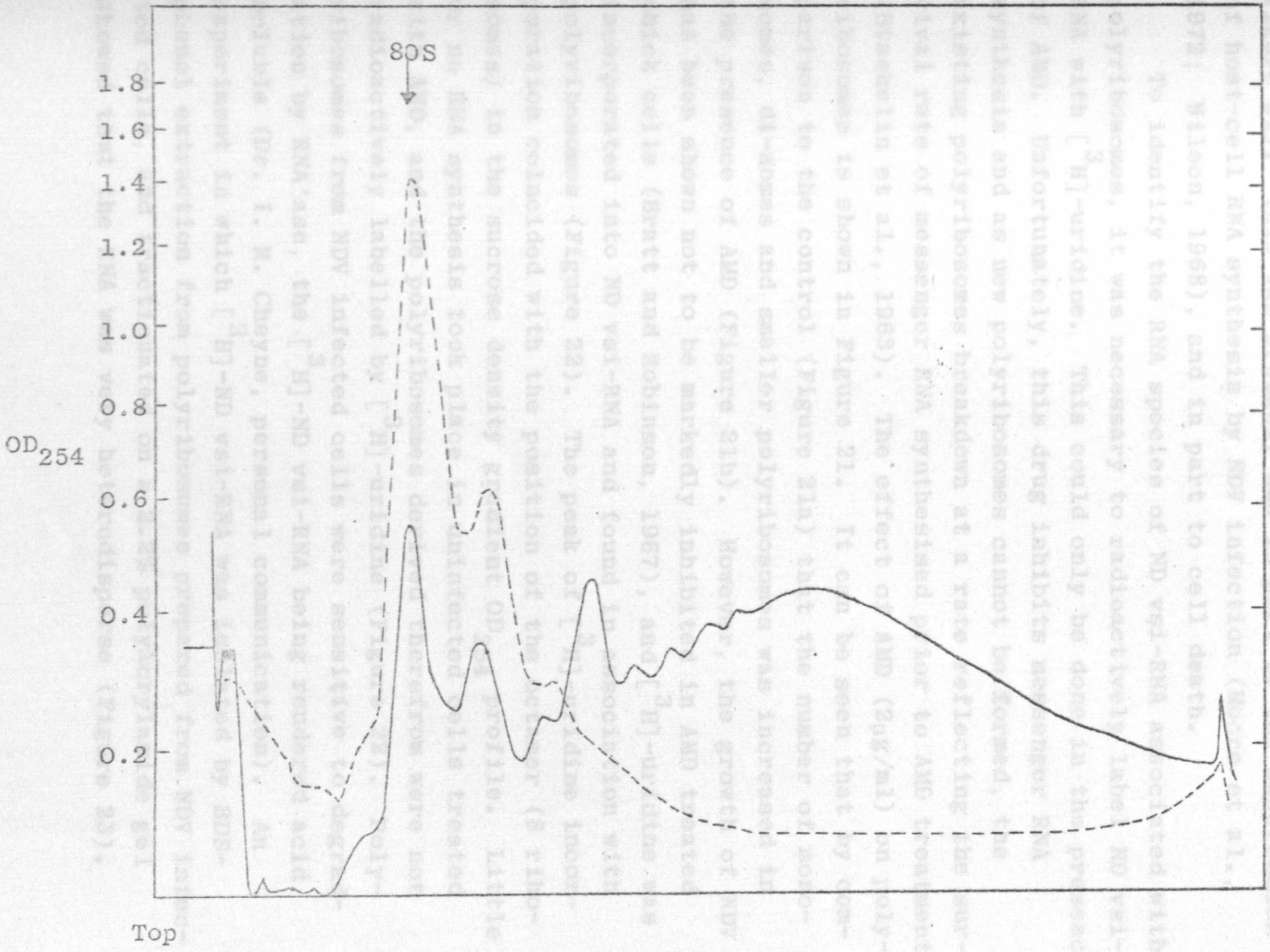

1.4
1.2
1.0
0.8
OD254
0.6
0.4
0.2
12
10
8
[3H]
c.p.m.
(x 10-1)
6
4
2
Top
1
10
20
30
40
Fraction Number

Figure 20. Sedimentation profile (OD_{254}) of polysomes isolated from chick embryo cells grown in monolayers. Polyribosomes were either (i) untreated ——, or (ii) treated with ribonuclease - - - -, before being centrifuged in 8 - 45% (w/v) sucrose density gradients buffered with TKM. The arrow indicates the position of the 80S ribosome.


80S
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
OD254
Top

When CEC monolayer cultures were infected with NDV strain Texas, the polyribosomes were unaltered as determined by their sucrose density gradient OD_{254} profile for up to 7 - 8h p.i.. However, by 9h p.i. the polyribosomes began to break down (not shown, but similar to breakdown induced by AMD - see Figure 21). This was probably due in part to the suppression of host-cell RNA synthesis by NDV infection (Moore et al., 1972; Wilson, 1968), and in part to cell death.

To identify the RNA species of ND vsi-RNA associated with polyribosomes, it was necessary to radioactively label ND vsi-RNA with [^{3}H]-uridine. This could only be done in the presence of AMD. Unfortunately, this drug inhibits messenger RNA synthesis and as new polyribosomes cannot be formed, the existing polyribosomes breakdown at a rate reflecting the survival rate of messenger RNA synthesised prior to AMD treatment (Staehelin et al., 1963). The effect of AMD (2μg/ml) on polyribosomes is shown in Figure 21. It can be seen that by comparison to the control (Figure 21a) that the number of monosomes, di-somes and smaller polyribosomes was increased in the presence of AMD (Figure 21b). However, the growth of NDV has been shown not to be markedly inhibited in AMD treated chick cells (Bratt and Robinson, 1967), and [^{3}H]-uridine was incorporated into ND vsi-RNA and found in association with polyribosomes (Figure 22). The peak of [^{3}H]-uridine incorporation coincided with the position of the octamer (8 ribosomes) in the sucrose density gradient OD_{254} profile. Little or no RNA synthesis took place in uninfected cells treated with AMD, and the polyribosomes derived therefrom were not radioactively labelled by [^{3}H]-uridine (Figure 22). Polyribosomes from NDV infected cells were sensitive to degradation by RNA'ase, the [^{3}H]-ND vsi-RNA being rendered acid soluble (Dr. I. M. Cheyne, personal communication). An experiment in which [^{3}H]-ND vsi-RNA was isolated by SDS-phenol extraction from polyribosomes prepared from NDV infected cells, and fractionated on a 2.2% polyacrylamide gel showed that the RNA was very heterodisperse (Figure 23).

Figure 21. Effect of actinomycin D on chick cell ribosomes. Cells were incubated 2 h in the absence (a) or presence (b) of AMD (2μg/ml) before disruption of the cells and isolation of polyribosomes. Equal amounts of polyribosomes isolated from untreated (a) or treated (b) cells were centrifuged in 8 - 45% (w/v) sucrose density gradients buffered with TKM, and the resultant OD_{254} sedimentation profiles are shown right. The arrow indicates the position of the 80S ribosome.

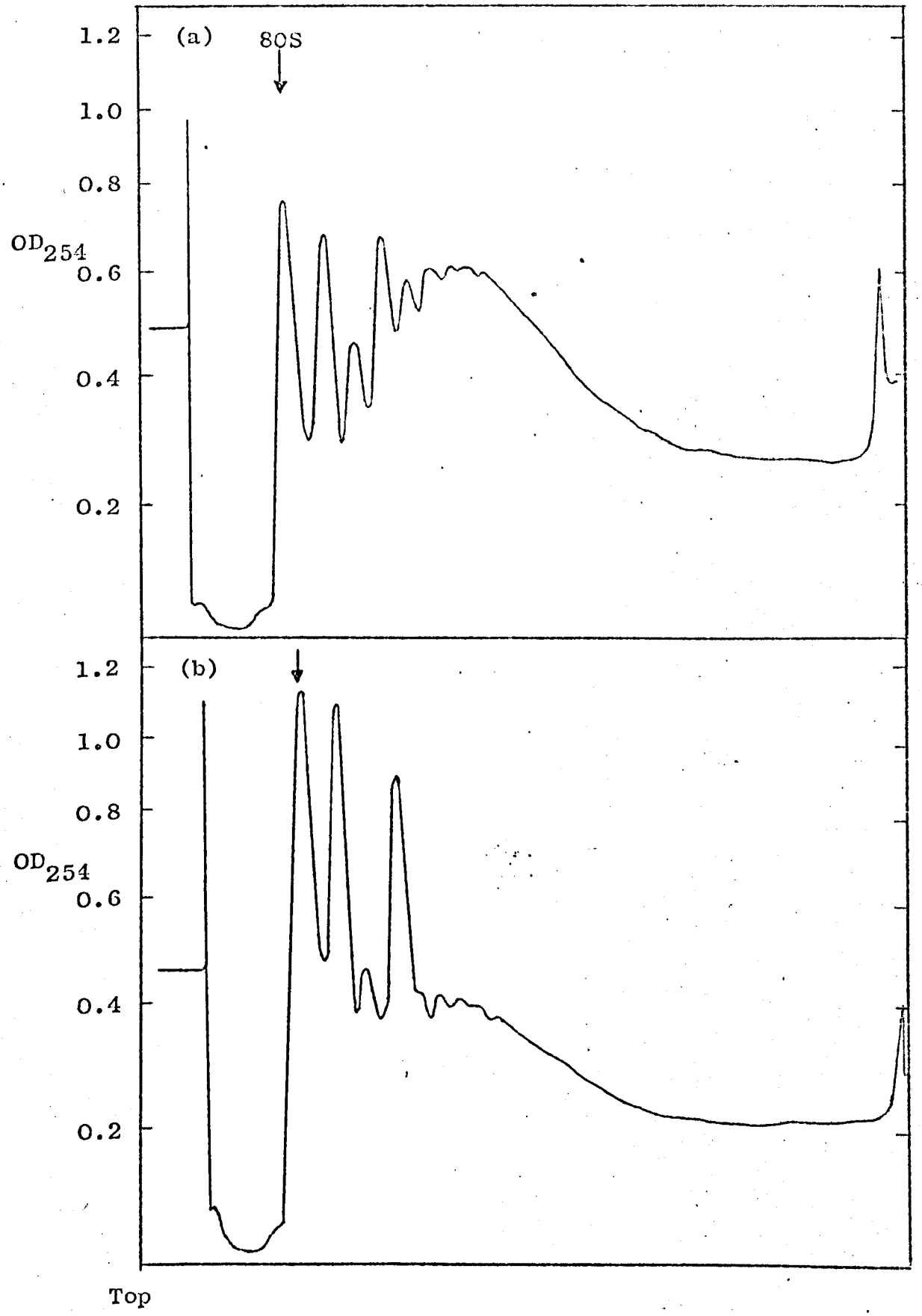
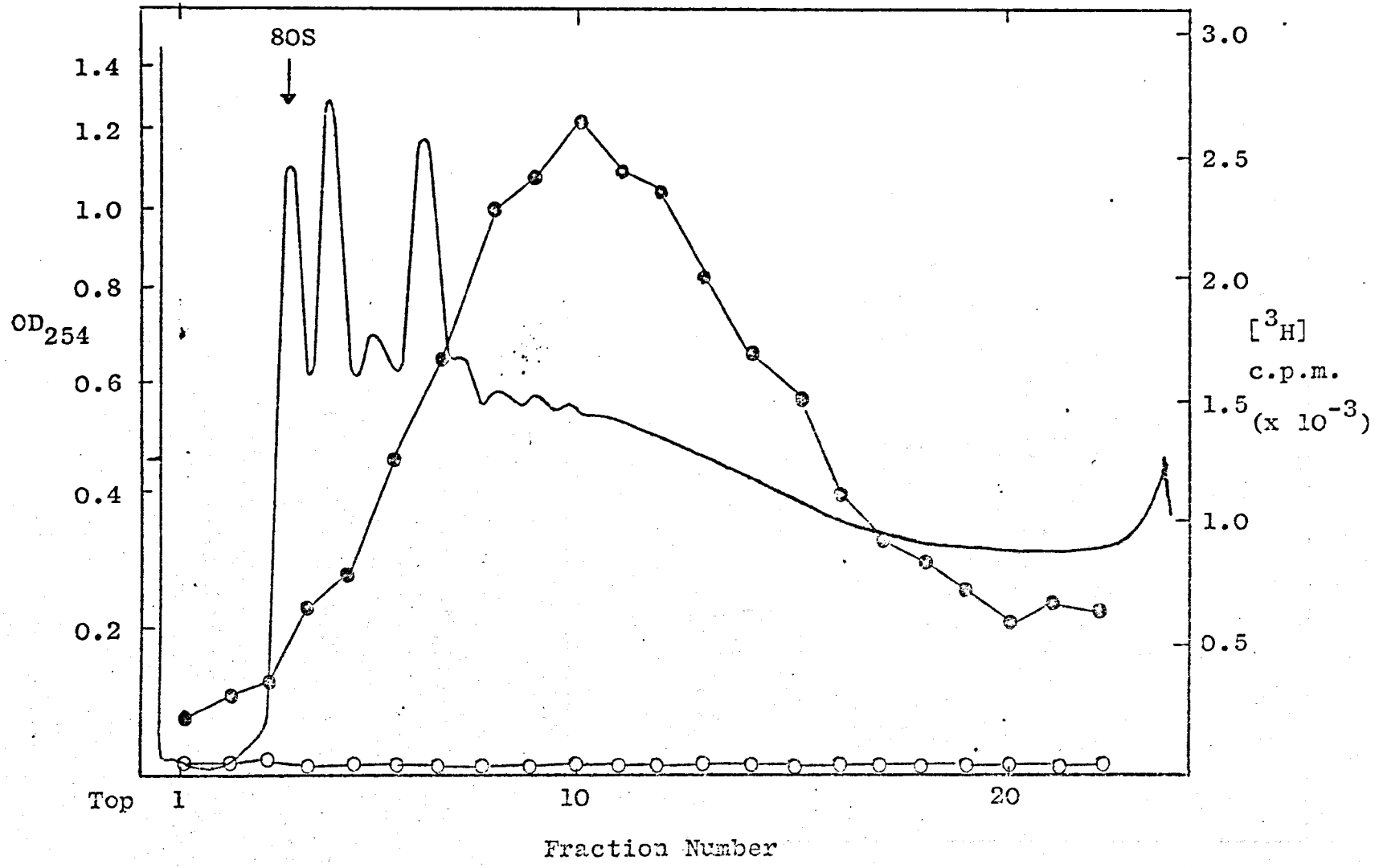

(a)
80S
1.2
1.0
0.8
0.6
0.4
0.2
OD254
(b)
1.2
1.0
0.8
0.6
0.4
0.2
OD254
Top

Figure 22. Incorporation of [^{3}H]-uridine into chick cell polyribosomes. CEC monolayers were either (a) uninfected, or (b) infected with NDV, strain Texas. Host-cell RNA synthesis was inhibited by the addition of AMD (2μg/ml) at 4.5 h p.i., and [^{3}H]-uridine (100μCi/culture) added at 5 h p.i.. At 7 h p.i. the cells were disrupted and polyribosomes isolated. Equal amounts of polyribosomes isolated from (a) uninfected and (b) infected cells were centrifuged in parallel 8 - 45% (w/v) sucrose density gradients buffered with TKM. The OD_{254} sedimentation profiles were almost identical and the solid line, ———— , represents this. Fractions collected from each gradient were treated with 10% TCA and acid-insoluble [^{3}H]-radioactivity determined. o———o [^{3}H]-radioactivity associated with polyribosomes isolated from uninfected cells; o———o [^{3}H]-radioactivity associated with polyribosomes isolated from infected cells. The arrow indicates the position of the 80S ribosome.


80S
1.4
1.2
1.0
0.8
0.6
0.4
0.2
OD254
3.0
2.5
2.0
1.5
1.0
0.5
[3H]
c.p.m.
(x 10-3)
Top
1
10
20
Fraction Number

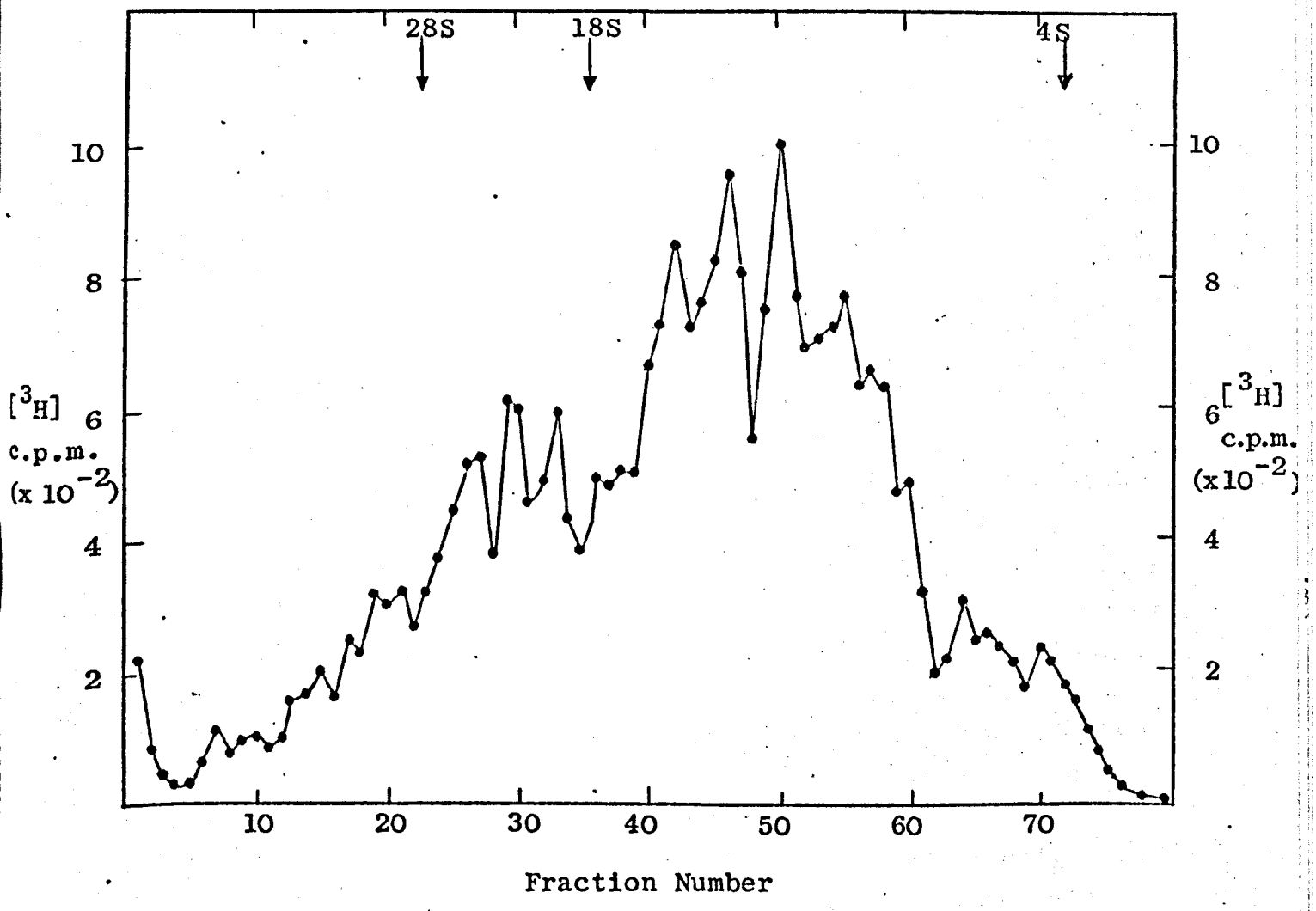
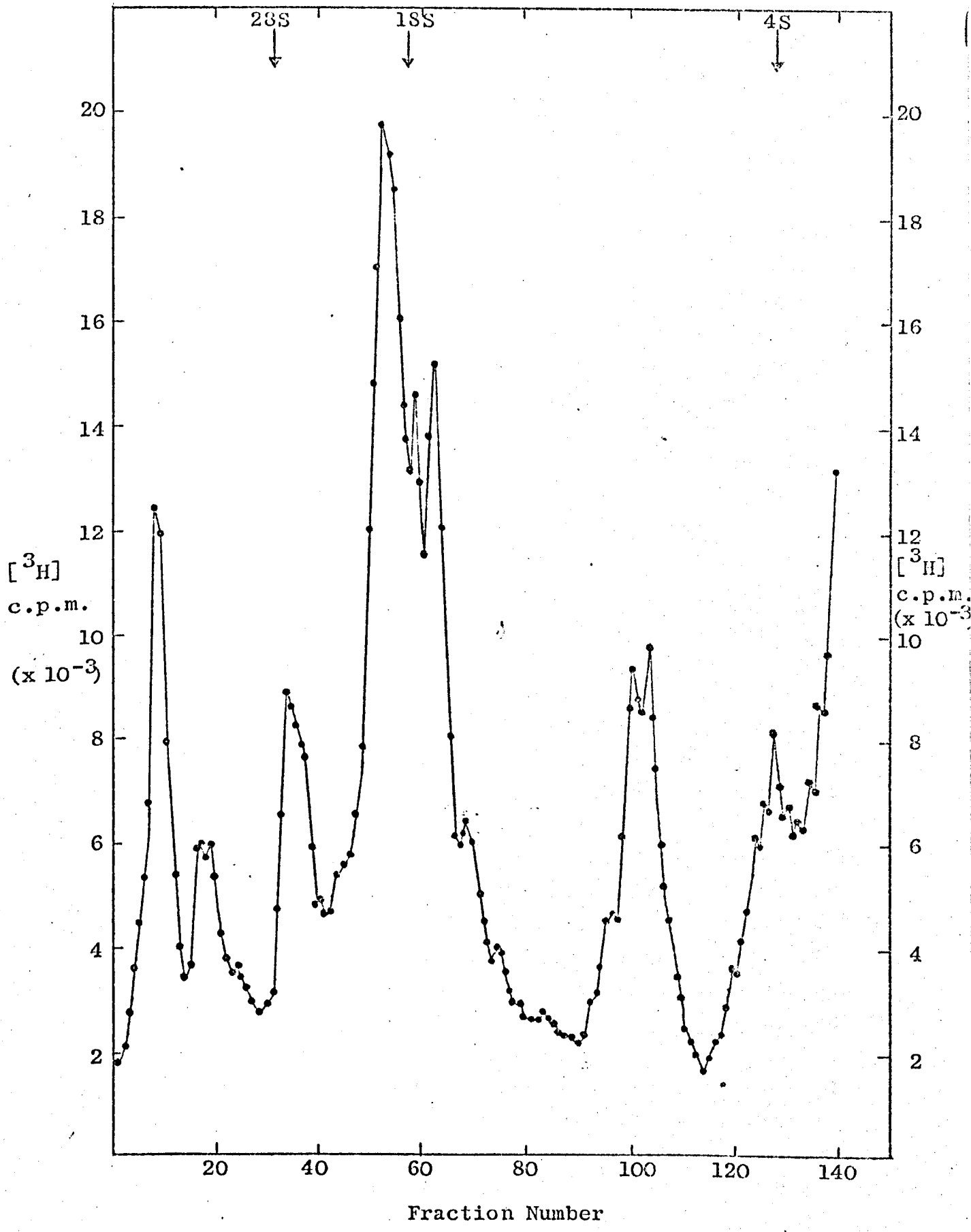


Figure 23. Polyacrylamide gel electrophoresis of [^{3}H]-polyribosomal associated RNA extracted from polyribosomes isolated from chick cells infected with NDV, strain Texas.* The RNA was fractionated on a 16 cm 2.2% polyacrylamide gel at 100V and 3mA/tube for 3 h. The arrows indicate the position of [^{32}P]-chick ribosomal RNA markers. (Loening system). *Conditions for infection and labelling of RNA were as described on page 38 of the Methods.

[^{3}H]-polyribosomal associated RNA was dissimilar to [^{3}H]-NDV vsi-RNA extracted from infected cell cytoplasm after distuption of the cells with Triton X-100 (Figure 24), and accounted for less than 1% of the radioactivity of this [^{3}H]-NDV vsi-RNA. The latter contained the usual RNA species, 50S, 35S, "22S" and "18S" RNAs found in infected cells (see Figures 11 and 15), plus some 8S RNA which could have resulted from degradation of the larger RNA species. However, only 24S and 22S RNAs of "22S" RNA, and 19S RNA of "18S" RNA were identifiable in [^{3}H]-polyribosomal associated RNA; these RNA species occurred at fractions 27, 29 and 33, respectively, in the gel electrophoretogram (Figure 23). Other RNA species, 13S (fraction 42), 11S (fraction 46), 10S (Fraction 50) and 8S (fraction 55) contained in [^{3}H]-polyribosomal associated RNA (Figure 23) were difficult to identify with any RNA species of [^{3}H]-NDV vsi-RNA from infected cell cytoplasm (Figure 24) because of the complexity of the gel electrophoretogram. These RNA species (i.e. 13S, 11S, 10S and 8S RNAs) could have resulted from degradation by RNA'ase and shearing forces during the preparation of polyribosomes, or from partial nucleolytic digestion of the presumptive NDV messenger RNA molecules (i.e. 35S, "22S" and "18S" RNAs) after being translated into viral polypeptides on the polyribosomes. [^{3}H]-polyribosomal associated RNA extracted in the same way from polyribosomes prepared from SFV infected cells also contained heterodisperse RNA (Dr. S.I.T. Kennedy, personal communication).

Blobel (1971) has demonstrated that the selective dissociation of ribosomes can be used to release messenger RNAs, which become detectable as free and protein-free molecules only after puromycin-induced disassembly of active ribosomes. Their subsequent isolation is possible because the only other naked RNA released is transfer RNA. All ribosomal RNAs are retained in the dissociated, but otherwise intact, functionally competent ribosomal subunits. Kennedy (1972) has used this method to dissociate polyribosomes from Semliki Forest virus (SFV) infected chick cells, and isolate an undegraded

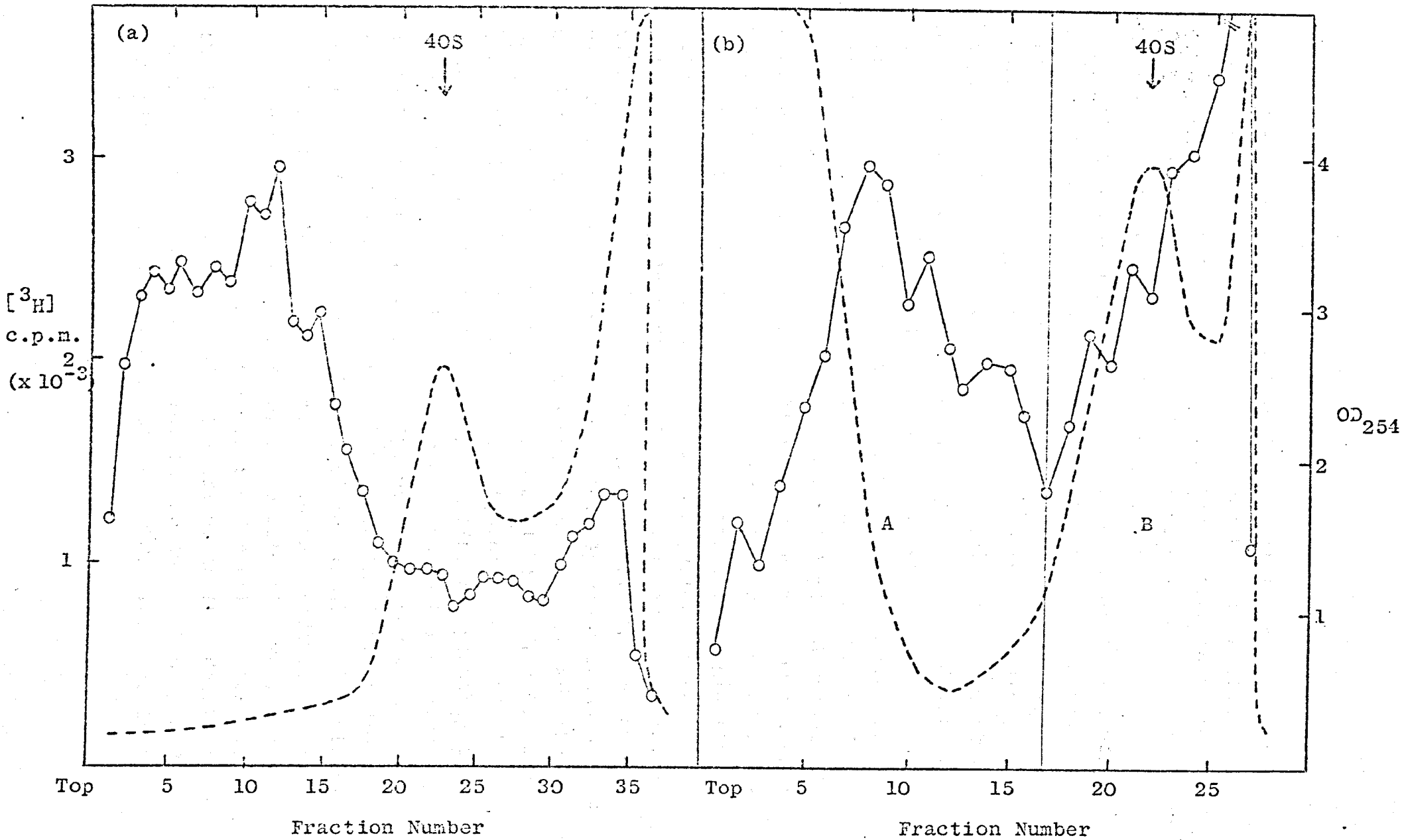
Figure 24. Polyacrylamide gel electrophoresis of [^{3}H]-ND (strain Texas) vsi-RNA extracted from infected cell cytoplasm.* Chick cells were disrupted with Triton X-100 (0.5% (v/v) final concentration) and the nuclei removed by centrifugation (2000g_{av} for 5 min at 4^{o}C). Infected cell cytoplasmic RNA was extracted with 1% SDS and phenol as described in the Methods. The RNA was fractionated on a 14cm 2.2% polyacrylamide gel at 100V and 3mA/tube for 2.35 h. The arrows indicate the position of [^{32}P]-chick ribosomal RNA species. (Loening system).
* Conditions for infection and labelling of RNA were as described on page 38 of the Methods.


28S
18S
4S
20
18
16
14
12
10
8
6
4
2
[3H]
c.p.m.
(x 10-3)
20
40
60
80
100
120
140
Fraction Number

26S RNA species. This he has suggested serves as the viral mRNA coding for the core protein and envelope protein, the two structural proteins, of SFV. Therefore, a similar attempt was made to isolate NDV mRNAs. Chick embryo cell monolayers (2.5 x 10^8 cells/petri dish) were infected with NDV, strain Texas (m.o.i. >10 p.f.u./cell). At 4.5h p.i. AMD was added to a final concentration of 2μg/ml. Then at 5.25h p.i. the maintenance medium was replaced with Earles-saline containing 2% (v/v) dialysed calf serum, AMD at 2μg/ml, [^{3}H]-uridine at 12.5μCi/ml, buffered to pH 7.3 with sodium bicarbonate (8 ml/petri dish). This medium was used in an attempt to increase the incorporation of [^{3}H]-uridine into ND vsi-RNA. The cells were harvested at 7.5h p.i., and polyribosomes isolated as described in the Methods. Polyribosome pellets were resuspended in ice-cold distilled water and combined. Compensating salt-buffer (2.25 ml of 1.0 M KCl, 0.1 M tris, 0.01 M $MgCl_2$, pH 7.5 at 20°C) was added to the total suspension (~1.8 ml). A small aliquot (0.45 ml) was withdrawn and 0.05 ml of water added. The rest of the suspension was made 2mM with respect to puromycin. Both preparations were incubated 15 min at 4°C, followed by 3 min at 37°C. Aliquots (0.3 ml) of each, and larger aliquots (1.8 ml) of puromycin treated material, were layered onto 12 ml linear 5 - 20% (w/v) sucrose gradients in 0.5 M KCl, 0.05 M tris, 0.005 M $MgCl_2$, pH 7.5 at 20°C, and centrifuged in a 6 x 14 ml titanium swing-out rotor at 38,000 rev/min (180,000g_{av}) for 2.5h at 4°C. A sucrose gradient containing puromycin treated polyribosomes unloaded at this stage showed that the 60S ribosomal subunit had sedimented to approximately half-way down the gradient. Although the optical density (OD_{254}) profile demonstrated that most polyribosomes had been disassembled to give ribosomal subunits and transfer RNA, the separation of the smaller subunit, 40S, from transfer RNA was insufficient. Therefore, the remaining gradients were centrifuged a further 7h at 27,500 rev/min (90,000g_{av}) at 4°C. Figure XI shows the OD_{254} sedimentation

Figure X1(a). Centrifugation of the polyribosome suspension treated with 0.5M KCl, 0.05M tris, 0.005M $MgCl_2$, pH 7.5 at 20°C, for 15 min at 4°C, followed by 3 min at 37°C, on a 5 - 20% (w/v) sucrose gradient containing the same buffer as described above. The conditions of NDV infection, radioactive labelling of vsi-RNA, and isolation of polyribosomes were as described in Results. An aliquot (0.3ml) of treated polyribosome suspension was centrifuged in a 5 - 20% (w/v) sucrose gradient at 180,000g_{av} for 2.5h, and then at 90,000g_{av} for 7h at 4°C. Fractions collected from the gradient were treated with 10% TCA and acid-insoluble [^{3}H]-radioactivity determined as described in Methods.
Both OD_{254} sedimentation, - - - - -, and acid-insoluble [^{3}H]-radioactivity, o——o, profiles have been normalised to the same sample volume (1.8 ml) as in (b) below. The arrow indicates the position of the 40S ribosomal subunit.

Figure X1(b). As (a), except that the polyribosome suspension was treated with 0.5M KCl, 0.05M tris, 0.005M $MgCl_2$, pH 7.5 at 20°C plus 2mM puromycin, and a 1.8 ml aliquot of treated polyribosome suspension was centrifuged in a 5 - 20% (w/v) sucrose gradient. OD_{254} ———, acid-insoluble [^{3}H]-radio activity o—o.


(a)
40S
(b)
40S
3
2
1
4
3
2
1
[3H]
c.p.m.
(x 10-3)
OD254
A
B
Top
5
10
15
20
25
30
35
Top
5
10
15
20
25
Fraction Number
Fraction Number

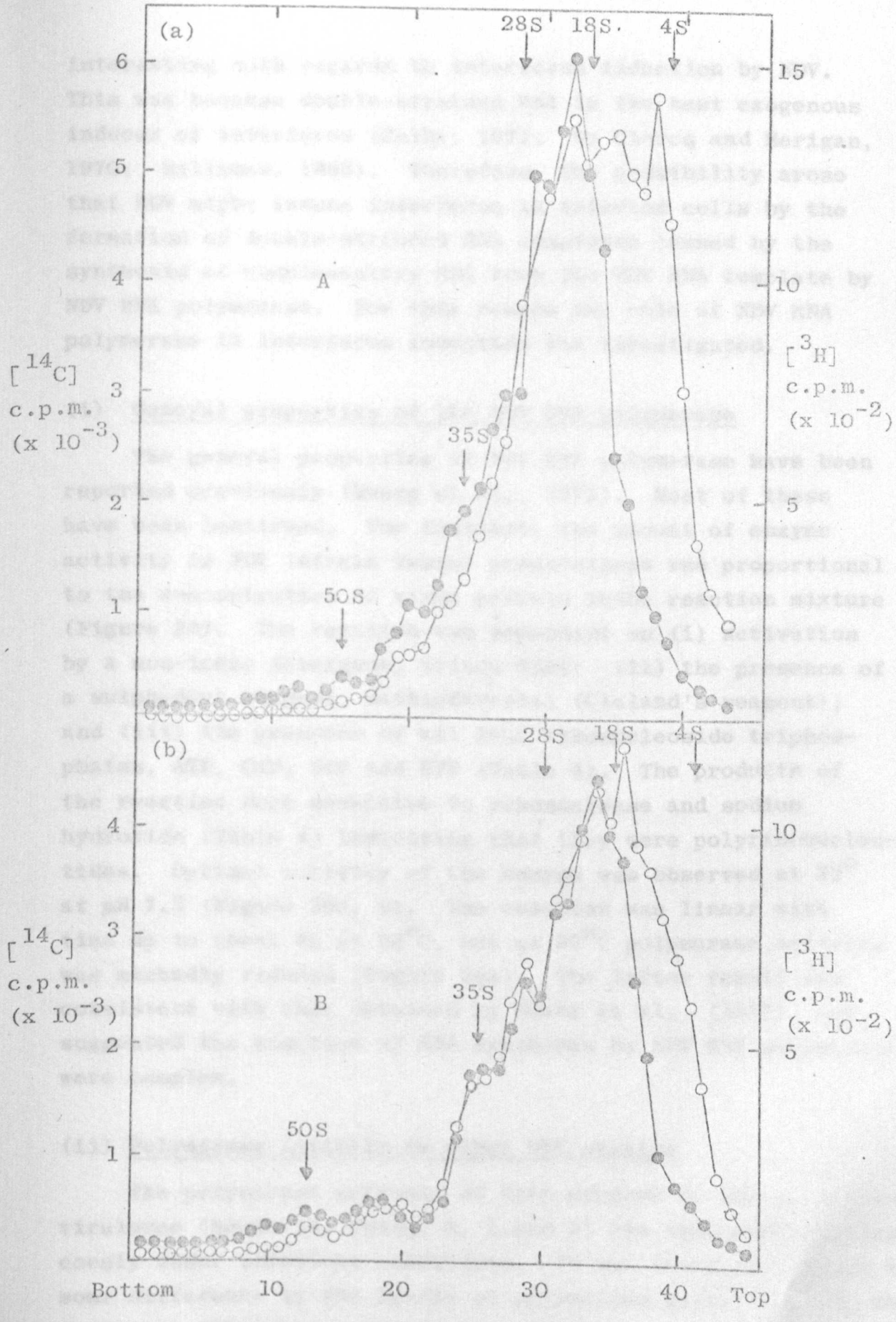
and [^{3}H]-acid-insoluble radioactivity profiles of (a) polyribosomes treated with high ionic strength buffer alone, and (b) polyribosomes treated with high ionic strength buffer plus puromycin. The observed dissociation into ribosomal subunits of material treated with high ionic strength buffer alone (Figure XI(a)) probably represented dissociation of non-active, chainless (i.e. no mRNA attached) ribosomes present in the polyribosome suspension (Blobel, 1971). There was no transfer RNA released under these conditions, so presumably the active ribosomes (polyribosomes) remained intact and sedimented to the bottom of the tube. However, there was some [^{3}H]-acid insoluble radioactivity released (Figure XI(a)). The majority of this sedimented more slowly than the 40S ribosomal subunit, and probably represented intact 18S, 22S and 35S RNAs of ND vsi-RNA plus degraded RNA derived from these species. It is unknown why, how and from what these RNA species were released. The presence of large amounts of transfer RNA at the top of the sucrose gradient after treatment of polyribosomes with high ionic strength buffer and puromycin indicated that active ribosomes (polyribosomes) had been disassembled (Figure XI(b)). The amount of 40S ribosomal subunit was approximately doubled as compared to non-puromycin treated polyribosomes (compare the OD_{254} sedimentation profile of Figure XI(b) to XI(a)). The [^{3}H]-acid insoluble radioactivity sedimenting more slowly than the 40S ribosome unit in Figure XI(b) was probably the same as that released by high ionic strength buffer alone, as in Figure XI(a). Surprisingly, puromycin caused no increase in the amount of this [^{3}H]-acid insoluble radioactivity. The majority of [^{3}H]-acid insoluble radioactivity specifically released by puromycin was fast sedimenting in the range 30 - 60S (Figure XI(b)). To determine what RNA species were contained in this fast sedimenting material, the fractions from an identical gradient to that described in Figure XI(b) were pooled from the region marked B, and precipitated with 2 vol. ethanol at -20°C. The fractions

from the region marked A (Figure XI(b)) were also pooled and ethanol precipitated. The precipitates were dissolved in 0.2% (w/v) SDS buffered to pH 7.4 with S buffer, [^{14}C]-ND vsi-RNA added as marker, and centrifuged in 5 - 25% (w/v) sucrose gradients as described in the Methods. Figure X2(a) and (b) represent the radioactive sedimentation profiles of [^{3}H]-RNA isolated from regions A and B, respectively. As expected, [^{3}H]-RNA isolated from region A probably contained 18S and 22S RNA, normally found in total ND vsi-RNA, plus a high proportion of RNA molecules sedimenting more slowly than 18S RNA, which were probably partially degraded virus specific RNAs (Figure X2(a)). The [^{3}H]-RNA isolated from region B contained all of the RNA species, i.e. 50S, 35S, 22S and 18S RNAs, found in total ND vsi-RNA, plus some RNA molecules sedimenting more slowly than 18S RNA (Figure X2(b)). The presence of 50S and 35S RNA might be expected as they would sediment as free RNA molecules into region B (Figure X1(b)). However, the presence of 22S and 18S RNA can only be explained if these were either still associated with the ribosomal sub-units, or complexed with protein so that they sediment faster than usual. Further work is needed to distinguish between these two possibilities.

NDV RNA dependent RNA polymerase

About eighteen months ago, Huang et al.. (1971) reported that NDV contained an enzyme that catalysed the incorporation of ribonucleotides into ribonucleic acid (RNA). This enzyme, NDV RNA-dependent RNA polymerase, has been shown to use NDV RNA as a template to synthesise RNA complementary in base sequence to NDV RNA (Huang et al., 1971). The isolation of partially double stranded structures, comprised of the template NDV RNA plus newly synthesised RNA, from the *in vitro* assay mixture for the enzyme (Huang et al. 1971) was particularly

Figure X2. Centrifugation of (a) [^{3}H]-RNA isolated from the pooled fractions of region A of the gradient depicted in Figure X1(b), and (b), [^{3}H]-RNA isolated from the pooled fractions of region B of same gradient, in a 5 - 25% (w/v) sucrose gradient buffered to pH 7.4 with S buffer. Both samples were combined with [^{14}C]-ND vsi-RNA isolated from chick cells infected with NDV, strain Texas, before centrifugation. [^{14}C]-ND vsi-RNA was obtained by incubation of one of the infected chick cell monolayer cultures used in the experiment described in Results with 50μCi of [^{14}C]-uridine instead of 100μCi of [^{3}H]-uridine. Total cellular RNA was extracted from these cells at 7.5h p.i. with 1% SDS and then as described in Methods. Fractions collected from each gradient were treated with 10% TCA and acid insoluble radioactivity determined as described in Methods. o——o, acid-insoluble [^{3}H]-radioactivity; ●——●, acid-insoluble [^{14}C]-radioactivity. The arrows show the position of chick cell ribosomal RNA species as determined from OD_{254} sedimentation profiles.


(a)
28S
18S
4S
A
35S
50S
[14C] c.p.m. (x 10-3)
[3H] c.p.m. (x 10-2)
(b)
28S
18S
4S
B
35S
50S
[14C] c.p.m. (x 10-3)
[3H] c.p.m. (x 10-2)
Bottom
10
20
30
40
Top
Fraction Number

interesting with regards to interferon induction by NDV. This was because double-stranded RNA is the best exogenous inducer of interferon (Colby, 1971; De Clercq and Merigan, 1970; Hilleman, 1968). Therefore, the possibility arose that NDV might induce interferon in infected cells by the formation of double-stranded RNA complexes caused by the synthesis of complementary RNA from the NDV RNA template by NDV RNA polymerase. For this reason the role of NDV RNA polymerase in interferon induction was investigated.

(i) General properties of the NDV RNA polymerase

The general properties of NDV RNA polymerase have been reported previously (Huang et al., 1971). Most of these have been confirmed. For instance, the amount of enzyme activity in NDV (strain Texas) preparations was proportional to the concentration of virus protein inthe reaction mixture (Figure 25). The reaction was dependent on (i) activation by a non-ionic detergent, Triton N101; (ii) the presence of a sulphydryl reagent, dithiothreitol (Cleland's reagent); and (iii) the presence of all four ribonucleoside triphosphates, ATP, CTP, GTP and UTP (Table 4). The products of the reaction were sensitive to ribonuclease and sodium hydroxide (Table 4) indicating that they were polyribonucleotides. Optimal activity of the enzyme was observed at 32^{o} at pH 7.3 (Figure 26a, b). The reaction was linear with time up to about 4h at 32^{o}C, but at 37^{o}C polymerase activity was markedly reduced (Figure 26a). The latter result was consistent with that obtained by Huang et al. (1971), and suggested the kinetics of RNA synthesis by NDV RNA polymerase were complex.

(ii) Polymerase activity in other NDV strains

The polymerase activity of five strains of NDV of different virulence (Herts 33, Texas, H, L and F) was measured simultaneously under identical conditions. It was found that there was some difference in the levels of polymerase activity among the different NDV strains (Table 5) which was reproducible, i.e.

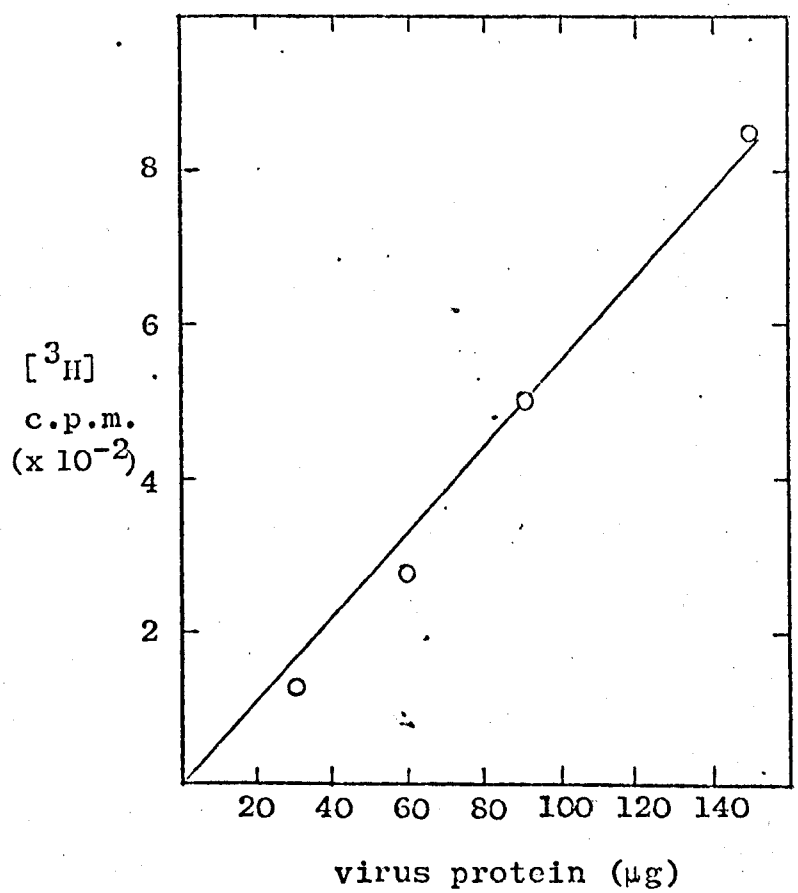


Figure 25. Dependence of NDV RNA-dependent RNA polymerase activity on amount of added virus protein. Amounts of NDV as shown were incubated for 2 h at 32°C in the standard reaction mixture and duplicate samples were then analysed for incorporation into acid-insoluble material as described in the Methods.

TABLE 4. REQUIREMENTS FOR NDV PARTICLE RNA POLYMERASE ACTIVITY†

Reaction mixture	c.p.m. per mg of virus protein
Complete	13,622
Complete incubated at 4°C	311
Minus ATP	825
Minus CTP	560
Minus UTP	1,915
Minus Triton N101	420
Minus dithiothreitol (Cleland's reagent)	500
*Plus ribonuclease (0.5μg)	500
*Plus sodium hydroxide (1M)	144

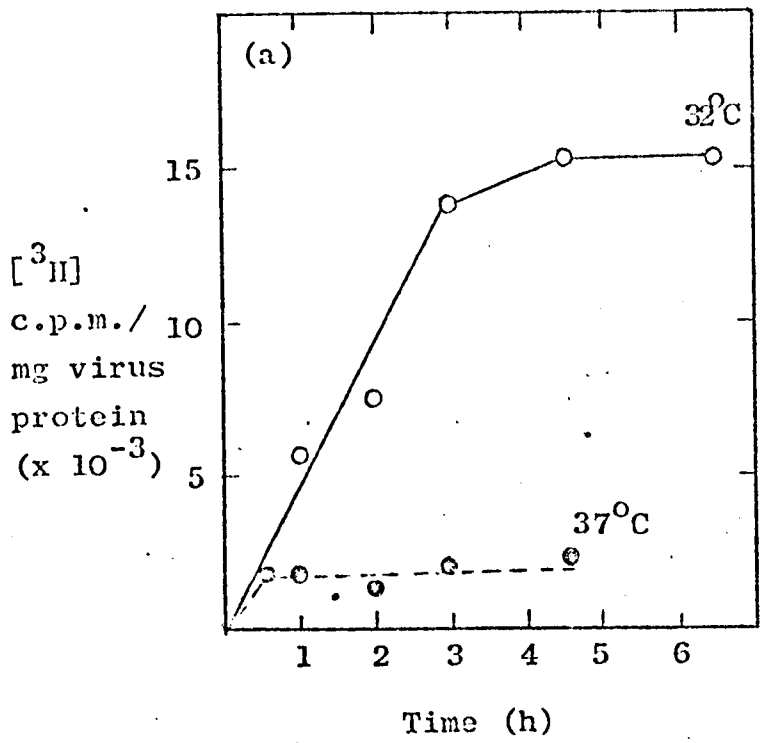
The reaction was carried out as described in the Methods section on page 54. An incubated sample without virions contained 40 c.p.m., and this value was subtracted from all experimental values.

*These reagents were added after incubation at 32°C for 2 h and in both cases incubation was continued for a further 15 min at 32°C before precipitation with trichloroacetic acid.

†The results shown in this table were obtained from joint-work carried out by the author and Dr. E. T. Sheaff (now at Department of Medical Microbiology, University of Toronto, Toronto, Ontario, Canada.).

Figure 26(a). Kinetics of NDV RNA-dependent RNA polymerase activity *in vitro*. Standard 0.23ml reaction mixtures were used containing 200µg of NDV protein and incubated for the indicated times at 32°C, o——o, or 37°C, ●——●.

(b). Dependence on pH of NDV RNA-dependent RNA polymerase activity. Standard reaction mixtures (0.23ml) with 10µmoles of tris at the different pH values and purified NDV (300µg) in TN buffers of corresponding pH values were incubated for 2 h at 32°C, and duplicate samples were then analysed for incorporation into acid-insoluble material as described in the Methods.


(a)
32°C
37°C
15
10
5
[3H] c.p.m./ mg virus protein (x 10-3)
1
2
3
4
5
6
Time (h)

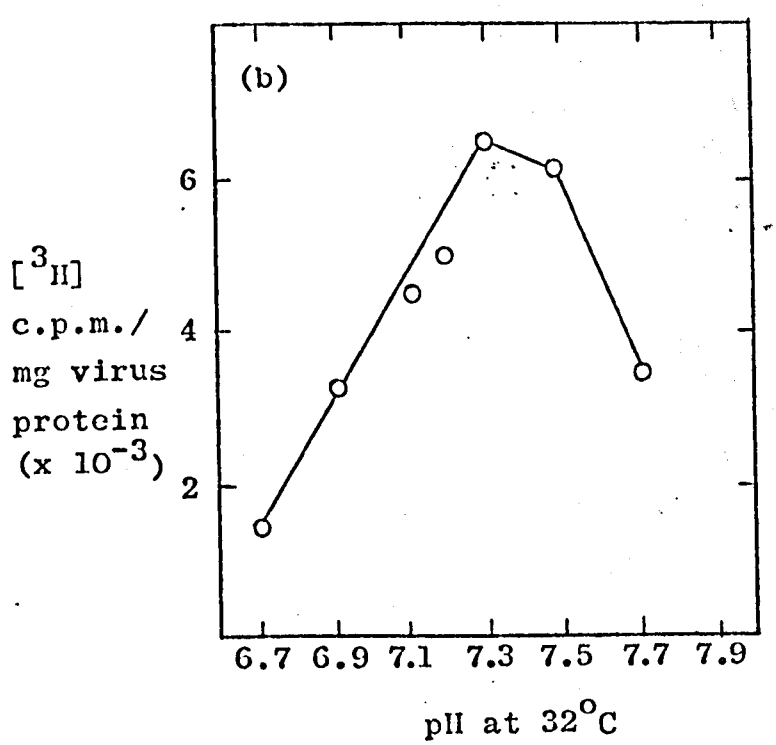

(b)
6
4
2
[3H] c.p.m./ mg virus protein (x 10-3)
6.7
6.9
7.1
7.3
7.5
7.7
7.9
pH at 32°C

TABLE 5. RNA POLYMERASE ACTIVITY OF DIFFERENT NDV STRAINS

NDV Strain	Virulence	c.p.m. per mg virus protein	c.p.m. per 10^{10} p.f.u.
Herts 33	Velogenic	10,500	2,800
Texas	Velogenic	21,400	3,300
L	Mesogenic	25,600	8,500
H	Mesogenic	2,100	740
* F	Lentogenic	8,600	-

All NDV strains were grown in embryonated eggs for 38 h and the allantoic fluid harvested after chilling the eggs for 4 h at 4°C. After simultaneous purification, the RNA polymerase activities of the concentrated virus suspensions were determined as described in the Methods section on page For each strain the radioactive value of virions incubated at 4°C in the reaction mixture was subtracted from the radioactive value of virions incubated at 32°C.

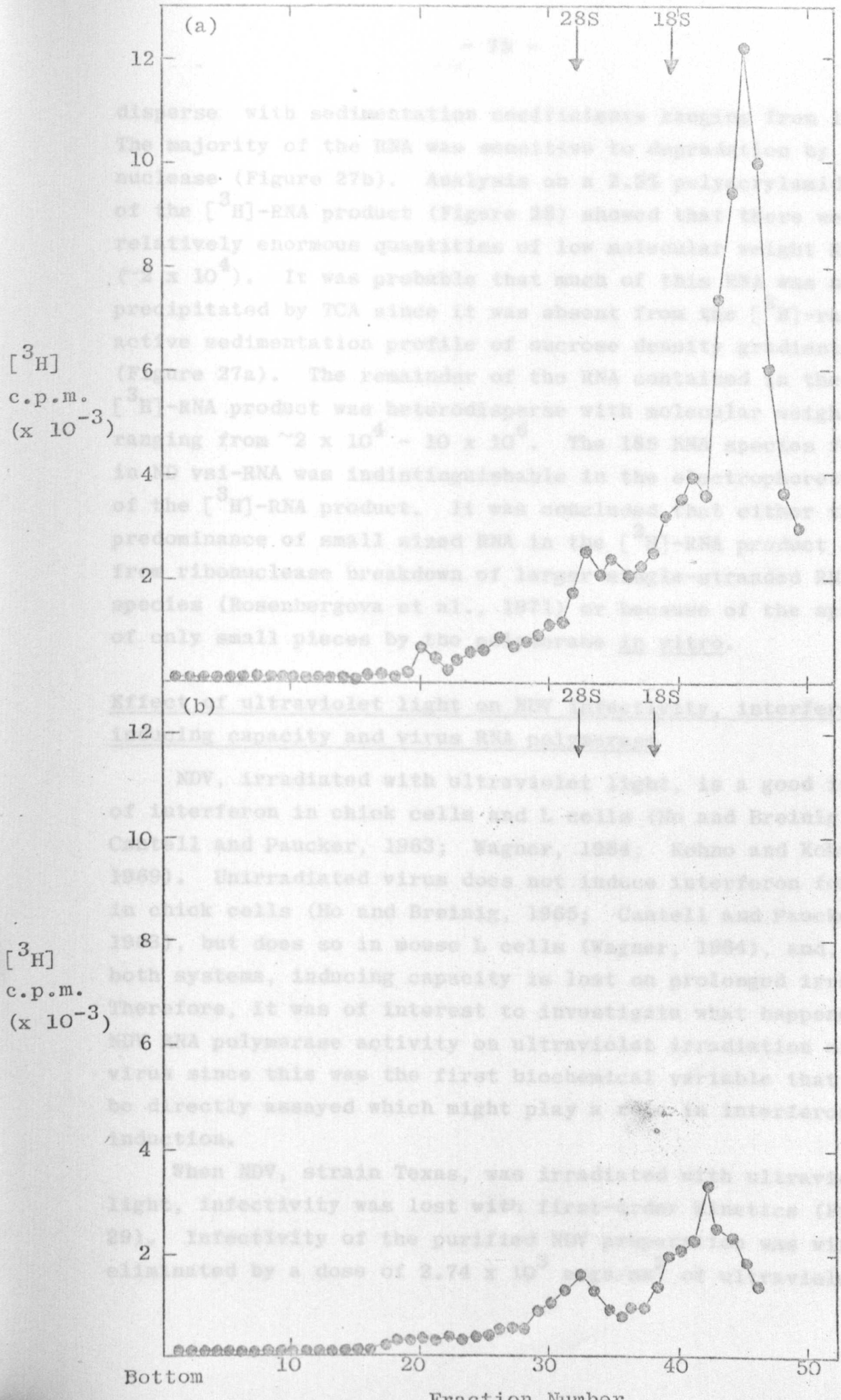
* The F strain is a non-plaque forming strain.

different preparations of the same NDV strain had a characteristic polymerase activity with respect to the other strains. This result was in contrast with that reported by Huang et al. (1971) who found one strain (NDV-HP) to have a polymerase specific activity of ~200 pmoles GMP incorporated per mg of virus protein per h, whilst three other strains (NDV - IM, NDV - RO and NDV - N) had lower enzyme specific activities of ~50 pmoles GMP per mg of virus protein per h. Enzyme specific activities as high as these were never found in any of the NDV strains used in this investigation, the highest being in NDV, strain L, which was approximately 9.5 pmoles per mg of virus protein per h. However, this discrepancy in polymerase specific activities might have resulted because of differences in purification methodology and the time taken for purifying virus. The latter was particularly important as polymerase activity of all NDV strains was unstable at 4^{o}. The decay of polymerase activity will be reported later in this section.

(iii) Products of the in vitro NDV RNA polymerase assay

Because of shortage of time, investigation into the products of the in vitro NDV RNA polymerase assay was not extensive. The volumes of reagents in the assay mixture were suitably scaled up (8 X), and the mixture incubated at 32^{o}C for 4h to obtain maximum incorporation of [^{3}H]-GMP. The RNA products were extracted using 1% SDS and then shaking with phenol as described for the extraction of NDV RNA on page 37 of the Methods section. This was precipitated with two volumes of ethanol at -20^{o}C using chick cell ribosomal RNA as carrier. The RNA was re-dissolved in a small volume of S buffer or 100mM NaCl, 50mM tris, 1mM EDTA, pH 7.4 at 20^{o}C, and analysed on 5 - 25% linear sucrose density gradients and 2.2% polyacrylamide gels, respectively. Figure 27a shows the sedimentation profile of the [^{3}H]-RNA products extracted from the polymerase assay mixture. The predominant feature was the presence of a large amount of slowly sedimenting RNA (~ 8S) of molecular weight ~1 x 10^{5}. Higher molecular weight RNA was hetero-

Figure 27. Sucrose density gradient analysis of NDV RNA-dependent RNA polymerase products labelled for 4 h at 32°C. The volumes of reagents in the in vitro assay mixture were suitably scaled up (8X) with the final mixture containing 2.4 mg NDV protein. After incubation for 4 h at 32°C, RNA was extracted with 1% SDS and phenol and precipitated with ethanol at -20°C using chick cell ribosomal RNA as carrier. Equal amounts of [^{3}H]-RNA in S buffer were centrifuged in 5 - 25% (w/v) sucrose density gradients buffered to pH 7.4 with S buffer. Fractions collected from the gradients were either (a) treated immediately with 10% TCA, or (b) treated with ribonuclease before TCA precipitation. Acid-insoluble radioactivity was determined in both cases. The arrows indicate the position of chick cell ribosomal RNA markers.


(a)
28S
18S
12
10
8
6
4
2
[3H]
c.p.m.
(x 10-3)
(b)
28S
18S
12
10
8
6
4
2
[3H]
c.p.m.
(x 10-3)
Bottom
10
20
30
40
50
Fraction Number

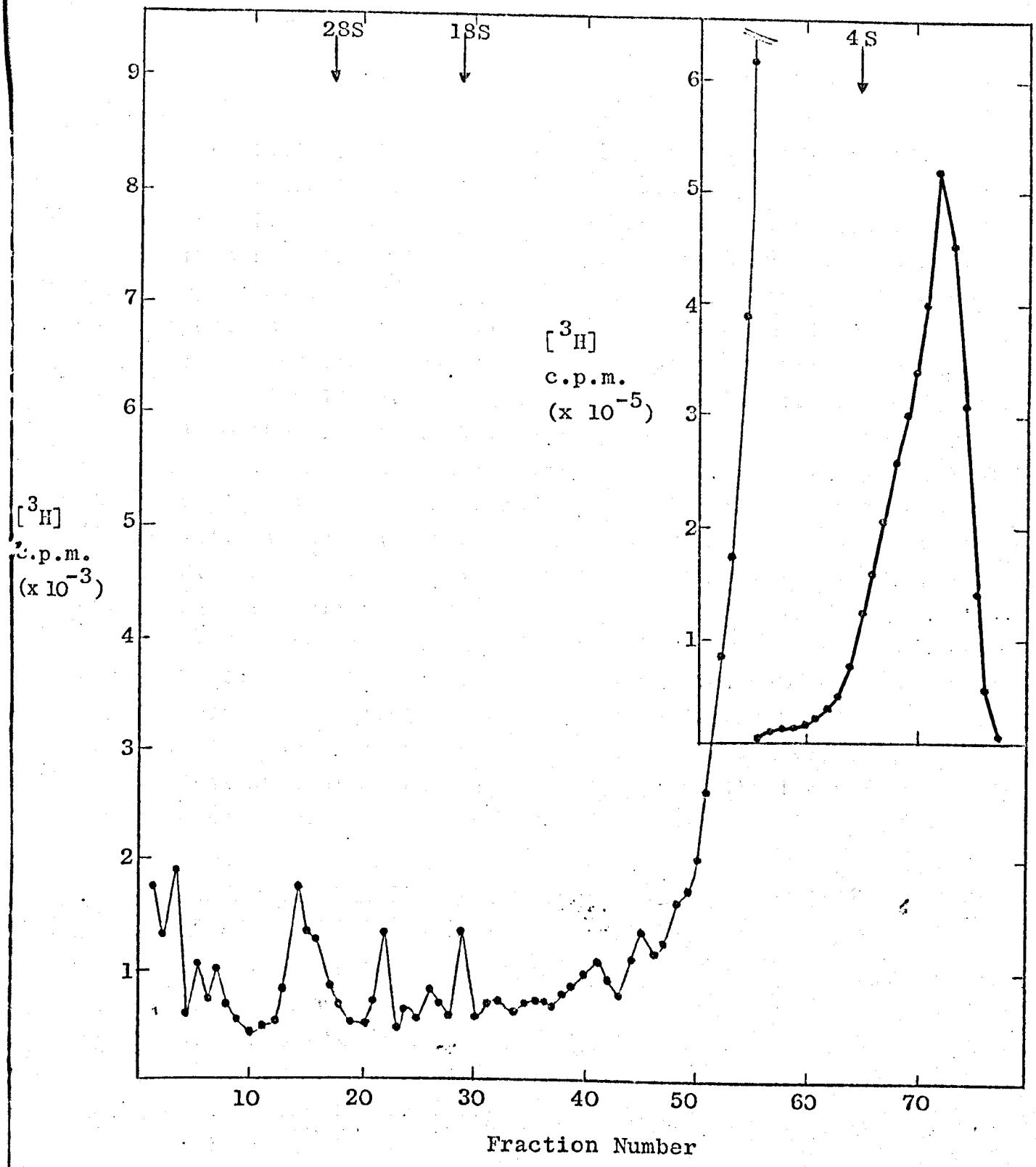
disperse with sedimentation coefficients ranging from 15 - 49S. The majority of the RNA was sensitive to degradation by ribonuclease (Figure 27b). Analysis on a 2.2% polyacrylamide gel of the [^{3}H]-RNA product (Figure 28) showed that there were relatively enormous quantities of low molecular weight RNA (~2 x 10^4). It was probable that much of this RNA was not precipitated by TCA since it was absent from the [^{3}H]-radioactive sedimentation profile of sucrose density gradients (Figure 27a). The remainder of the RNA contained in the [^{3}H]-RNA product was heterodisperse with molecular weights ranging from ~2 x 10^4 - 10 x 10^6. The 18S RNA species found in ND vsi-RNA was indistinguishable in the electrophoretogram of the [^{3}H]-RNA product. It was concluded that either the predominance of small sized RNA in the [^{3}H]-RNA product resulted from ribonuclease breakdown of larger single-stranded RNA species (Rosenbergova et al., 1971) or because of the synthesis of only small pieces by the polymerase *in vitro*.

Effect of ultraviolet light on NDV infectivity, interferon inducing capacity and virus RNA polymerase

NDV, irradiated with ultraviolet light, is a good inducer of interferon in chick cells and L cells (Ho and Breinig, 1965; Cantell and Paucker, 1963; Wagner, 1964; Kohno and Kohase, 1969). Unirradiated virus does not induce interferon formation in chick cells (Ho and Breinig, 1965; Cantell and Paucker, 1963), but does so in mouse L cells (Wagner, 1964), and, in both systems, inducing capacity is lost on prolonged irradiation. Therefore, it was of interest to investigate what happened to NDV RNA polymerase activity on ultraviolet irradiation of the virus since this was the first biochemical variable that could be directly assayed which might play a role in interferon induction.

When NDV, strain Texas, was irradiated with ultraviolet light, infectivity was lost with first-order kinetics (Figure 29). Infectivity of the purified NDV preparation was virtually eliminated by a dose of 2.74 x 10^3 ergs/mm^2 of ultraviolet

Figure 28. Polyacrylamide gel electrophoresis of NDV RNA-dependent RNA polymerase products labelled for 4 h at 32°C. Reaction mixture and conditions of assay were as described in Figure 27. The polymerase products ([^{3}H]-RNA) were fractionated on a 15 cm 2.2% polyacrylamide gel by electrophoresis at 115V and 3mA/tube for 2.65h. The arrows indicate the position of [^{32}P]-chick cell ribosomal RNA markers. Note that the inset scale for radioactivity only applies to the dots joined by red-line. (Loening system).


28S
18S
4S
[3H]
c.p.m.
(x 10-3)
[3H]
c.p.m.
(x 10-5)
Fraction Number

irradiation. Interferon inducing capacity (Figure 29) was not apparent until the NDV preparation had received more than 1.53×10^3 ergs/mm^2 of ultraviolet irradiation, and then rose to a maximum with increasing doses of irradiation up to 3.66×10^3 ergs/mm^2 (1 min under UV lamp). Longer periods of UV irradiation led to a slower decline of interferon inducing capacity, and this was virtually eliminated by 2.26×10^4 ergs/mm^2 (6 min under UV lamp). Investigation into the effect of UV irradiation on NDV RNA polymerase activity showed that initially polymerase activity was lost rapidly (but at a rate 10X slower than the rate of loss of infectivity) with increasing doses of UV irradiation up to 3.66×10^3 ergs/mm^2 (Figure 29). This was followed by a slower decline in polymerase activity with longer periods of UV irradiation. After 2.26×10^4 ergs/mm^2 (6 min under UV lamp) of UV irradiation, approximately 10% of the original polymerase activity remained. From Figure 29 it is seen that polymerase activity and interferon inducing capacity were lost at similar rates, suggesting that the two were possibly connected.

Effect of β-propiolactone (BPL) on NDV infectivity, interferon inducing capacity and virus RNA polymerase

β-propiolactone (BPL) is an alkylating agent which has long been used as a virucide and in the preparations of vaccines (Mangun et al., 1951; LoGrippo, 1960). It has been reported to render Sendai virus non-infective while leaving unaffected the capacity of the virus to fuse cells rapidly in culture (Neff and Enders, 1968; Pedreira and Tauraso, 1969; Wainberg et al., 1971). Its primary site of action, therefore, is almost certainly on the viral genome (Prinzie et al., 1960). The effect of BPL inactivation of NDV on interferon production was investigated as a result of a suggestion of B. Lomniczi (Veterinary Medical Research Institute of the Hungarian Academy of Sciences, Budapest, Hungary). It was found that by using very low concentrations of BPL (0 - 0.016%) an infectivity

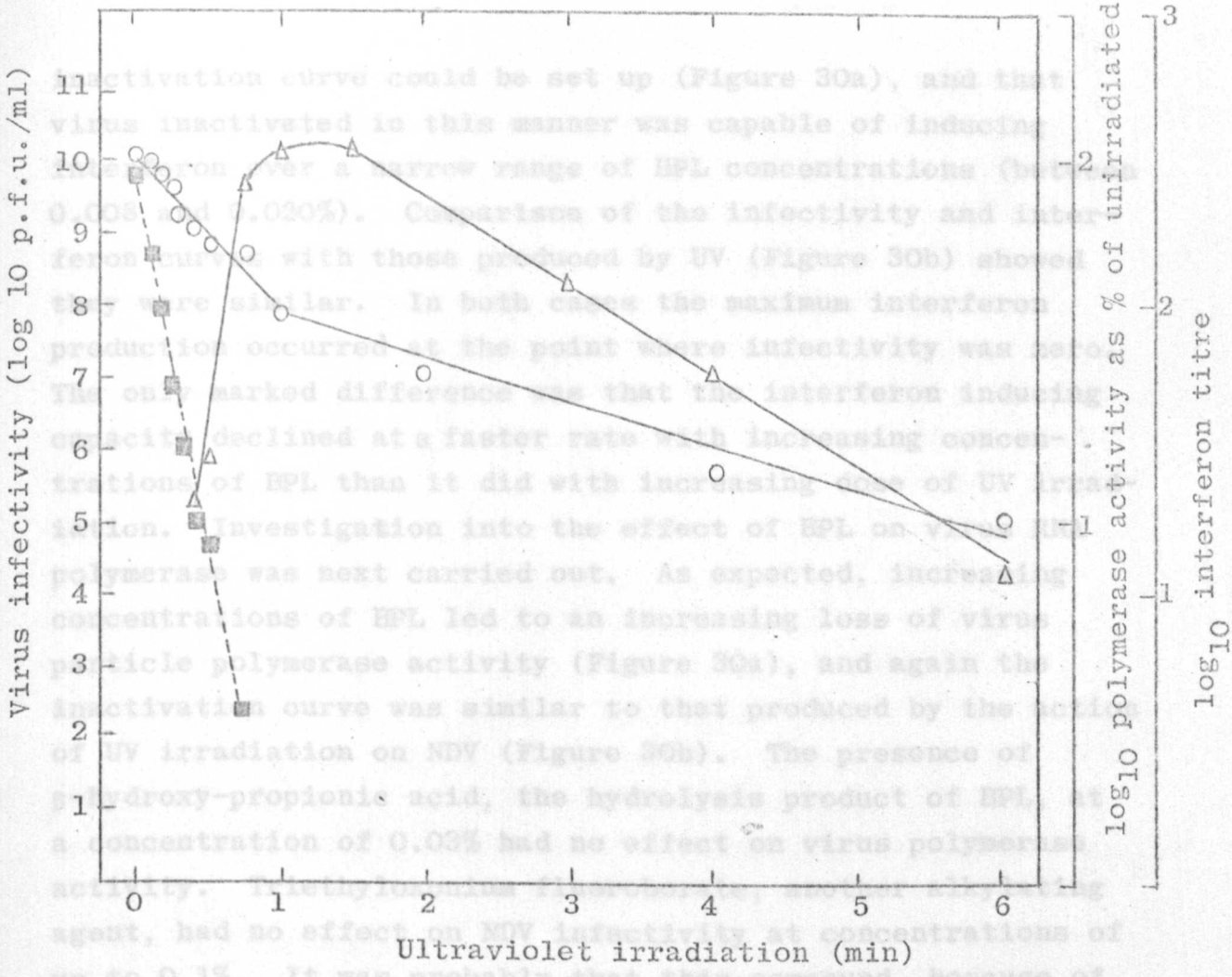


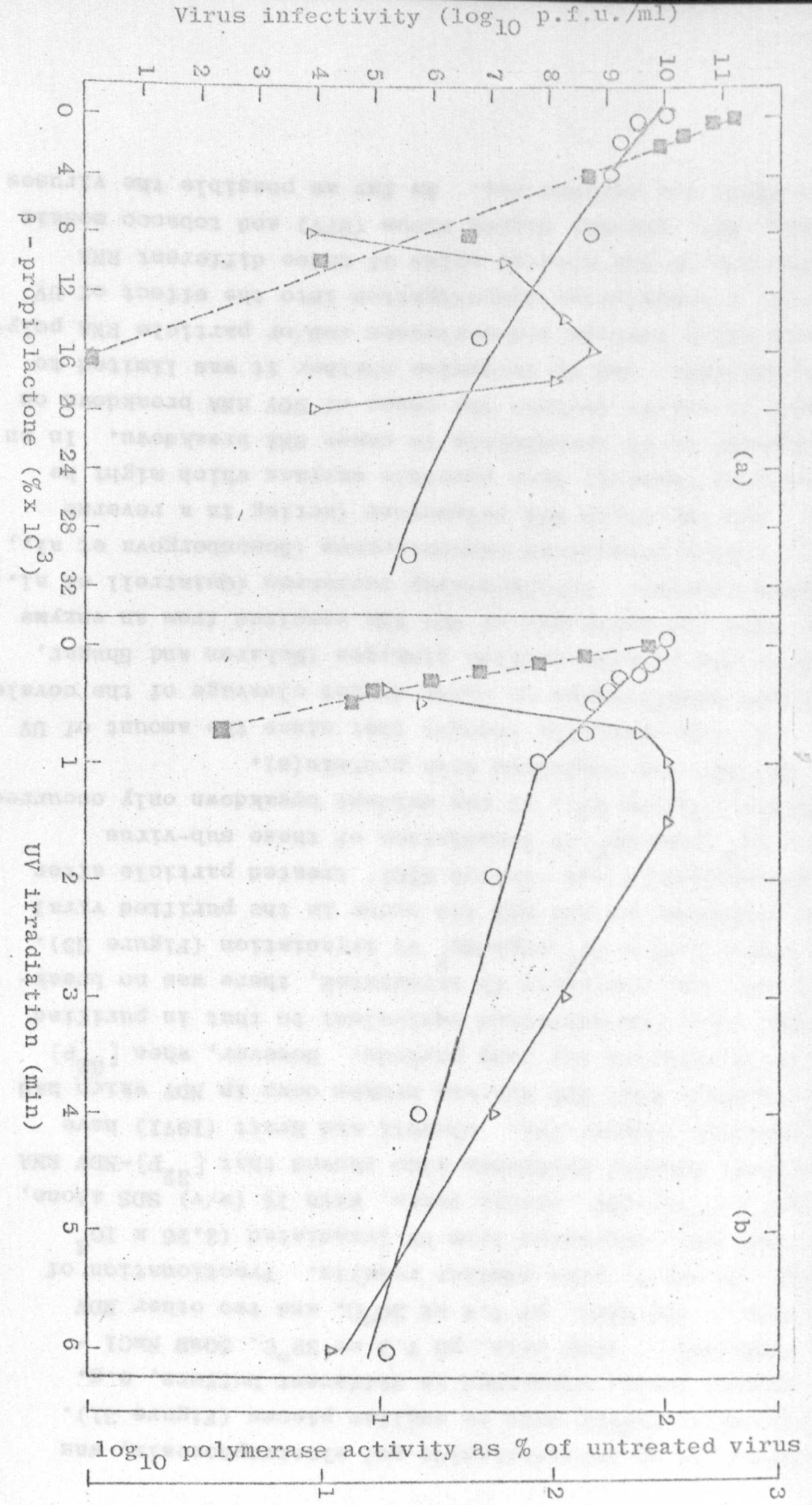
Figure 29. Effect of UV-irradiation on the infectivity (■- - - -■), interferon-inducing capacity (Δ———Δ), and RNA-dependent RNA polymerase activity (o——o) of purified NDV (Texas strain). The specific activity of the RNA polymerase of unirradiated virus was 1.2×10^4 c.p.m./mg virus protein. The virus received 61 ergs/mm^2/s UV-irradiation.

inactivation curve could be set up (Figure 30a), and that virus inactivated in this manner was capable of inducing interferon over a narrow range of BPL concentrations (between 0.008 and 0.020%). Comparison of the infectivity and interferon curves with those produced by UV (Figure 30b) showed they were similar. In both cases the maximum interferon production occurred at the point where infectivity was zero. The only marked difference was that the interferon inducing capacity declined at a faster rate with increasing concentrations of BPL than it did with increasing dose of UV irradiation. Investigation into the effect of BPL on virus RNA polymerase was next carried out. As expected, increasing concentrations of BPL led to an increasing loss of virus particle polymerase activity (Figure 30a), and again the inactivation curve was similar to that produced by the action of UV irradiation on NDV (Figure 30b). The presence of β-hydroxy-propionic acid, the hydrolysis product of BPL, at a concentration of 0.03% had no effect on virus polymerase activity. Triethyloxonium fluoroborate, another alkylating agent, had no effect on NDV infectivity at concentrations of up to 0.1%. It was probably that this compound, because of its ionic nature, was unable to penetrate the viral envelope which contained lipid. On the other hand, BPL was lipid soluble, and was able to attack NDV RNA after penetration through the viral envelope.

Effect of ultraviolet irradiation on the nucleic acid of NDV particles

The effect of UV irradiation on the nucleic acid of NDV particles was investigated as it had been suggested by Gandhi and Burke (1970) that the decline in interferon inducing capacity of NDV after prolonged UV irradiation (Figure 29) might be due to breakdown of NDV RNA. It was shown using [^{32}P]-labelled NDV that extraction of [^{32}P] NDV RNA from purified [^{32}P]-NDV, strain Texas, which had been UV irradiated with doses greater than 3.66×10^3 ergs/mm^2, followed by

Figure 30. Effect of (a) β-propiolactone and (b) UV-irradiation on the infectivity (□ ---□), interferon-inducing capacity (Δ——Δ), and RNA-dependent RNA polymerase activity (o——o) of purified NDV (strain Texas). Figure 30(b) is the same as Figure 29 and reproduced here for comparative purposes. The specific activity of the RNA polymerase of untreated virus in (a) was 1.43×10^4 c.p.m./mg. virus protein.


Virus infectivity ($\log_{10}$ p.f.u./ml)
β - propiolactone (% x 10^3)
(a)
(b)
UV irradiation (min)
$\log_{10}$ polymerase activity as % of untreated virus
$\log_{10}$ interferon titre

fractionation by polyacrylamide gel electrophoresis, was progressively broken down to smaller pieces (Figure 31). NDV, strain Texas, suspended in different buffers, e.g. PBS, 30mM NaCl - 10mM tris, pH 7.3 at 32°C, 50mM NaCl - 10mM tris - 1mM EDTA, pH 7.4 at 20°C, and two other NDV strains, H and F, gave similar results. Fractionation of [^{32}P]-NDV RNA, extracted from UV irradiated (2.26×10^4 ergs/mm^2) [^{32}P]-NDV, strain Texas, with 1% (w/v) SDS alone, on sucrose density gradients also showed that [^{32}P]-NDV RNA was degraded (Figure 32). Clavell and Bratt (1971) have also reported that NDV RNA was broken down in NDV which had been UV irradiated for long periods. However, when [^{32}P] NDV RNA, at a concentration equivalent to that in purified [^{32}P]-NDV, was similarly UV irradiated, there was no breakdown after 2.26×10^4 ergs/mm^2 UV irradiation (Figure 33). Since breakdown of NDV RNA did occur in the purified viral ribonucleoprotein and 'Triton N101' treated particle after 2.26×10^4 ergs/mm^2 UV irradiation of these sub-virus particles (Figure 33), it was evident breakdown only occurred when NDV RNA was complexed with protein(s).

It was initially thought that since the amount of UV light was insufficient to cause direct cleavage of the covalent bonds of the internucleotide linkages (McLaren and Shugar, 1964) that the breakdown of NDV RNA resulted from an enzyme mediated process. Contaminating nucleases (Quintrell et al., 1971), virion associated endonucleases (Rosenbergova et al., 1971), and the virus RNA polymerase (acting in a reverse nucleolytic fashion) were possible enzymes which might be "activated" on UV irradiation to cause RNA breakdown. In an attempt to define further the cause of NDV RNA breakdown on UV irradiation, and to determine whether it was limited to viruses which contain endonucleases and/or particle RNA polymerases, a comparative investigation into the effect of UV irradiation on the nucleic acids of three different RNA viruses, NDV, Semliki Forest virus (SFV) and tobacco mosaic virus (TMV) was carried out. As far as possible the viruses

Figure 31. The effect of ultraviolet irradiation on the molecular weight of RNA extracted from the Texas strain of NDV. Virus nucleic acid was extracted from purified irradiated virus and fractionated on 2.2% polyacrylamide gels as described in the Methods. The arrows indicate the position of [^{3}H]-chick cell ribosomal RNA markers. (a), Unirradiated control; (b), 15s irradiation; (c), 30s irradiation; (d), 60s irradiation; (e), 180s irradiation; (f), 360s irradiation. Purified virus received 61 ergs/mm^{2}/s UV-irradiation. (Loening system was used for polyacrylamide gel electrophoresis).

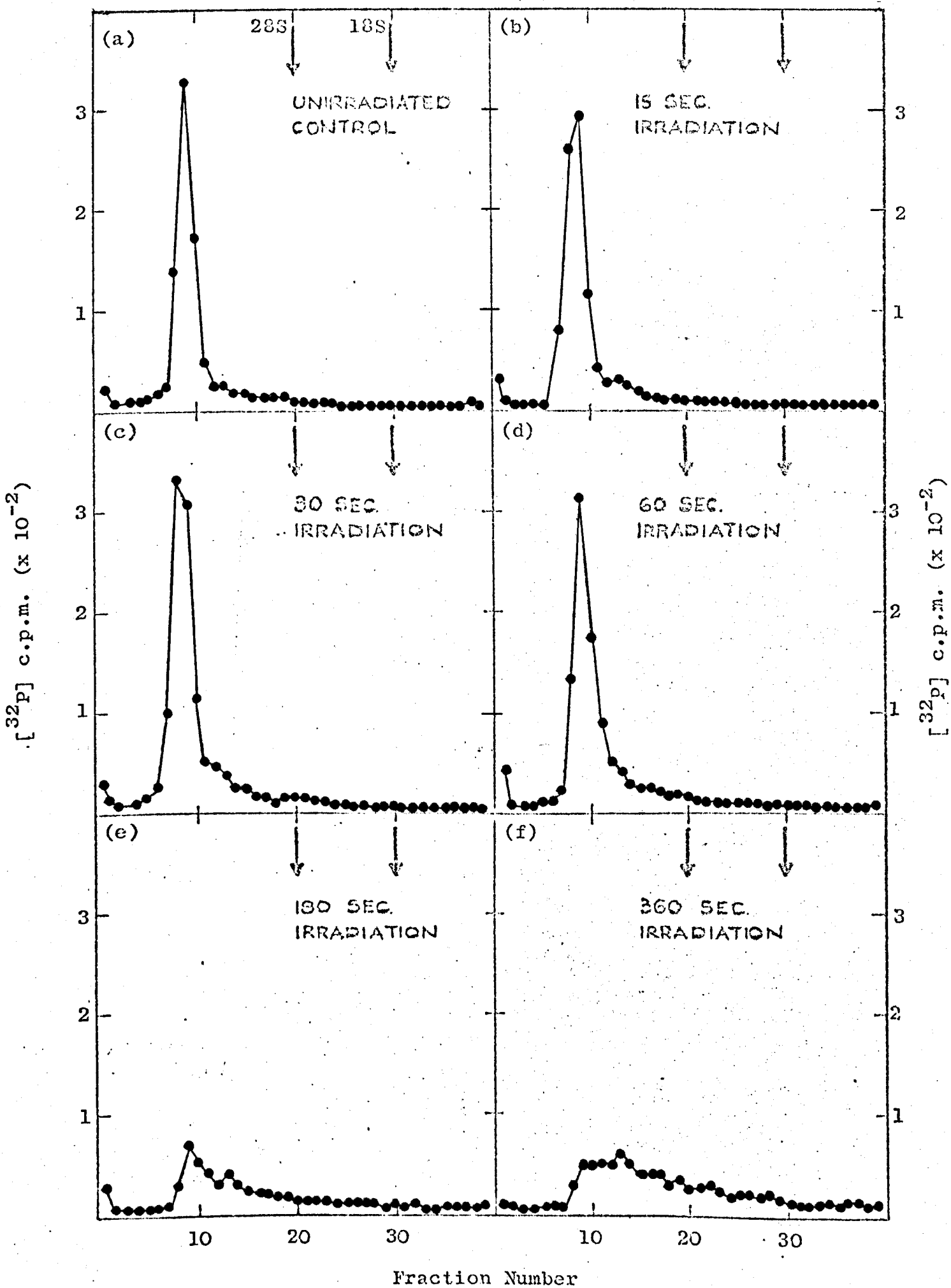

(a)
28S
18S
UNIRRADIATED CONTROL
(b)
15 SEC. IRRADIATION
(c)
30 SEC. IRRADIATION
(d)
60 SEC. IRRADIATION
(e)
180 SEC. IRRADIATION
(f)
360 SEC. IRRADIATION
[32P] c.p.m. (x 10-2)
Fraction Number

Figure 32. The effect of ultraviolet irradiation on the molecular weight of RNA extracted from the Texas strain of NDV. Virus nucleic acid was extracted from purified irradiated virus using 1% SDS alone and centrifuging on 5 - 25% (w/v) sucrose density gradients buffered to pH 7.4 with S buffer as described in the Methods. The arrows show the position of added chick ribosomal RNA markers. (a), Unirradiated control; (b), 360s irradiation. Purified virus received 61 ergs/mm^2/s UV-irradiation.

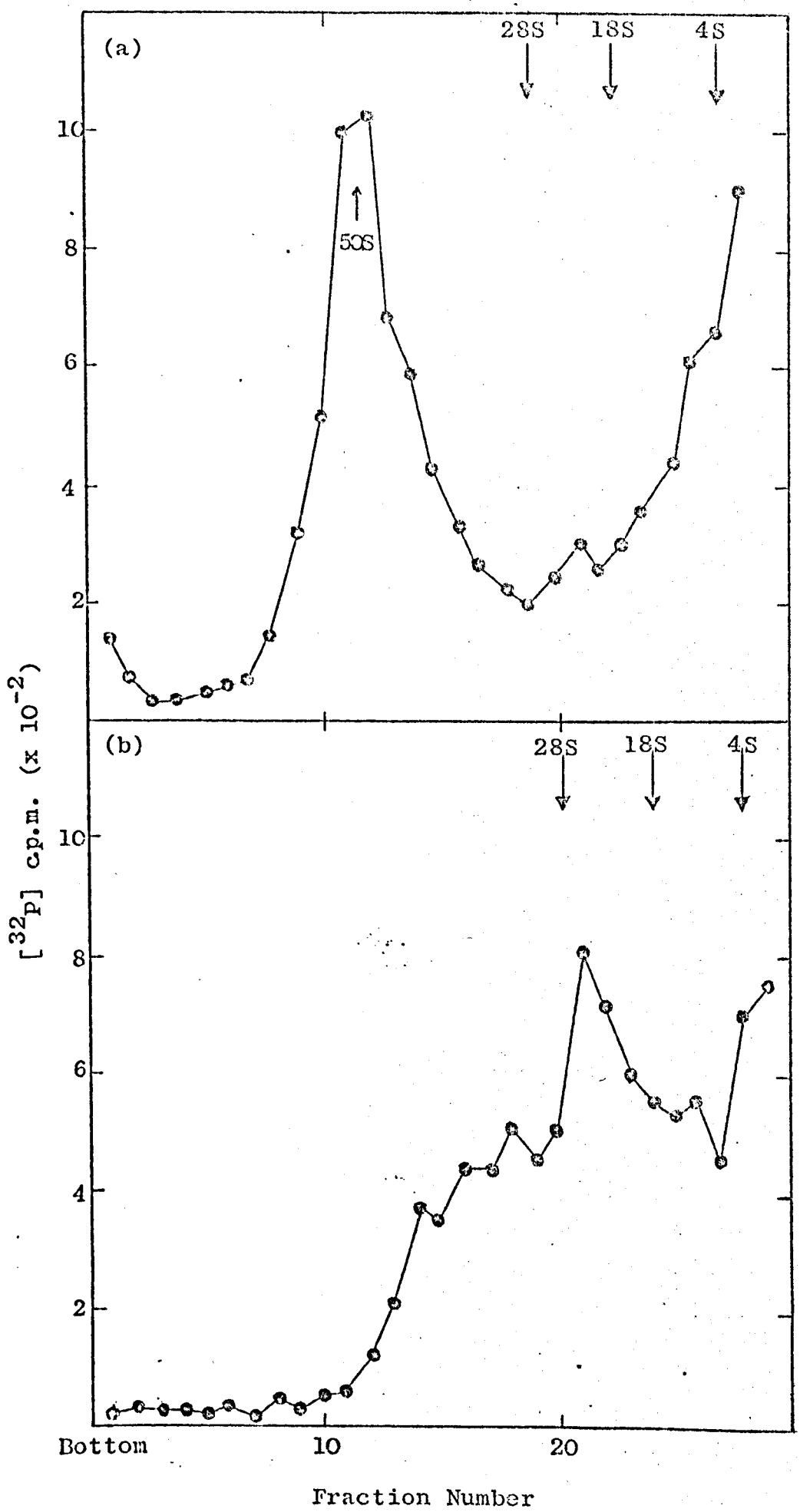
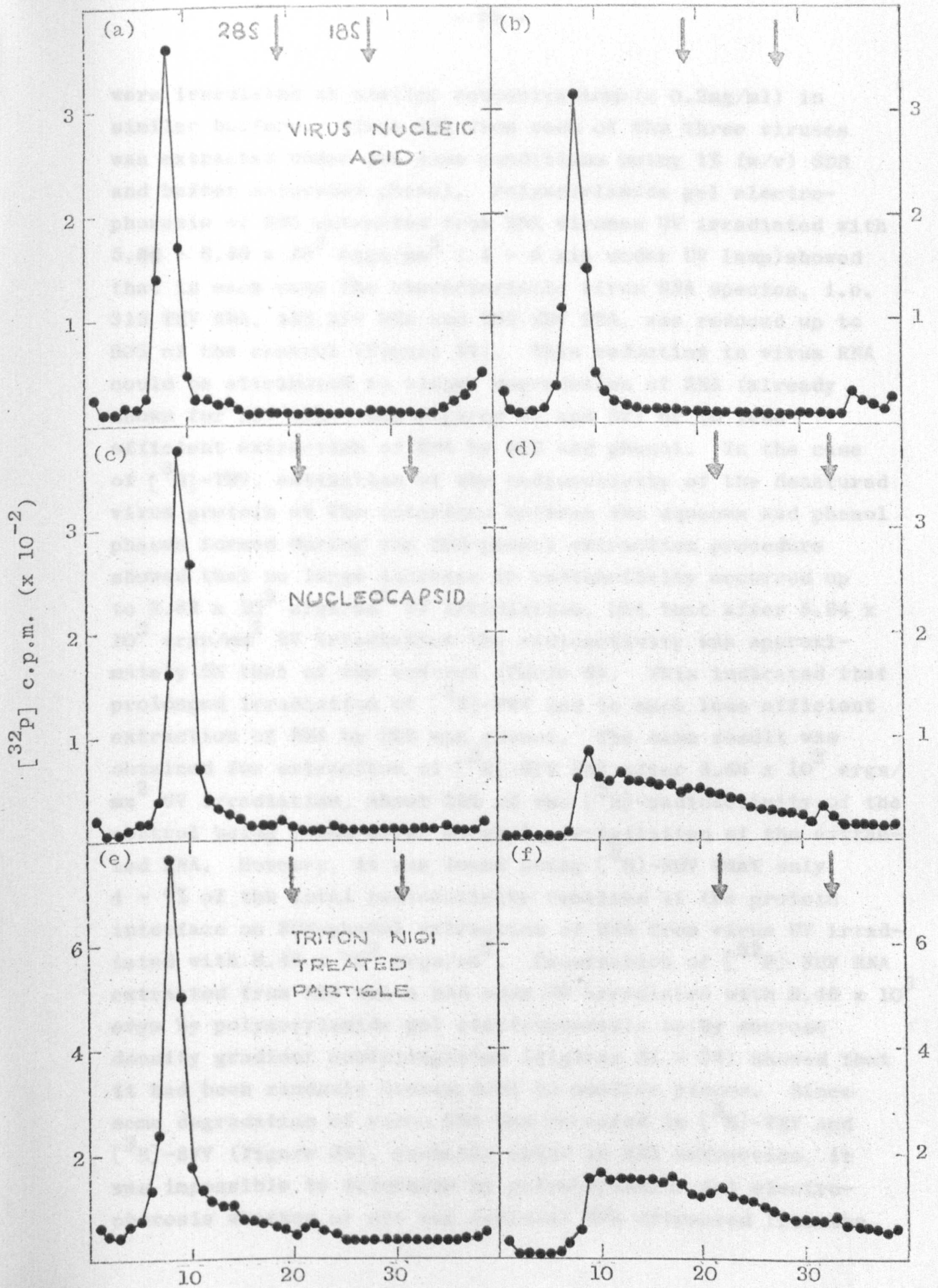
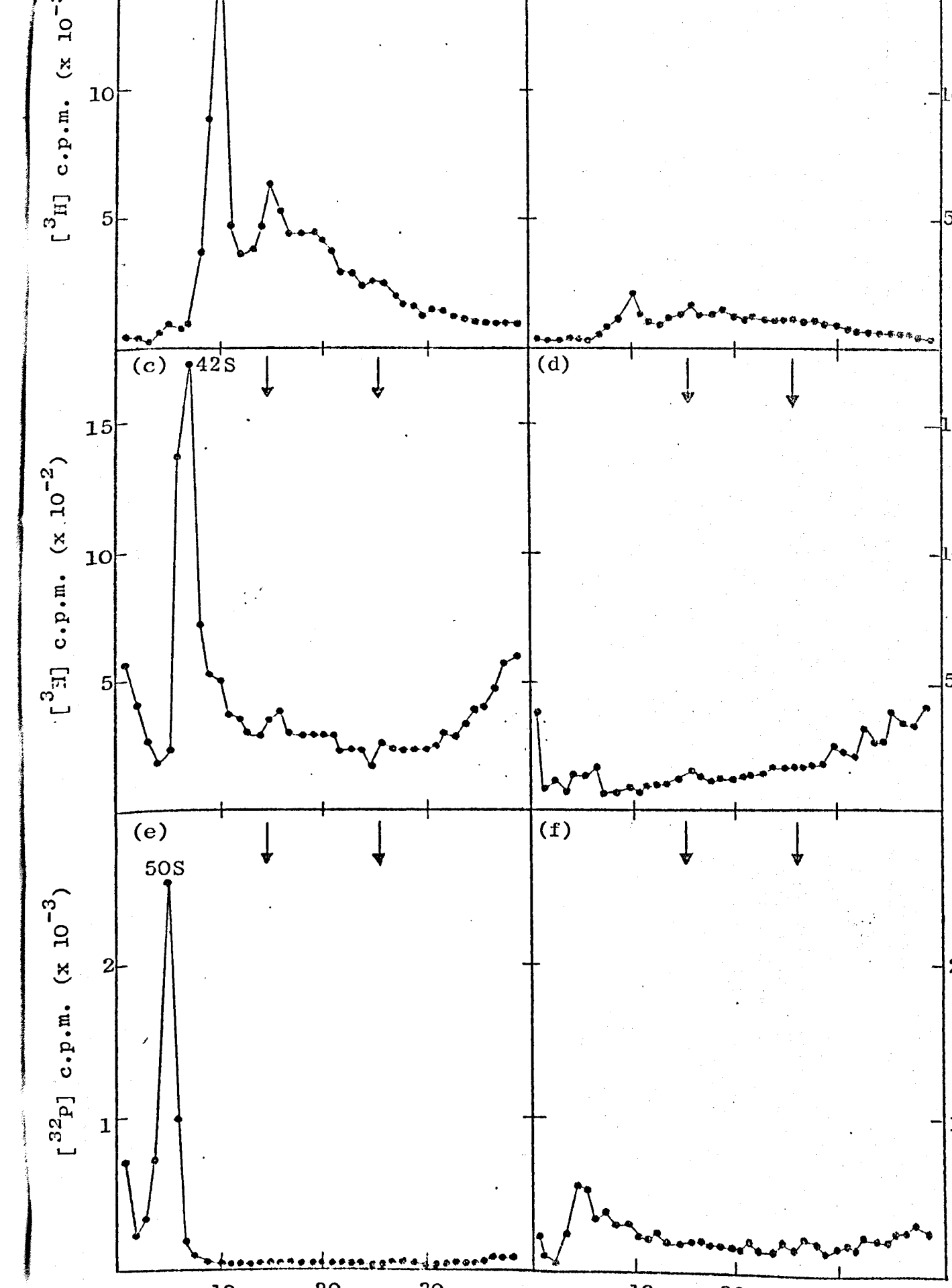

(a)
28S
18S
4S
50S
10
8
6
4
2
(b)
28S
18S
4S
10
8
6
4
2
[32P] cp.m. (x 10-2)
Bottom
10
20
Fraction Number

Figure 33. The effect of ultraviolet light on the molecular weight of NDV RNA, and on the RNA extracted from the virus' nucleocapsids and the 'Triton N101'-treated particles. The nucleic acid was fractionated by polyacrylamide gel electrophoresis (2.2% gels) before (a, c and e) and after 360s irradiation (b, d and f). Virus nucleic acid was extracted from virus particles before irradiation (a and b), or from the virus' nucleocapsids obtained from purified virus as described in the Methods (c and d), or from the sub-viral particles obtained by treatment of purified virus with 0.08% (w/v) Triton N101 at 37°C for 20 min (e and f). The latter ('Triton N101'-treated particles) were separated from detergent as described in the Methods. The arrows show the position of the [^{3}H]-chick cell ribosomal RNA markers. (Loening system).


(a)
28S
18S
VIRUS NUCLEIC ACID
(b)
(c)
NUCLEOCAPSID
(d)
(e)
TRITON NIOI TREATED PARTICLE
(f)
[32P] c.p.m. (x 10-2)
Fraction Number

were irradiated at similar concentrations (< 0.2mg/ml) in similar buffers. Virus RNA from each of the three viruses was extracted under the same conditions using 1% (w/v) SDS and buffer saturated phenol. Polyacrylamide gel electrophoresis of RNA extracted from RNA viruses UV irradiated with 5.64 - 8.46 x 10^3 ergs/mm^2 (4 - 6 min under UV lamp)showed that in each case the characteristic virus RNA species, i.e. 31S TMV RNA, 45S SFV RNA and 50S NDV RNA, was reduced up to 90% of the control (Figure 34). This reduction in virus RNA could be attributed to either degradation of RNA (already shown for NDV RNA - see Figures 31 and 32) or to less efficient extraction of RNA by SDS and phenol. In the case of [^{3}H]-TMV, estimation of the radioactivity of the denatured virus protein at the interface between the aqueous and phenol phases formed during the SDS-phenol extraction procedure showed that no large increase in radioactivity occurred up to 2.82 x 10^3 ergs/mm^2 UV irradiation, but that after 5.64 x 10^3 ergs/mm^2 UV irradiation the radioactivity was approximately 5X that of the control (Table 6). This indicated that prolonged irradiation of [^{3}H]-TMV led to much less efficient extraction of RNA by SDS and phenol. The same result was obtained for extraction of [^{3}H]-SFV RNA after 8.46 x 10^3 ergs/mm^2 UV irradiation, about 25% of the [^{3}H]-radioactivity of the control being found after alcohol precipitation of the extracted RNA. However, it was found using [^{3}H]-NDV that only 4 - 7% of the total radioactivity remained at the protein interface on SDS-phenol extraction of RNA from virus UV irradiated with 8.46 x 10^3 ergs/mm^2. Examination of [^{32}P]- NDV RNA extracted from NDV which had been UV irradiated with 8.46 x 10^3 ergs by polyacrylamide gel electrophoresis or by sucrose density gradient centrifugation (Figures 31 - 34) showed that it had been randomly brokem down to smaller pieces. Since some degradation of virus RNA had occurred in [^{3}H]-TMV and [^{3}H]-SFV (Figure 34), probably prior to RNA extraction, it was impossible to determine by polyacrylamide gel electrophoresis whether or not the residual RNA extracted from the

Figure 34. Polyacrylamide gel electrophoresis (2.2% gels) of RNA extracted from unirradiated [^{3}H] TMV (a), [^{3}H] SFV (c), [^{32}P] NDV (e), and from [^{3}H] TMV after 4 min UV-irradiation (b), [^{3}H] SFV (d) and [^{32}P] NDV (f) after 6 min UV-irradiation. Purified RNA viruses received 23.5 ergs/mm^{2}/s UV-irradiation. The arrows show the position of the [^{3}H]-or [^{32}P]-chick ribosomal RNA markers. (Loening system).


(c)
42S
(d)
(e)
50S
(f)
[3H] c.p.m. (x 10-2)
[32P] c.p.m. (x 10-3)
10
5
15
10
5
2
1

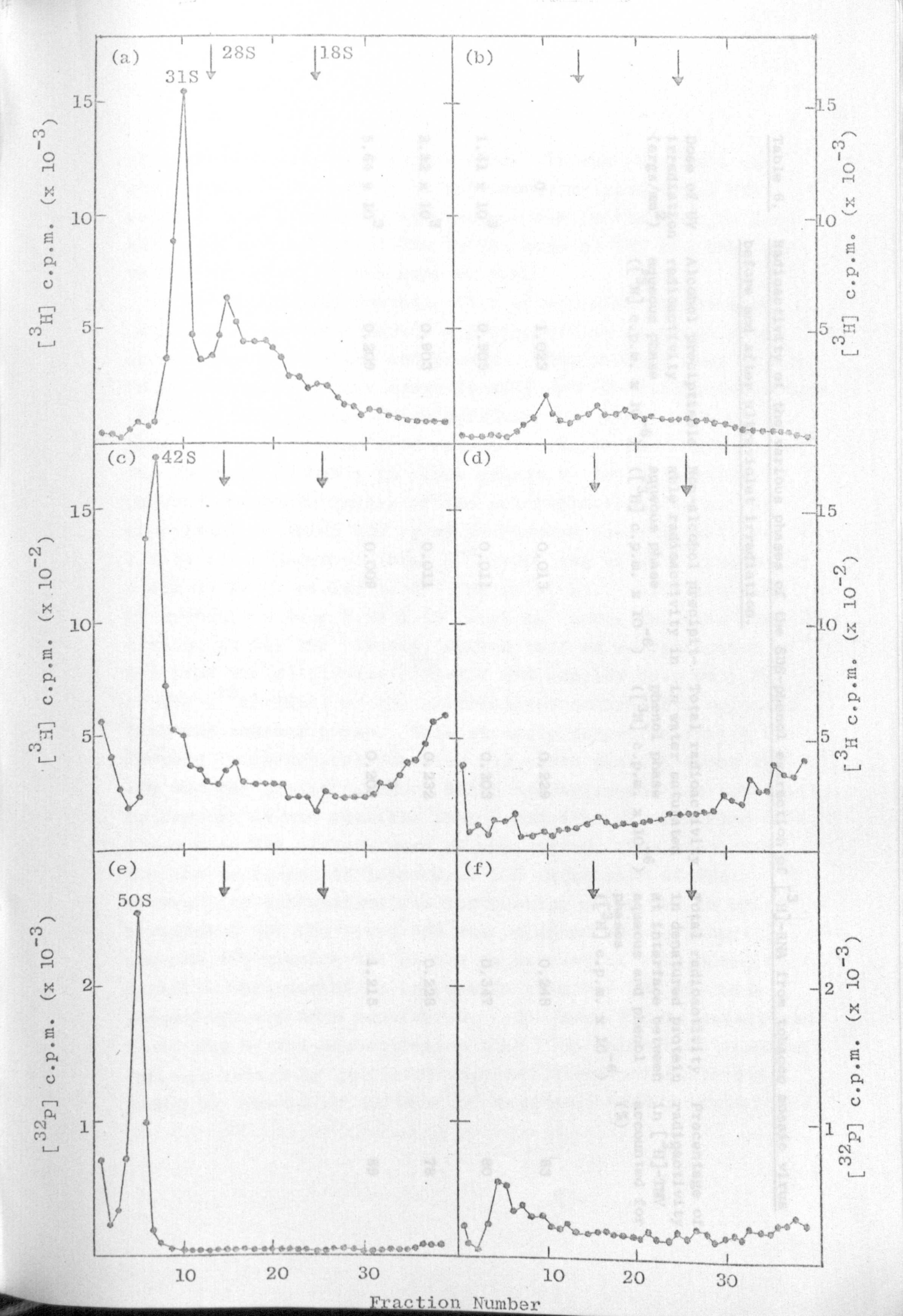

(a)
28S
18S
31S
(b)
(c)
42S
(d)
(e)
50S
(f)
[3H] c.p.m. (x 10-3)
[3H] c.p.m. (x 10-2)
[32P] c.p.m. (x 10-3)
15
10
5
2
1
10
20
30
Fraction Number

Table 6. Radioactivity of the various phases of the SDS-phenol extraction of [^{3}H]-RNA from tobacco mosaic virus before and after ultraviolet irradiation.

Dose of UV irradiation (ergs/mm^2)	Alcohol preciptiable radioactivity aqueous phase ([^{3}H] c.p.m. x 10^{-6})	Non-alcohol precipitable radioactivity in aqueous phase ([^{3}H] c.p.m. x 10^{-6})	Total radioactivity in water saturated phenol phase ([^{3}H] c.p.m. x 10^{-6})	Total radioactivity in denatured protein at interface between aqueous and phenol phases ([^{3}H] c.p.m. x 10^{-6})	Precentage of radioactivity in [^{3}H]-TMV accounted for (%)
0	1.023	0.013	0.229	0.248	83
1.41×10^3	0.905	0.011	0.203	0.347	80
2.82×10^3	0.903	0.011	0.232	0.228	78
5.64×10^3	0.209	0.009	0.203	1.215	89

irradiated viruses was broken down. It was concluded that the reason for reduction of the characteristic virus RNA species from irradiated viruses was due in the main to less efficient extraction of RNA in the case of TMV and SFV, and to RNA breakdown in the case of NDV.

It was thought possible that ultraviolet irradiation of RNA viruses might induce a photochemical reaction which cross-linked virus RNA and protein. The protein most likely to be involved in this cross-linking was the ribonucleoprotein (RNP) or core protein which surrounds the RNA. On a charge basis the basic amino acid residues (lysine and arginine) of the RNP were probably in close proximity to the negatively charged phosphate groups of the polynucleotide chain. An experiment in which 300 µg of polylysine (average mol. wt. = 1×10^5) was added to 135µg [^{32}P]-NDV RNA at 22°C, incubated 5 min at 22°C, cooled to 4°C (Idoine et al., 1971) and then UV-irradiated with 8.46×10^3 ergs/mm^2 under the same conditions as the RNA viruses, showed that on re-extraction of RNA from the polylysine [^{32}P]-NDV RNA complex that only 5% of the [^{32}P]-label of the unirradiated control was released into the aqueous phase. This strongly suggested that a UV-induced photochemical reaction had taken place between the RNA and the lysine residues which cross-linked them covalently. By analogy it was possible that a reaction of this kind took place when RNA viruses were UV irradiated, thus accounting for the decreased efficiency of SDS extraction of RNA. However, no explanation was forthcoming as to why in UV irradiated NDV the virus RNA was released and as heterogeneous sub-genomic RNA pieces on extraction with SDS. The possible involvement of enzymes in this NDV RNA breakdown phenomenon was next considered. Nuclease contamination was ruled out by the demonstration that [^{3}H] chick cell ribosomal RNA was intact by polyacrylamide gel electrophoresis after being UV irradiated (8.46×10^3 ergs/mm^2) with purified NDV.

Rosenbergova et al. (1971) have reported that NDV preparations do contain endonuclease activity, but probably at the temperature of UV irradiation (4 - 10°C) used in this series of experiments endonuclease activity was negligible. Kingsbury and Darlington (1968) have shown that NDV nucleocapsids were resistant to ribonuclease, and therefore the participation of endonucleases, contaminating or virion associated, in breaking down NDV RNA was considered to be very remote. Both the virus and the sub-viral particle prepared by treatment of NDV with Triton N101 contain RNA polymerase activity before UV irradiation (see Table 8). Robinson (1971b) has reported similar findings for Sendai virus and sub-viral particles including nucleocapsids which apparently had about 30% of the polymerase activity of the virus. NDV RNA polymerase activity was absent or present at an unmeasurable level in the nucleocapsids prepared from purified NDV as described on page 36. This fact coupled with the demonstration that NDV RNA polymerase activity was destroyed by UV irradiation (Figure 29) made the involvement of polymerase in breaking down NDV RNA unlikely.

Effect of β-propiolactone on the nucleic acid of NDV particles

Since UV light causes the breakdown of NDV RNA within purified virions this could be a contributing cause to the loss of interferon inducing capacity on prolonged UV irradiation of the virus (see Figure 29). However, treatment of [^{32}P] NDV with 0.025% BPL, a concentration at which interferon inducing capacity was eliminated, and then extraction followed by fractionation of [^{32}P]-NDV RNA by polyacrylamide gel electrophoresis, showed that it was not broken down (Figure 35a). This indicated that breakdown of virus RNA was not responsible for the decline of interferon inducing capacity in BPL-inactivated virus. Also since interferon was not induced by virus treated with this concentration of BPL, NDV RNA cannot itself be the inducer of interferon.

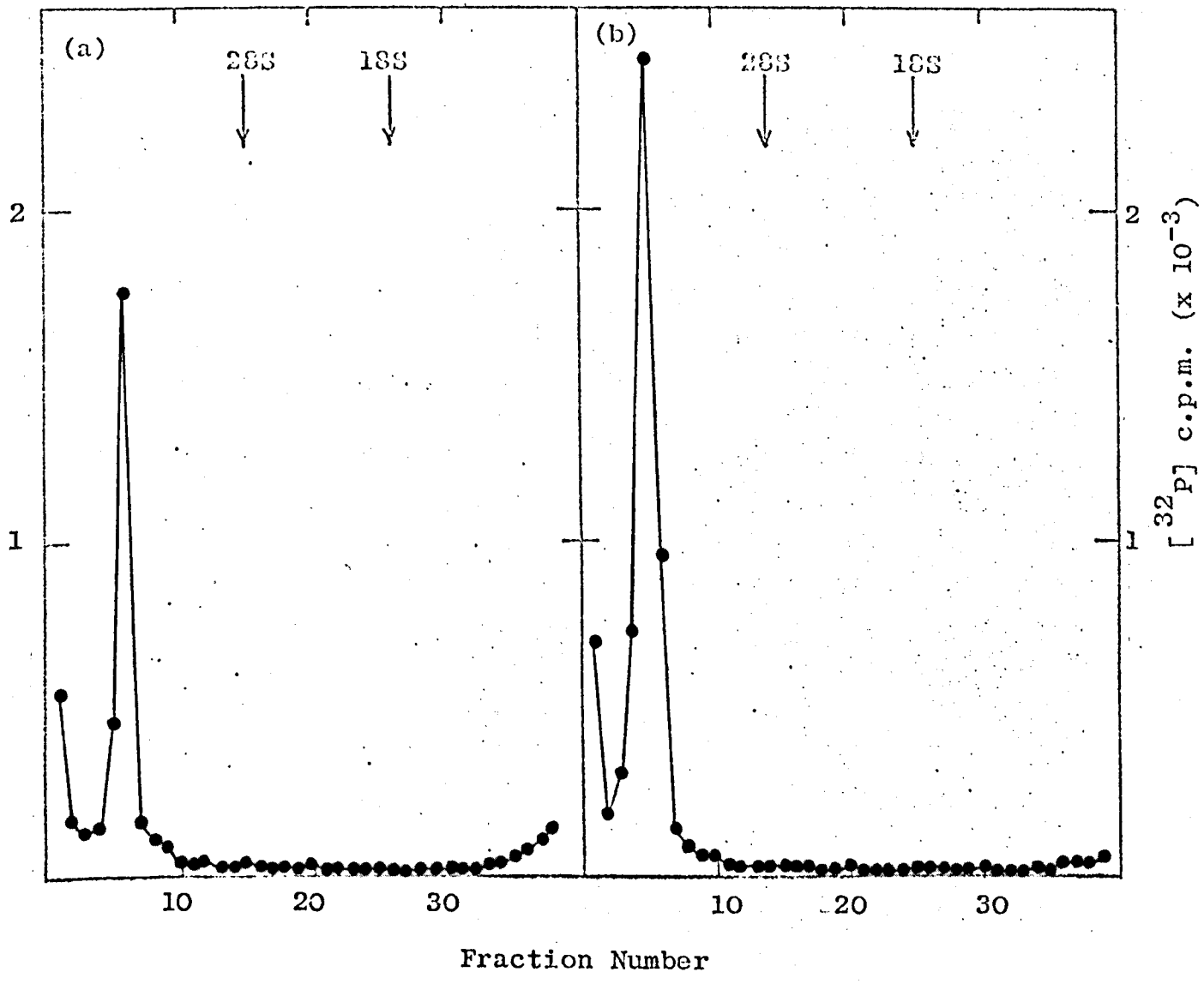


Figure 35. Polyacrylamide gel electrophoresis (2.2% gels) of RNA extracted from purified [^{32}P]-NDV (strain Texas) (a) treated with 0.025% BPL or (b) heated at 56^{o}C for 12 min. The arrows show the position of [^{3}H]-chick cell ribosomal RNA markers. (Loening system).

Effect of heat at 56°C on the nucleic acid of NDV particles

The effect of heat at 56°C on NDV has been investigated and reported by Kohno et al. (1969) and Sheaff et al. (1972). It was found that brief heating at 56°C destroyed infectivity (but at a slower rate than did UV irradiation or BPL) and led to virus capable of inducing interferon. The interferon inducing capacity was lost on heating at 56°C for longer times. NDV RNA polymerase activity was destroyed by heat at 56°C with first-order kinetics at the same rate as infectivity suggesting that polymerase activity was necessary for infectivity. Fractionation of NDV RNA extracted from NDV heated for 12 min at 56°C, at which time interferon inducing capacity had almost disappeared and RNA polymerase activity was at an undetectable level, on polyacrylamide gels showed that it was not broken down (Figure 35b). This indicated that, as with BPL inactivated virus, breakdown of NDV RNA was not responsible for the loss of interferon inducing capacity in heat inactivated virus.

It was also shown that breakdown of NDV RNA by UV irradiation of purified NDV was independent of BPL - or heat at 56°C - inactivation of the virus. That is, virus inactivated by 0.025% BPL or heated at 56°C for 12 min and subsequently UV irradiated (8.46 x 10^3 ergs/mm^2) was found to contain degraded RNA.

Preliminary investigation into the structural basis of NDV particle RNA polymerase

Although many of the enzymological properties of NDV particle RNA polymerase have been elucidated (Huang et al., 1971; Clavel and Bratt, 1971; Sheaff et al., 1972), nothing was known about the structural basis of polymerase activity. Therefore, an investigation of the correlation between enzyme activity and the presence of different virus proteins was commenced. During the course of these experiments, it became clear that both enzyme activity, measured in the *in vitro* assay, and the ability to induce interferon depended on the conditions under which the purified virus had been stored.

Action of Triton N101 on purified NDV

(i) Effect on infectivity and haemagglutinin titre

NDV, strain Texas, was incubated in 0.08% (v/v) Triton N101 for 1h at 32°C, and the sub-viral particles deposited by centrifugation as described on page 36 of the Methods section. The isolated sub-viral particles so produced had 0.01 - 0.10% of the infectivity and about 3% of the haemagglutinating activity of untreated virus.

(ii) Characterisation by electron microscopy of the sub-viral particles produced by treatment with Triton N101

The electron micrograph of the purified NDV suspension showed a homogeneous population of roughly spherical particles covered by fringes or spikes on their surfaces (Figure 1). However, the isolated sub-viral particles produced by treatment of purified NDV with Triton N101 were aggregated in solution, and the predominate feature in electron micrographs of these particles was large areas of electron dense virus cores aggregated together (Figure 36a). The virus cores were probably comprised of nucleocapsids surrounded by outer protein sacs, and were similar in appearance to some of the core structures isolated after detergent treatment of avian myeloblastosis virus (Stromberg, 1972). A few virus particles, with their spikes apparently intact, were also seen, many beginning to fuse with the electron dense virus cores (Figure 36b).

(iii) Sedimentation coefficients and buoyant density of sub-viral particles produced by treatment with Triton N101

The sedimentation characteristics of the sub-viral particles, isolated after treatment of purified NDV with Triton N101, were found to be in keeping with heterogeneity observed by electron microscopy. Using sucrose density gradients containing Triton N101, which prevented aggregation of sub-viral particles, and [^{32}P]-labelled NDV it was found that the sub-viral particles sedimented as a broad band (Figure 37a). Under the centrifugation conditions used

Figure 36(a). Electron micrograph showing an area of electron dense virus cores aggregated together. The virus cores, comprising nucleocapsids and outer protein sacs, the majority without spikes, were produced by treatment of purified virus with Triton N101, removal of the detergent by centrifugation, and preparation of the resultant sub-viral particles for electron microscopy.

(b). Electron micrograph of apparently intact virus particles with spikes fusing together and with aggregated cores. This was an unusual feature of the stained preparation used for Figure 36(a).

Fig.36

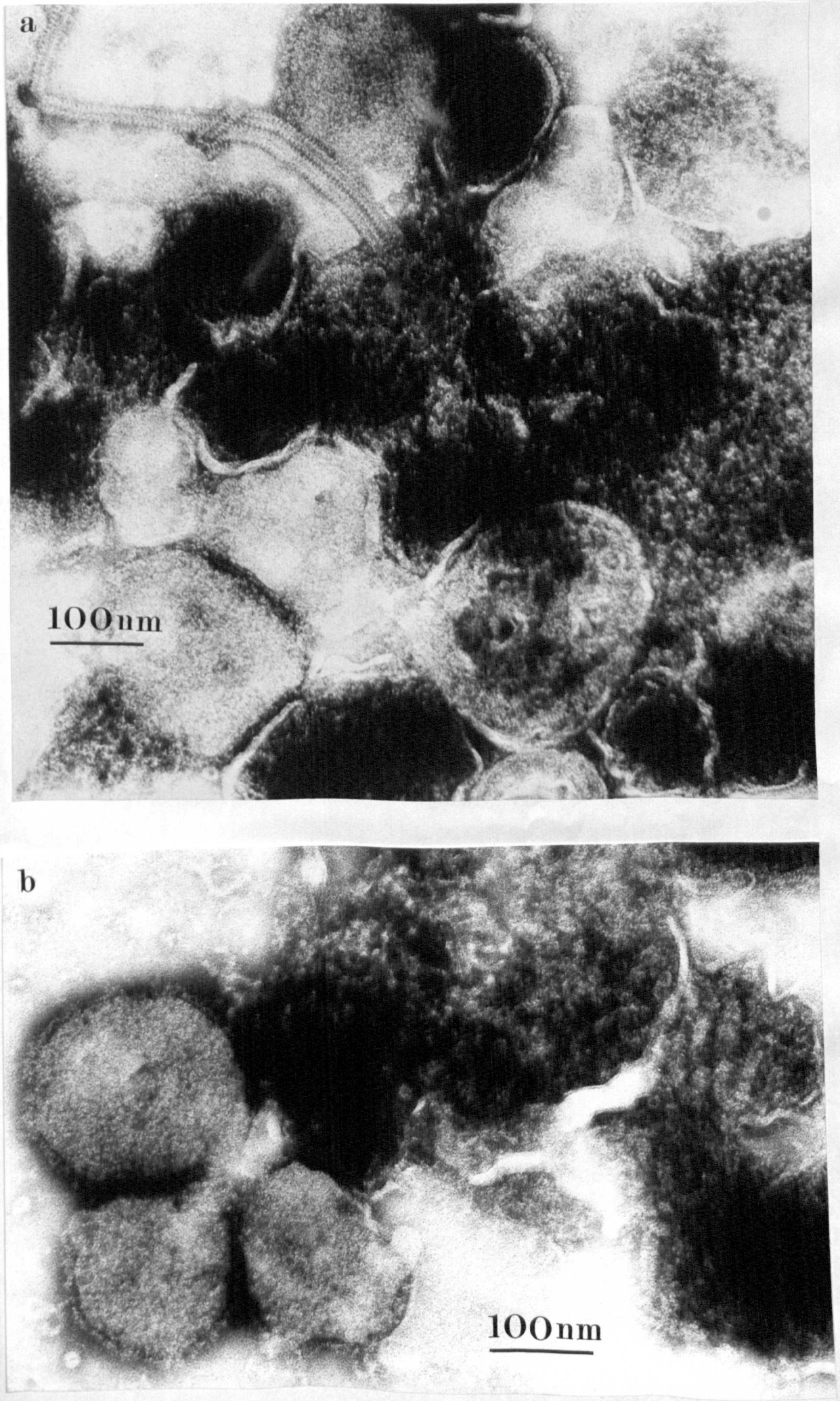
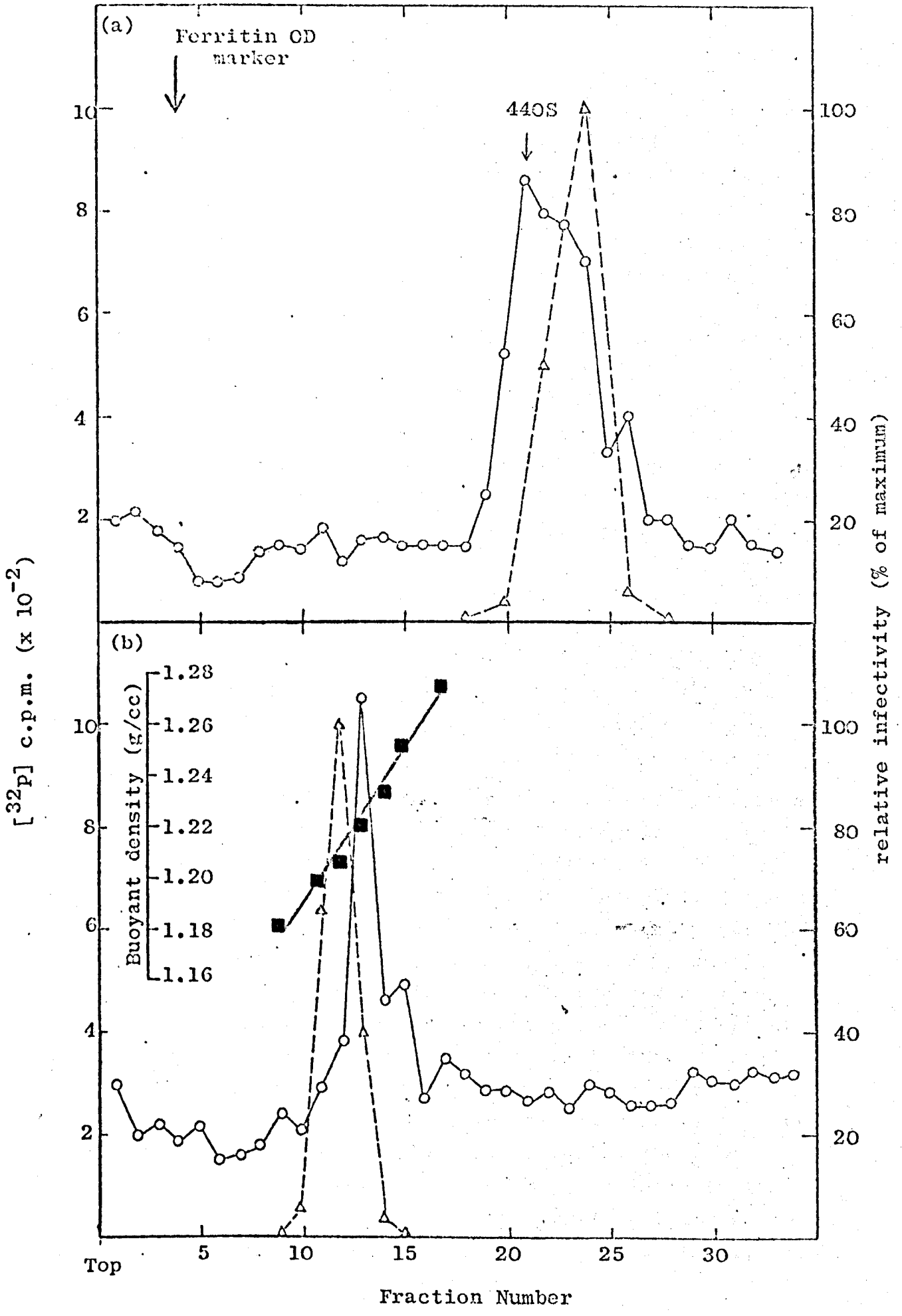


Figure 37. Centrifugation of [^{32}P]-labelled NDV (strain Texas) treated with Triton N101 in (a) a 15-65% (w/v) sucrose density gradient buffered to pH 7.3 with TN buffer and containing 0.08% (w/v) Triton N101 and (b) a 15 - 60% (w/v) sodium potassium tartrate gradient buffered to pH 7.3 with TN buffer. Radioactivity o———o, infectivity Δ- - -Δ, density ▫———▫. The maximum infectivity was 1.2 x 10^6 p.f.u./ml in (a) and 3.5 x 10^4 p.f.u./ml in (b).

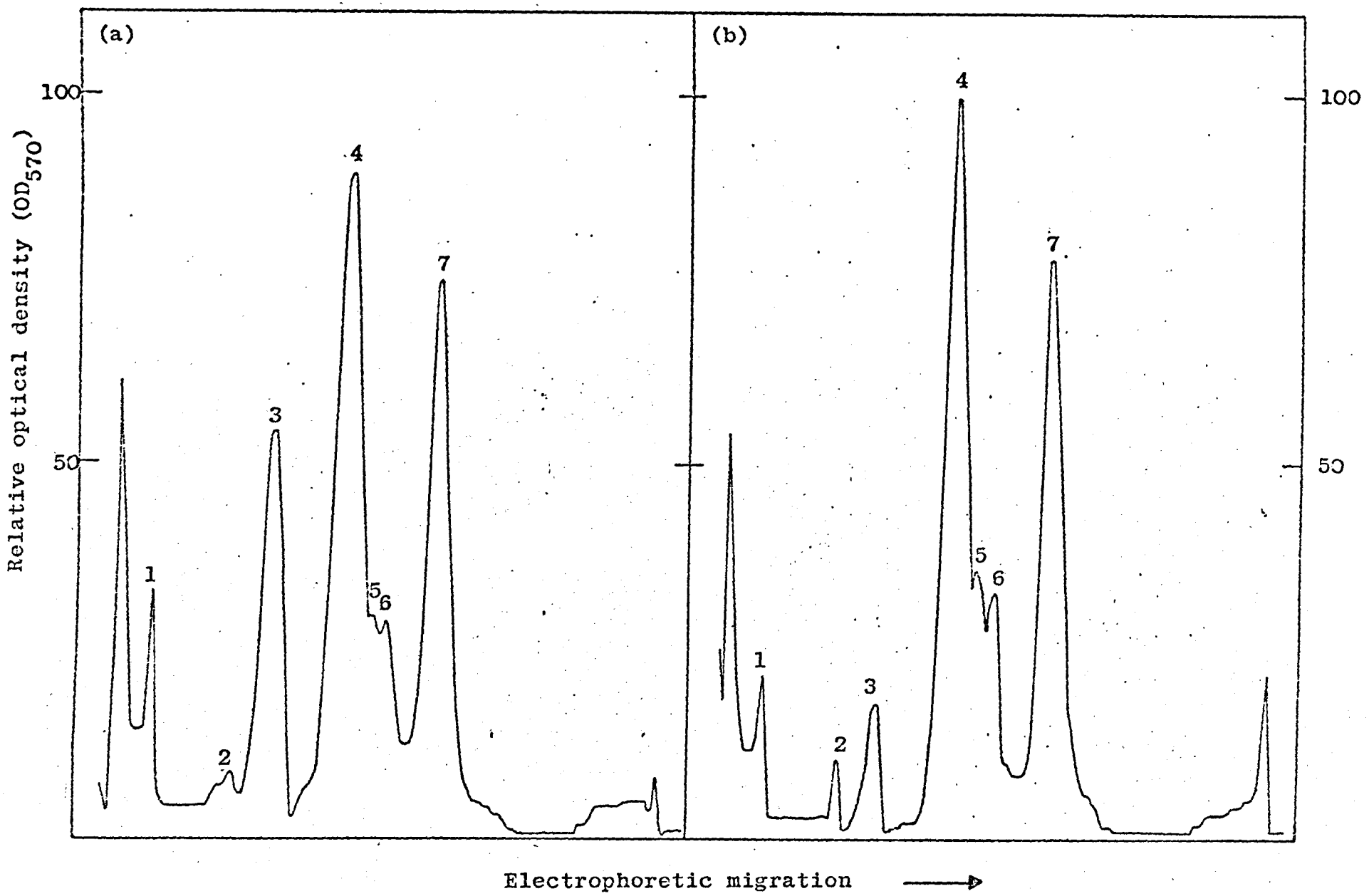

(a)
Ferritin OD marker
44OS
(b)
Buoyant density (g/cc)
1.28
1.26
1.24
1.22
1.20
1.18
1.16
[32P] c.p.m. (x 10-2)
relative infectivity (% of maximum)
Top
Fraction Number

(93,000g_{av} for 1h at 4°C) the few remaining infectious virus particles in the suspension reached their equilibrium position, but not the sub-viral particles. The majority of the sub-viral particles had an $S_{20,w}$ value of approximately 440S calculated using ferritin, a macromolecule of known sedimentation coefficient ($S_{20,w}$ of ferritin = 70S) as a marker (Martin and Ames, 1961). Using potassium sodium tartrate density gradients and centrifugation conditions under which all particles reached equilibrium (78,000g_{av} for 16h at 4°C), it was shown that the buoyant density of the majority of sub-viral particles, as judged by [^{32}P]- label, was slightly greater than that of infectious virus (1.226g/cc as compared to 1.210g/cc) (Figure 37b). This result was consistent with increased buoyant density of sub-viral particles produced by treatment of purified Sendai virus with Non-idet P40, a non-ionic detergent similar to Triton N101, reported by Robinson (1971b).

(iv) Characterisation of sub-viral particles produced by treatment with Triton N101 by protein polyacrylamide gel electrophoresis

Figure 38(a) illustrates the electrophoretic pattern of NDV strain Texas structural proteins after staining with Coomassie blue. A photograph of a typical stained gel containing virus proteins is shown in Figure 39(a). It was found that at least 7 protein species could be identified. Comparison with two other NDV strains, H and F, showed that 6 of these proteins (VP1, VP3, VP4, VP5, VP6 and VP7) were common to them all. There were always present a few faint bands between VP1 and VP2 on Coomassie blue stained gels (Figure 39(a)), but these probably represented protein aggregates as their number, relative mobility and intensity of staining varied from gel to gel. Three of the protein species (VP3, VP4 and VP7) were present in relatively large amounts and have been described by others (Bikel and Duesberg, 1969; Evans and Kingsbury, 1969; Haslam et al., 1969).

Figure 38. Polyacrylamide gel electrophoresis of (a) the proteins extracted from NDV strain Texas and (b) the proteins extracted from sub-viral particles produced by treatment of NDV with Triton N101. The particle concentration of virus and sub-viral particles was the same before SDS solubilisation. The gels were stained with Coomassie blue and scanned as described in Methods.


(a)
(b)
100
50
Relative optical density (OD_{570})
1
2
3
4
5
6
7
Electrophoretic migration

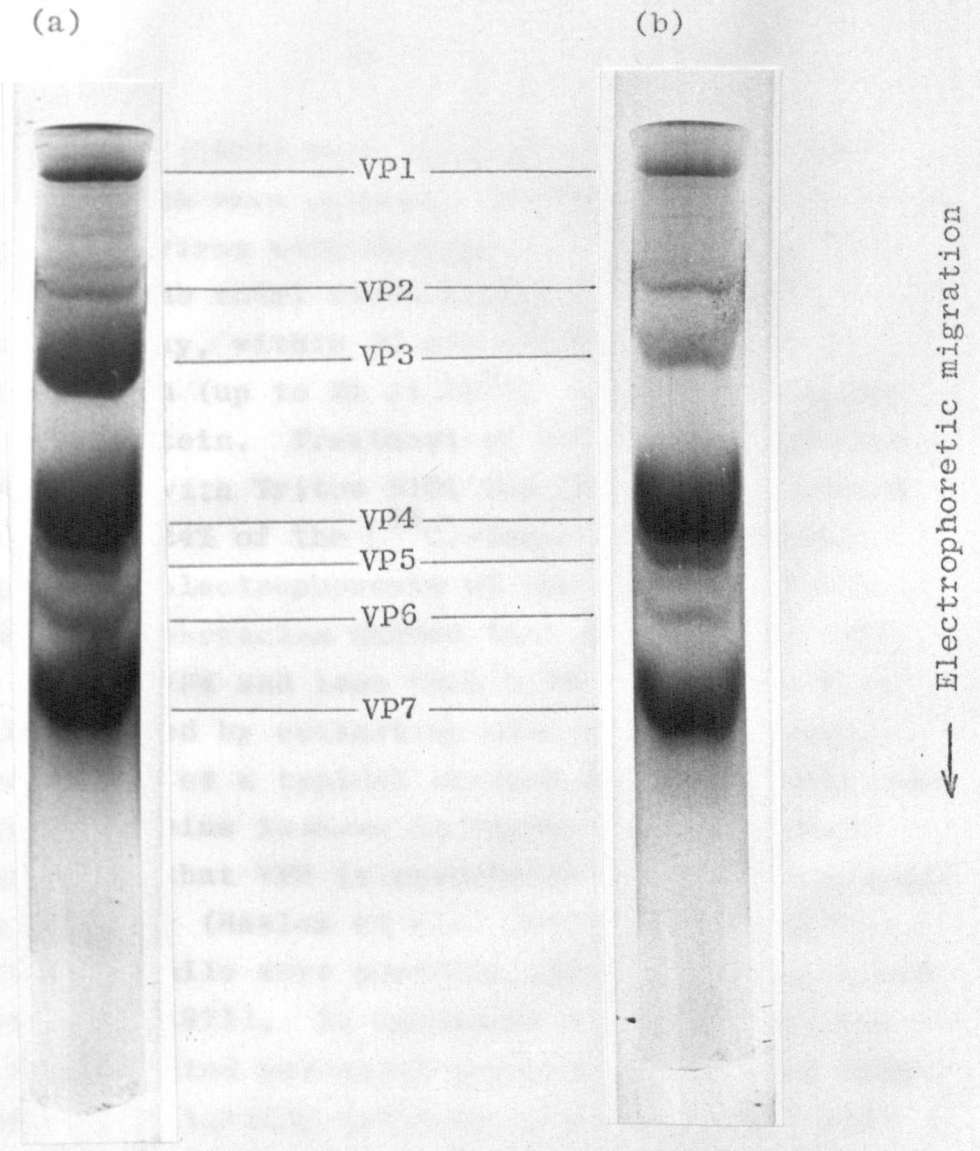
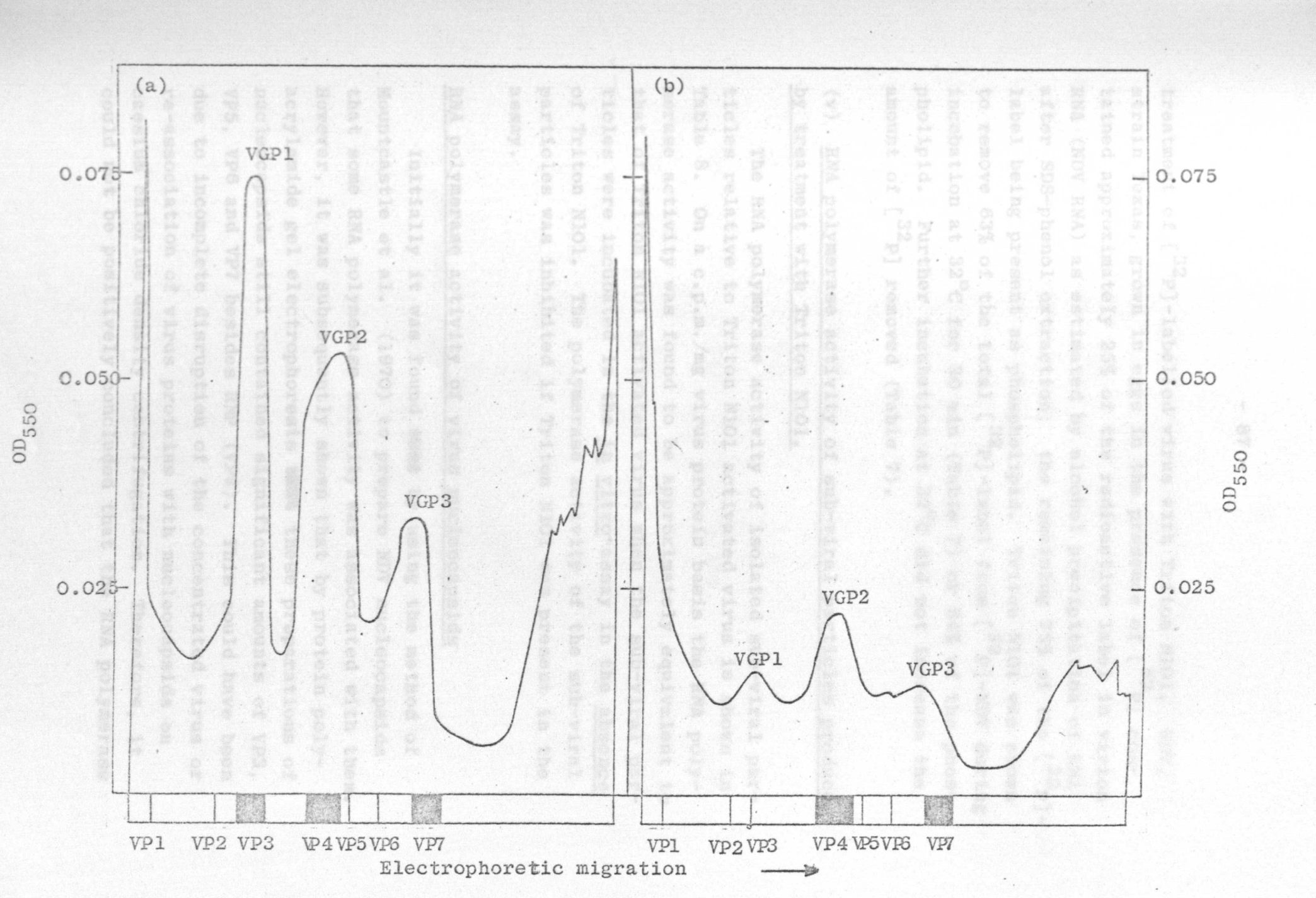


Figure 39. Photographs of Coomassie blue stained polyacrylamide gels containing (a) the proteins extracted from NDV strain Texas and (b) the proteins extracted from sub-viral particles produced by treatment of NDV with Triton N101. Note the bowed shape of the protein bands due to a tube-wall effect and heavy loading (~250µg virus protein/gel).

Mountcastle et al. (1971) have also described two minor protein species which were probably identical with VP5 and VP6. Treatment of virus with Triton N101 removed approximately 16 - 23% of the total virus proteins, as estimated by colorimetric assay, within 30 min incubation at 32°C. Prolonged incubation (up to 2h at 32°C) apparently did not remove any more protein. Treatment of the virus, labelled with [^{14}C]-valine, with Triton N101 for 1h at 32°C, showed that approximately 24% of the [^{14}C]-label was released. Polyacrylamide gel electrophoresis of the structural proteins of sub-viral particles showed that about 40% of VP1, 80% of VP3, 15% of VP4 and less than 1.5% of VP7 had been removed as calculated by estimating peak areas (Figure 38b). A photograph of a typical stained gel containing sub-viral particle proteins is shown in Figure 39b. Indirect evidence has shown that VP3 is associated with virus haemagglutinating activity (Haslam et al., 1969; Evans and Kingsbury, 1969), while more positive evidence was obtained by Iinuma et al. (1971). In agreement with this, it was found that the isolated sub-viral particles contained only 3% of the haemagglutinating activity of intact virus particles, and because of the predominance of spikeless particles as shown by electron microscopy (Figure 36a,b) VP3 was implicated as a major component of virus spikes. Mountcastle et al. (1971), using virus labelled with [^{3}H]-glucosamine and [^{14}C]-amino acids, have shown that NDV contains two glycoproteins, the faster migrating one of which was masked by the ribonucleoprotein (RNP) peak. It was probable that the 15% decrease in staining of VP4 (Figure 38b) represented removal of the faster migrating glycoprotein. To confirm this it was necessary to be able to detect carbohydrates. Satisfactory labelling of virus when it was grown in chick cells in the presence of [^{3}H]-glucosame could not be obtained, and therefore a periodic acid-Schiff staining technique was used to identify NDV glycoproteins on SDS-acrylamide gels (Kobylka et al., 1972). This staining

procedure was relatively insensitive and required up to 450µg of virus protein per gel. The magenta coloured bands also faded quickly. It was found that at least 3 glycoproteins could be identified (Figure 40a) in three strains of NDV (Texas, H and F). The relative positions of the glycoproteins were identified by restaining the gels with Coomassie blue. Two of the glycoproteins, VGP1 and VGP2, were probably the same as those identified by Mountcastle et al. (1971). The third glycoprotein (VGP3), which had the lowest molecular weight and was present in the smallest amount, has not been described before. Its mobility was approximately the same as that of VP7. On most Schiff's stained gels containing virus proteins a sharp minor band in the region of VP1 was visible by eye, but frequently this was not detected by the spectrophorometer. The bottoms of the gels containing proteins from purified virus were irregularly stained, probably due to the presence of glyco- or phospho- lipid which also reacts with periodic acid (Carraway et al., 1972). Treatment of the virus with Triton N101 reduced the amounts of all carbohydrate staining bands (very approximately, 75% of VGP1 removed and 65% of VGP2, VGP3 removed as determined by peak area measurements) including the stain at the bottom of the gels (Figure 40b), indicating that Triton N101 removed both the external virus glycoproteins and the phospholipid. VGP1 was probably the same as VP3 since the reduction in Coomassie blue stain and Schiff's stain in this region of gels containing proteins of sub-viral particles as compared to gels containing proteins of virus particles was about the same, i.e. 75 - 80%. By the same criteria VGP2 was probably not the same as VP4 (15% of Coomassie blue, 65% of Schiff's stain removed), nor VGP3 the same as VP7 (1.5% of Coomassie blue, 65% of Schiff's stain removed). In agreement with this Mountcastle et al. (1971) showed that the ribonucleoprotein did not contain glucosamine. The removal of phospholipid was confirmed by

Figure 40. Polyacrylamide gel electrophoresis of (a) the glycoproteins extracted from NDV strain Texas, and (b) the glycoproteins extracted from sub-viral particles produced by treatment of NDV with Triton N101. The particle concentration of virus and sub-viral particles was the same before SDS solubilisation. The gels were stained using the periodate-Schiff method as described in Methods. An accurate diagram of the migration of NDV proteins as determined by subsequent Coomassie blue staining of each gel is also shown.


(a)
(b)
VGP1
VGP2
VGP3
0.075
0.050
0.025
OD_{550}
VP1 VP2 VP3 VP4 VP5 VP6 VP7
Electrophoretic migration

treatment of [^{32}P]-labelled virus with Triton N101. NDV, strain Texas, grown in eggs in the presence of [^{32}P] contained approximately 25% of the radioactive label in virion RNA (NDV RNA) as estimated by alcohol precipitation of RNA after SDS-phenol extraction; the remaining 75% of the [^{32}P]-label being present as phospholipid. Triton N101 was shown to remove 63% of the total [^{32}P]-label from [^{32}P]-NDV during incubation at 32°C for 30 min (Table 7) or 84% of the phospholipid. Further incubation at 32°C did not increase the amount of [^{32}P] removed (Table 7).

(v) RNA polymerase activity of sub-viral particles produced by treatment with Triton N101.

The RNA polymerase activity of isolated sub-viral particles relative to Triton N101 activated virus is shown in Table 8. On a c.p.m./mg virus protein basis the RNA polymerase activity was found to be approximately equivalent to that of Triton N101 activated virus when the sub-viral particles were incubated in the *in vitro* assay in the *absence* of Triton N101. The polymerase activity of the sub-viral particles was inhibited if Triton N101 was present in the assay.

RNA polymerase activity of virus nucleocapsids

Initially it was found by using the method of Mountcastle et al. (1970) to prepare NDV nucleocapsids that some RNA polymerase activity was associated with them. However, it was subsequently shown that by protein polyacrylamide gel electrophoresis these preparations of nucleocapsids still contained significant amounts of VP3, VP5, VP6 and VP7 besides RNP (VP4). This could have been due to incomplete disruption of the concentrated virus or re-association of virus proteins with nucleocapsids on caesium chloride density centrifugation. Therefore, it could not be positively concluded that the RNA polymerase

TABLE 7. REMOVAL OF RADIOACTIVITY FROM [^{32}P]-LABELLED NDV STRAIN TEXAS BY TRITON N101

Incubation at 32°C (min)	Total [^{32}P] in virus or sub-viral particles (c.p.m.)	Total [^{32}P] in supernatant after isolation of sub-viral particles (c.p.m.)	Total [^{32}P] in virus/sub-viral particles + supernatant (c.p.m.)
0	2,158		2,158
30	756	-	-
60	742	1,224	1,966
120	818	1,377	2,195

- Not done.

TABLE 8. RNA POLYMERASE ACTIVITY OF NDV STRAIN TEXAS AND THE SUB-VIRAL PARTICLE OBTAINED BY TREATMENT WITH TRITON N101

Enzyme Source	RNA polymerase activity (c.p.m./mg virus protein)	
	+ Triton in assay	- Triton in assay
Virus	1,125	247
Sub-viral particle	700	1,462

activity in these preparations (approximately 10% of the polymerase activity of Triton N101 activated virus) was due to nucleocapsids (defined as the viral genome surrounded by RNP (VP4) alone). The Tween-ether disruption of NDV described by De The and O'Connor (1966) also did not completely remove all external virus proteins. In fact, it was found that even under the more severe conditions described in the Methods section of page 36 that a preparation consisting only of ribonucleoprotein (VP4) could not be achieved. The particles resulting after detergent treatment had a buoyant density of 1.27g/cc (Figure 41) which was characteristic of nucleocapsids on sucrose - D_2O density gradients (Robinson, 1971b; Blair, 1968). However, polyacrylamide gel electrophoresis of these nucleocapsids showed that VP5 did not appear to be removed at all, and that VP6 and VP7 were always present in small amount (Figure 42). Figure 43a shows a photograph of a typical gel stained with Coomassie blue containing proteins from these nucleocapsids which demonstrates the presence of small amounts of VP3, VP6 and VP7 besides VP4 (RNP) and VP5. A photograph of a gel containing the proteins from isolated sub-viral particles produced by treatment of purified NDV with Triton N101 (Figure 43b) is shown for comparison. Nucleocapsids having a buoyant density of 1.27g/cc on sucrose-D_2O density gradients and containing the proteins shown in Figures 42 and 43a had no measurable RNA polymerase activity.

Decay of NDV RNA-dependent RNA polymerase on storage of purified virus

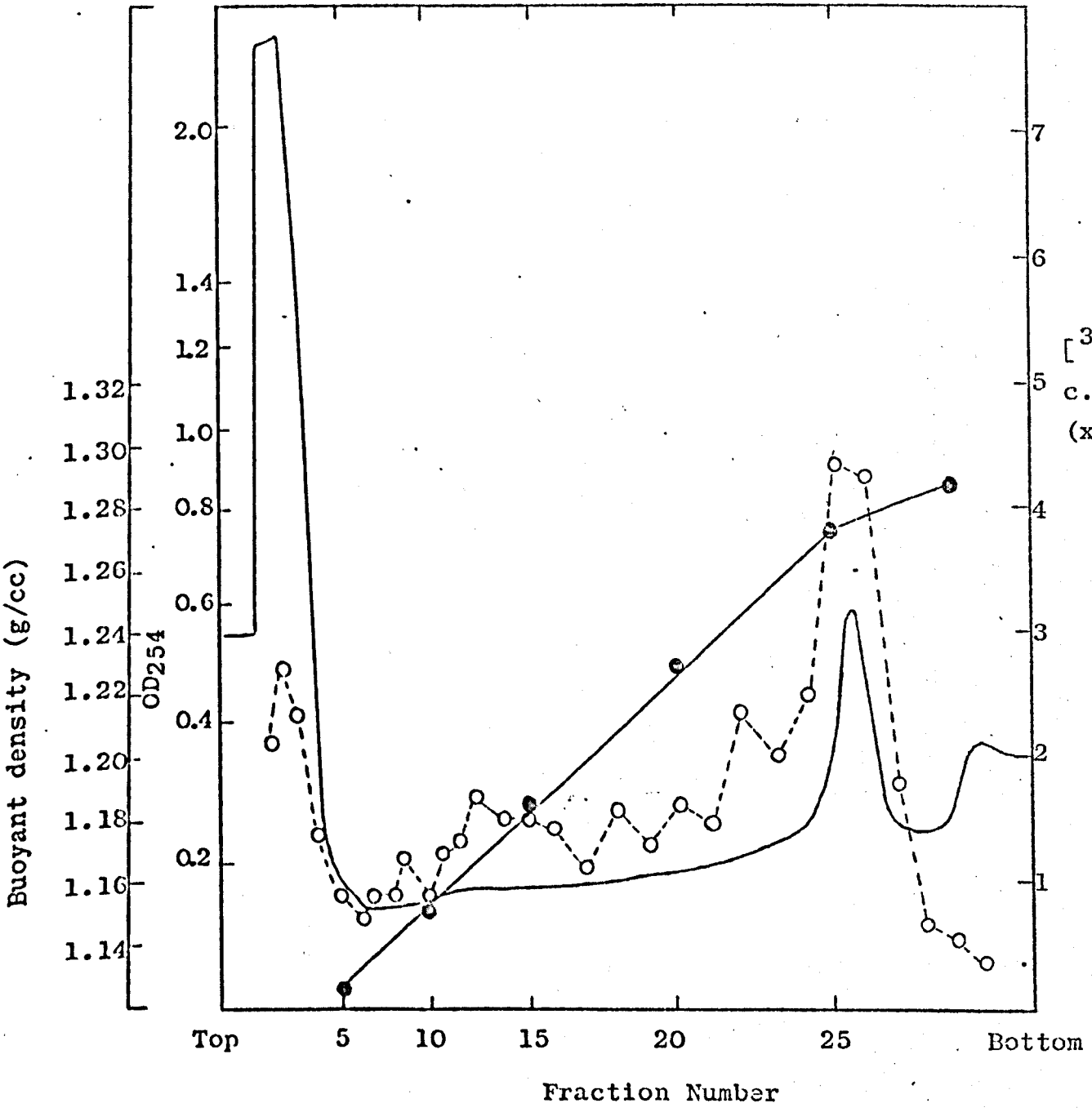
During the course of this work, it was noticed that purified strains of NDV rapidly lost RNA polymerase activity on storage at 4°C (Figure 44 and Table 9, columns 3 and 5). Concomitantly, there was some loss of infectivity in these preparations, the virulent strains, Herts 33 and Texas, being most unstable in this respect (Table 9, columns 2 and 4).

Figure 41. Centrifugation of [^{3}H]-NDV nucleocapsids produced as described in Methods in a linear 20 - 65% (w/v) sucrose density gradient containing D_2O buffered with TN buffer to pH 7.3. Generally, unlabelled NDV nucleocapsids contained in the OD_{251} peak at 1.27g/cc were collected, re-treated with DOC and re-centrifuged to equilibrium on an identical sucrose-D_2O gradient as described in Methods before use.

————, OD_{254}.

o————o, acid insoluble [^{3}H]-radioactivity.

•————•, buoyant density (g/cc).


Buoyant density (g/cc)
1.32
1.30
1.28
1.26
1.24
1.22
1.20
1.18
1.16
1.14
OD254
2.0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
[3H]
c.p.m.
(x 10-2
7
6
5
4
3
2
1
Top
5
10
15
20
25
Bottom
Fraction Number

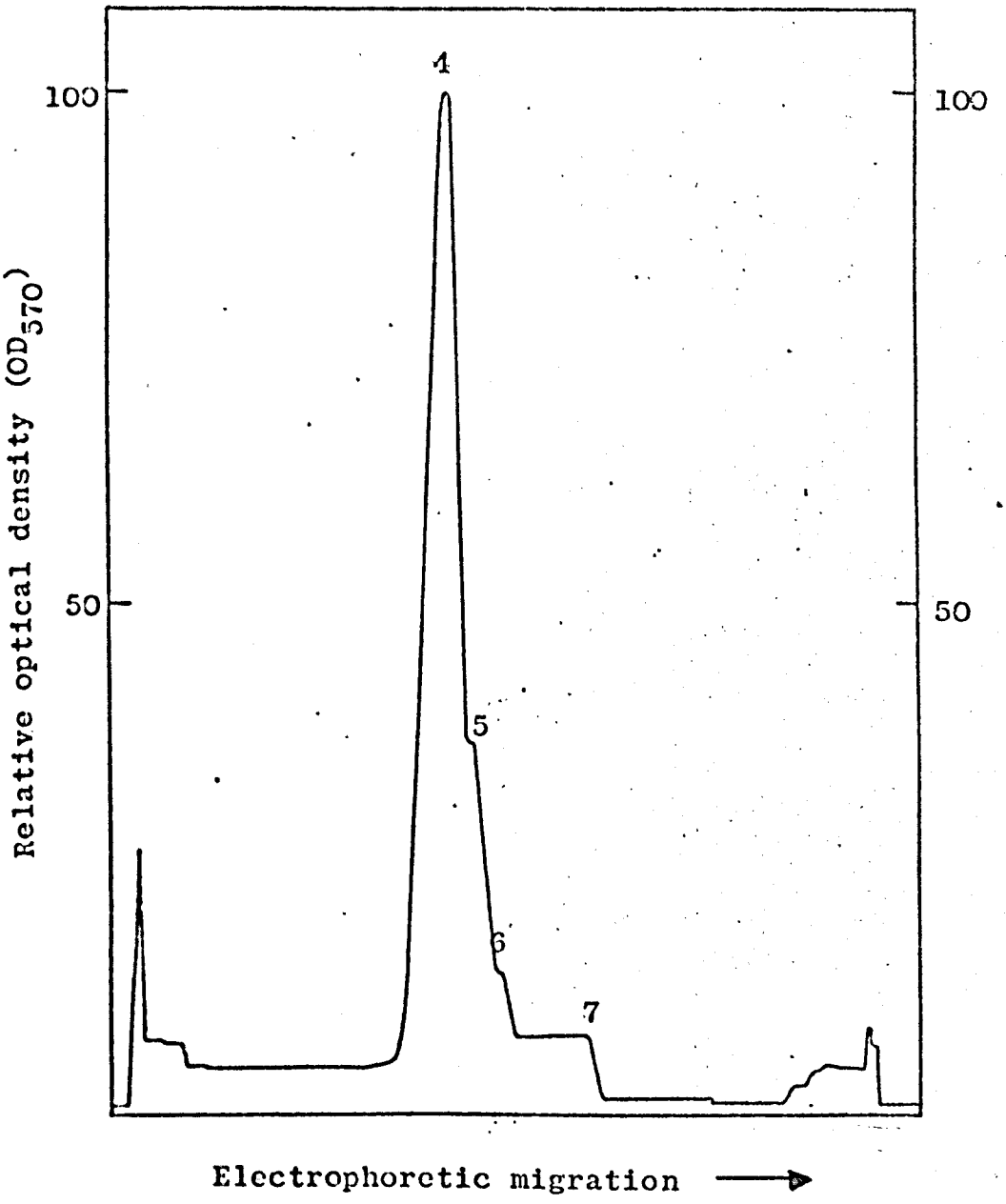


Figure 42. Polyacrylamide gel electrophoresis of the proteins extracted from purified NDV nucleocapsids isolated from NDV strain Texas as described in Methods. The gel was stained with Coomassie blue and scanned as described in Methods.

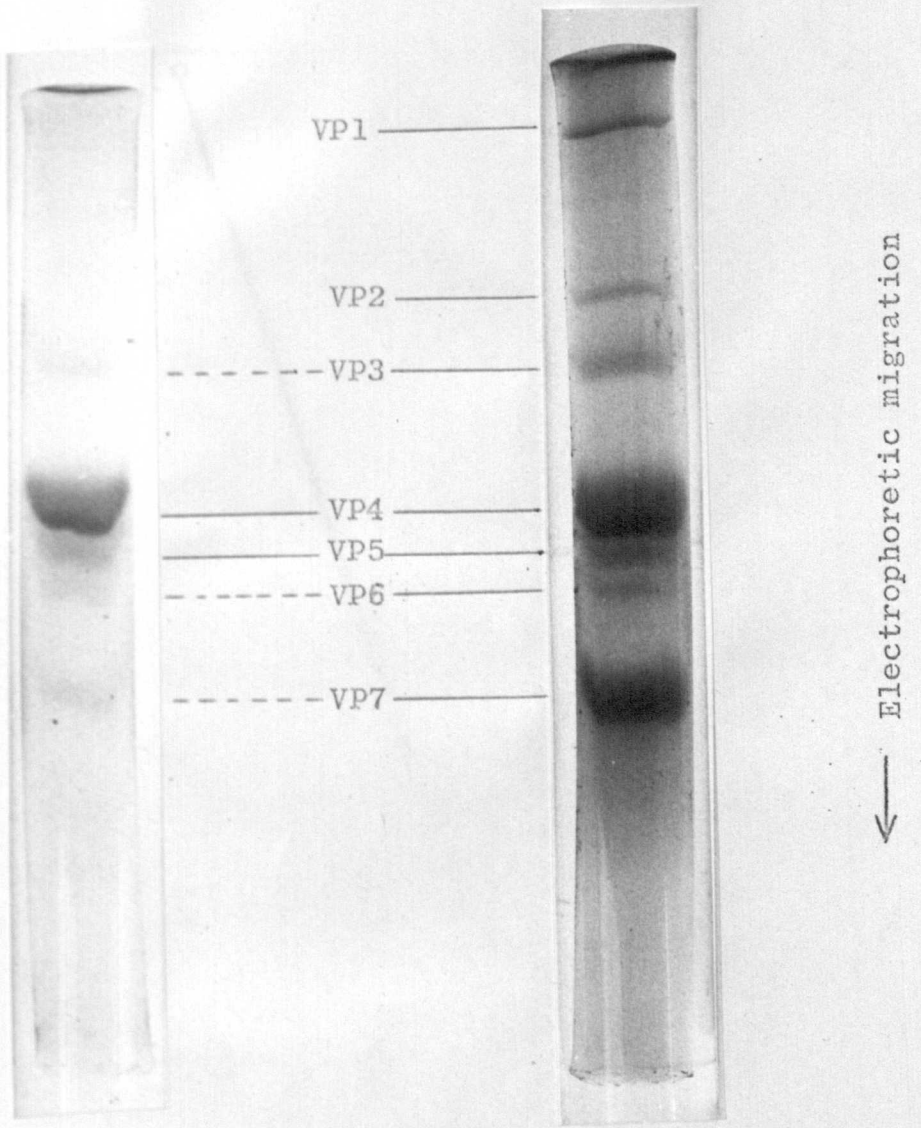


Figure 43(a). Photograph of a Coomassie blue stained polyacrylamide gel containing proteins extracted from purified NDV nucleocapsids isolated from NDV strain Texas as described in Methods.

(b). Photograph of a Coomassie blue stained polyacrylamide gel containing proteins extracted from sub-viral particles produced by treatment of NDV (strain Texas) with Triton N101 as described in Methods, included as a reference for the electrophoretic migration of virus proteins.

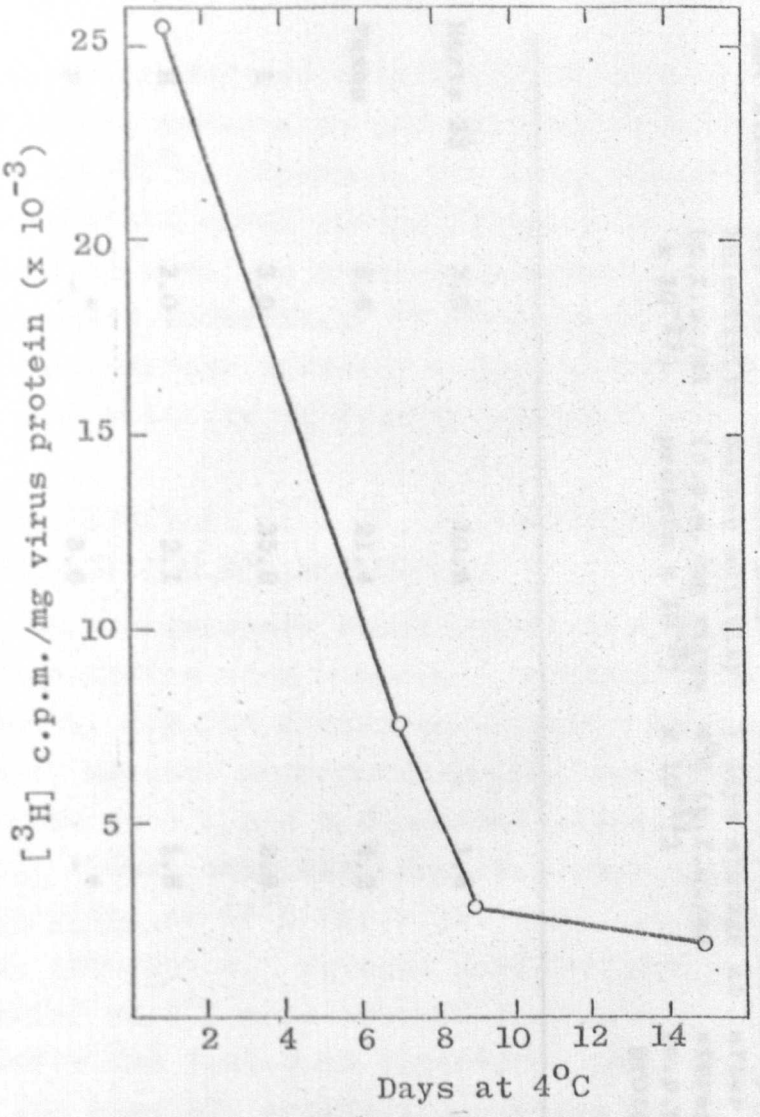


Figure 44. The effect of storage of NDV strain L at 4°C on the RNA polymerase activity. Standard 0.23ml reaction mixtures were used containing 500µg of NDV protein and incubated for 2 h at 32°C. Duplicate samples were analysed for incorporation into acid-insoluble material as described in Methods.

Table 9. Decay of NDV infectivity and RNA polymerase activity on storage of different strains at 4°C and "activation" of polymerase activity after incubation of virus at 37°C for 48 h.

NDV strain	Initial infectivity (p.f.u./ml x 10^{-11})	Initial polymerase activity (c.p.m./mg virus protein x 10^{-3})	Infectivity after 7 days storage at 4°C (p.f.u./ml x 10^{-11})	Polymerase activity after 7 days storage at 4°C (c.p.m./mg virus protein x 10^{-3})	Polymerase activity after incubation at 37°C for 48h (c.p.m./mg virus protein x 10^{-3})	
					5th-7th day after purification	13th-15th day after purification
Herts 33	3.0	10.5	1.6	0.6	11.2	4.5
Texas	5.6	21.4	4.2	3.1	12.4	2.0
L	3.0	25.6	2.9	7.6	12.2	16.3
H	2.0	2.1	1.8	0.3	23.4	7.5
F	-*	8.6	-*	2.2	16.2	7.8

* The F strain is a non-plaque forming strain.

The Texas strain of NDV had only 1 - 10% of its original infectivity and no measurable RNA polymerase activity after being frozen at -70°C. However, the addition of 20% (v/v) glycerol to purified virus before freezing to prevent ice-crystal formation gave, on thawing, preparations with only slightly decreased infectivity (~10% reduction), but again with reduced polymerase activity (~5% activity remained as compared to the activity of freshly purified virus).

Effect of incubation at 37°C on NDV infectivity, interferon inducing capacity and RNA polymerase

It has been previously shown that interferon production by NDV was associated with a reduced polymerase activity of virus particles, e.g. UV irradiated virus. It was therefore of interest to measure interferon production by strains which had been stored at 4°C and had lowered polymerase activity. Kohno et al. (1969) have also reported that when NDV was incubated *in vitro* at 37°C for a few days, it became capable of producing interferon. Several purified NDV strains which had been stored at 4°C were assayed for infectivity, polymerase activity and tested as interferon inducers. Simultaneously, the same NDV preparations which had been incubated at 37°C for 48h were also assayed for infectivity, polymerase activity and interferon inducing ability. None of the purified strains which had been stored at 4°C throughout, and had lost up to 90% of their polymerase activity, were interferon inducers (Tables 9 and 10). However, it was found that whereas all strains lost a considerable amount of infectivity during incubation at 37°C, only two strains, Herts 33 and H, became capable of inducing interferon (Table 10). The only strain which induced high titres of interferon (strain H) was also the one that at low m.o.i. (1 - 10p.f.u./cell) produced low titres of interferon without any treatment (Lomniczi and Burke, 1971). When RNA polymerase activity of these preparations was assayed it was found that, in all strains, activity was increased by incubation at 37°C after prior incubation at 4°C (Table 9, columns 5 and 6). In some

Table 10. Effect of incubation for 48h at 37°C on infectivity and interferon inducing capacity of different NDV strains

NDV strain	Infectivity of virus stored at 4°C (p.f.u./ml x 10^{-11})	Interferon inducing capacity of virus stored at 4°C (interferon units)	Infectivity of virus incubated at 37°C for 48h (p.f.u./ml x 10^{-8})	Interferon inducing capacity of virus incubated at 37°C for 48h (interferon units)
Herts 33	0.46	15	<1.0	170
Texas	2.8	<10	8.0	<10
L	2.8	<10	52.0	<10
H	1.6	<10	4.8	1,600
F	-*	<10	-*	<10

* The F strain does not plaque

TABLE 11. RNA POLYMERASE ACTIVITY OF NDV STRAIN TEXAS AND ITS SUB-VIRAL PARTICLE AFTER (a) STORAGE AT 4°C THROUGHOUT, (b) STORAGE AT 4°C FOLLOWED BY INCUBATION FOR 48h AT 37°C

Enzyme source	RNA polymerase activity (c.p.m./mg virus protein)
Virus stored at 4°C throughout	1,125
Virus incubated for 48h at 37°C	6,900
Sub-viral particle isolated from virus stored at 4°C throughout	1,462
Sub-viral particle isolated from virus stored at 4°C followed by incubation for 48h at 37°C	17,850

Triton N101 was added to the assay when virus was used as a source of enzyme, but not when the sub-viral particle was used.

instances, (i.e. the H and F strains) polymerase activity was higher than that of virus assayed immediately after purification (Table 9, columns 1 and 5). In general, the longer the virus was left at 4°C before incubating at 37°C for 48h, the smaller the re-activation of RNA polymerase (Table 9, columns 5 and 6). The change in activity was not due to any loss of virus structural proteins, since incubation of the virus at 37°C for 48h did not alter the protein polyacrylamide gel electrophoretic pattern of the virus. In keeping with the elevated polymerase activity of virus incubated at 37°C for 48h, it was found that the isolated sub-viral particles produced from this virus by treatment with Triton N101 had elevated polymerase activity (Table 11). It was also demonstrated by protein polyacrylamide gel electrophoresis that these sub-viral particles had the same proteins in the same amounts as sub-viral particles isolated from virus incubated at 4°C throughout after Triton N101 treatment. This suggested that the increase in polymerase activity after incubation at 37°C was due to an effect on the internal components of the virus.

DISCUSSION

DISCUSSION

The RNA of Newcastle Disease virus particles and Newcastle Disease virus specific RNA synthesis *in vivo* and *in vitro*

Previous workers have shown that NDV RNA, i.e. the RNA contained in virus particles, could be separated into two components, a single high molecular weight RNA species and heterogeneous low molecular weight RNA (Duesberg and Robinson, 1965; Kingsbury, 1966a). The high molecular weight RNA, which is the viral genome, had a sedimentation coefficient (S_{20},w) of 57S in 0.1M salt (sodium chloride), and is single-stranded (Duesberg and Robinson, 1965). The heterogeneous low molecular weight RNA was probably a mixture of degraded 57S RNA and contaminating cellular RNA, and had a sedimentation coefficient of about 4S under the same conditions (Duesberg and Robinson, 1965). Using the Spirin formula (Spirin, 1963), which is an empirical relation between the S_{20},w and the molecular weight (M) of TMV RNA in 0.1M salt, i.e. $M = 1{,}550\ (S_{20},w)^{2.1}$, Duesberg and Robinson (1965) calculated a molecular weight of 7.5×10^6 for 57S NDV RNA. However, Strauss and Sinsheimer (1963) have demonstrated that the Spirin formula applies accurately only to some single-stranded RNAs, probably because different RNA molecules have different frictional coefficients under the same conditions. Since the frictional coefficient of 57S NDV RNA is unknown, the estimate of its molecular weight based on sedimentation coefficient alone is uncertain. In addition, the frictional coefficients, and hence the sedimentation volocities, of single-stranded RNAs are determined by ionic strength. In the case of high molecular weight paramyxovirus RNA it has been shown that the S_{20},w can vary from 40S when salt is absent (Compans and Choppin, 1968; Barry and Bukrinskaya, 1968), to 50S in 0.05M salt (Compans and Choppin, 1968; Kingsbury, 1966a), and 57S in 0.1M salt (Duesberg and Robinson, 1965; Blair and Robinson, 1968; Barry and Bukrinskaya, 1968). The latter value, i.e. 57S, may be questioned as an S_{20},w of 50S in 0.1M salt has also

been reported for high molecular weight SV5 RNA (Compans and Choppin, 1968) and NDV RNA (Clavell and Bratt, 1971), and this value was obtained by analytical sucrose density gradient centrifugation as has been reported in this thesis. It is perhaps unfortunate that further work using the analytical ultracentrifuge has not been done to confirm the early value of 57S for NDV RNA reported by Duesberg and Robinson (1965).

The figure of 4.8×10^6 for high molecular weight NDV RNA determined by polyacrylamide gel electrophoresis reported in this thesis is somewhat lower than values based on the sedimentation coefficient of this RNA. However, there are certainly fewer assumptions involved in the determination of molecular weight from calibrated gels. For instance, it is known that, with few exceptions, RNA molecules of different sizes conform to the linear relation between log (molecular weight) and electrophoretic mobility (Bishop et al., 1967; Loening, 1969; Weinberg and Penman, 1970) and that the relation is independent within wide limits of salt or gel concentration (Loening, 1969). The major problem arises from using assumed molecular weights for the RNA species used to calibrate gels. For example, Petermann and Pavlovec (1966) reported molecular weights of 1.65×10^6 and 0.65×10^6 for the RNAs of the large and small subunits of HeLa cell ribosomes, respectively, whilst Hamilton (1967) found molecular weights of up to 1.75×10^6 and 0.7×10^6 for these two RNA species. Nor has the molecular weight of the HeLa cell 45S ribosomal RNA precursor been definitely agreed upon, although it probably lies in the range of $4.0 - 4.5 \times 10^6$ (Weinberg et al., 1967; McConkey and Hopkins, 1969; Weinberg and Penman, 1970). Therefore, the molecular weight of NDV RNA (N.B. NDV RNA will mean only the <u>high molecular weight RNA species</u> isolated from virus particles from this point in the discussion) estimated by calibration of polyacrylamide gels will depend on the choice of assumed molecular weights of other RNA molecules. Since also NDV RNA is bigger than 45S ribosomal RNA precursor, it becomes difficult to estimate accurately just by how much bigger it is using this method.

Loening, using the molecular weights of Hamilton (1967) for HeLa cell ribosomal RNA and 4.4×10^6 for 45S RNA, estimated a molecular weight of 5.2×10^6 for NDV RNA (Dr. U. E. Loening, personal communication), and a value much in excess of this is not likely. If a molecular weight of approximately 5×10^6 for NDV RNA is assumed to be correct, then the expected sedimentation coefficient using the Spirin formula (assuming this is valid) should be 47.5S. This value is in fair agreement with the 50S value reported in this thesis and by others (Kingsbury, 1966a; Compans and Choppin, 1968; Clavell and Bratt, 1971).

The sedimentation characteristics of Newcastle Disease virus specific intracellular RNA (ND vsi - RNA) were similar to those reported by Bratt and Robinson (1967, 1971), Blair and Robinson (1968), and Bratt (1969a,b). The resolution by sucrose density centrifugation of ND vsi-RNA into its component RNA species, i.e. 50S, 35S, 22S and 18S RNAs was never very satisfactory and if one component was in excess, e.g. 18S RNA, then the components were hardly separated at all. Virtually all ND vsi-RNA was sensitive to degradation by ribonuclease under the conditions described in this thesis, but Bratt and Robinson (1971), using very short pulses of [^{3}H]-uridine, have shown some ribonuclease resistance in the 35S RNA indicating that a significant amount of this RNA is base-paired. The decrease in amount of this base-paired RNA with longer pulses of [^{3}H]-uridine has led them to suggest that 35S RNA contained the replicative intermediate from which single-stranded RNA was synthesised. Similar evidence for the existence of base-paired RNA (replicative form and replicative intermediates) in NDV-infected cells has been presented by Zhdanov and Kingsbury (1969) and Zhdanov et al. (1970).

The resolution of high molecular weight RNA species achieved by analytical RNA polyacrylamide gel electrophoresis was much superior to that achieved by sucrose density gradient centrifugation. Application of polyacrylamide gel electrophoresis to ND vsi-RNA improved the relative separation of its component RNA species, i.e. 50S, 35S, 22S and 18S RNAs, so that several problems relating to ND vsi-RNA synthesis could

be examined. The first problem investigated was whether ND vsi-RNA synthesis was related to virus virulence. The sub-genomic, virus specific intracellular RNA species, i.e. 35S, 22S and 18S RNAs, found in NDV infected cells have been shown to be complementary in base sequence to NDV RNA (Kingsbury, 1966b; Bratt and Robinson, 1967; Portner and Kingsbury, 1970). Since these sub-genomic, complementary RNA species were present in relatively large amounts compared to NDV RNA (50S RNA), and have been found in association with polyribosomes in infected cells, it has been suggested that they are the viral messenger RNAs (Kingsbury, 1966b; Bratt and Robinson, 1967). Thus it may be postulated that the velogenic (most virulent) strains of NDV by synthesising more viral mRNAs than the lentogenic strains are able to make more of the proteins causing cell death. It was found, in agreement with Bratt (1969a), that NDV strains of different virulence synthesised all of the sub-genomic, complementary RNA species, but that the relative amounts of these varied. In general, the velogenic strains made more of the 18S RNA species than did the lentogenic strains, suggesting that the velogenic strains could potentially make more virus proteins. Since it is unknown whether velogenic strains do make more virus proteins than lentogenic strains, no satisfactory explanation of the correlation between 18S RNA synthesis and virus virulence can yet be given.

The second investigation concerned the estimation of the molecular weights of the sub-genomic, complementary RNA species contained in ND vsi-RNA using calibrated polyacrylamide gels. By this time improvements made in the technique of analytical RNA polyacrylamide gel electrophoresis had shown that both the 18S RNA and 22S RNA species were somewhat heterogeneous. The "18S" RNA was resolved into at least three RNA components, and the "22S" RNA into two RNA components. Calibration of polyacrylamide gels with respect to molecular weight using HeLa cell nucleolar RNA as markers made possible the estimation of all the component RNA species of ND vsi-RNA. The molecular weights are as follows:- 2.5×10^6 for 35S RNA, 1.2×10^6 and 1.0×10^6 for the two components of "22S" RNA, and 0.70×10^6, 0.55×10^6 and 0.45×10^6 for the three com-

ponents of "18S" RNA. All of the sub-genomic RNA species contained in ND vsi-RNA have been shown to be complementary to NDV RNA (Bratt and Robinson, 1967). Assuming NDV RNA is completely transcribed, the sum of the molecular weights of the sub-genomic RNA species should be the same as the molecular weight of NDV RNA. However, the sum of the above molecular weights is 6.4×10^6 which is in excess of the molecular weight of NDV RNA, i.e. $\sim 5 \times 10^6$. This discrepancy can only be accounted for if some of the smaller RNA molecules are processed from 35S RNA or are degradation products of it. Consistent with this was the finding by Bratt and Robinson (1967) that both 35S RNA and mixtures containing 35S RNA, 22S RNA and 18S RNA accounted for 70% of the sequences of NDV RNA by hybridisation. It is puzzling though that an RNA molecule of molecular weight 2.5×10^6, half the size of NDV RNA, accounts for 70% of the sequences of NDV RNA. It must be presumed that as neither the absolute concentration or purity of intracellular RNA components (e.g. possible contamination with NDV RNA molecules) nor the specific activity of $[^{32}P]$-NDV RNA were known in these hybridisation experiments by Bratt and Robinson (1967) that the figures arrived at were only approximate. Therefore, it is suggested, assuming the hybridisation data of Bratt and Robinson (1967) to be approximately right, that some, but not all, of the RNA molecules smaller than 35S RNA are derived from it. Even more surprising was the finding that 18S RNA hybridised to 50% of the base sequences of NDV RNA (Bratt and Robinson, 1967). Kingsbury (1970) has pointed out that this hybridisation data demonstrates that either 18S RNA is heterogeneous with respect to nucleotide sequences, the total population making up sequences complementary to 50% of the genome, or that all the 18S RNA molecules are identical with half the genome redundant for their sequences. Again assuming the validity of the hybridisation data, the present finding that 18S RNA does contain more than one RNA species makes the first possibility seem the more likely. It is interesting that the rhabdovirus, vesicular stomatitis virus (VSV), which also synthesises sub-genomic, complementary RNA in infected cells has a 14S size-class of RNA which has also proved to be heterogeneous (Mudd and Summers, 1970).

As mentioned earlier it has been suggested by several authors that the sub-genomic, complementary RNA species made in paramyxovirus infected cells are the viral messenger RNA molecules which are translated in the polyribosomes (Kingsbury, 1966b; Bratt and Robinson, 1967; Blair and Robinson, 1970). The evidence for this is slight. Bratt and Robinson (1967) provided positive, though circumstantial, evidence indicating a messenger role for complementary RNA by the finding that 18S and 35S RNA species are associated with polyribosomes. However, as pointed out by Kingsbury (1970), any complementary RNA found associated with polyribosomes might be synthesised there, or found there for some adventitious reason, rather than being captured by the investigator in a translational act.

Noll (1969) has criticised many so-called polyribosome sedimentation patterns, frequently encountered in the literature, which show an indication of a ribosome (monosome) peak followed by an amorphous broad band of UV-absorbing (or light scattering) material. Rightly, he states that in the absence of the progression of peaks characteristic of polyribosomes, identification of the material corresponding to the broad band as polyribosomes with any degree of certainty is not possible, and the unresolved patterns could well be attributed to any other polydisperse material that happens to absorb or scatter light at 260nm, as for example glycogen or unspecific aggregates of ribosomes. This criticism may be levelled at the polyribosomal sedimentation patterns of Bratt and Robinson (1967) and Blair and Robinson (1970). Nor did these workers include in the polyribosome isolation procedure a step in which the cytoplasmic extract or post mitochondrial supernatant spun through a 0.5/2.0 M sucrose double layer. This step is useful in that under suitable conditions of centrifugation, the pellet will contain mostly polyribosomes with almost no monosomes because the viscosity barrier at the 0.5/2.0 M interface retards the single ribosomes and a portion of the smaller oligomers sufficiently to prevent them from reaching the bottom. Sedimentation of the polyribosomes

through 2M sucrose also results in a significant purification, since most of the soluble proteins of the cytoplasm remain at the interface between the 0.5 and 2M sucrose layers. With these points in mind the association of the sub-genomic species of ND vsi-RNA with polyribosomes was re-investigated.

Chorioallantoic membranes provided large amounts of intact polyribosomes. Unfortunately, ND vsi-RNA was not labelled sufficiently in the presence of [^{3}H]-uridine and AMD for it to be detected in the cells of chorioallantoic membranes. It is suggested that the failure to detect ND vsi-RNA could have resulted from one or a combination of the following:- (i) The membranes were less sensitive to AMD (Barry et al., 1962; Barry, 1964b) so that cellular RNA synthesis was not sufficiently depressed for ND vsi-RNA synthesis to be detected; (ii) the nucleotide pool size in the infected cells was large so that the added [^{3}H]-uridine was diluted out too far for its incorporation into ND vsi-RNA to be detected; (iii) the rate of ND vsi-RNA synthesis was very low in membrane cells despite the fact virus grew quite well in these.

The development of a procedure for isolating intact polyribosomes from chick embryo cell (CEC) monolayer cultures has proved itself of great potential with regard to the identification of viral messenger RNAs. For example, by using puromycin and high potassium chloride concentrations to release mRNA from polyribosomes, it has been possible to identify a 26S RNA species in SFV polyribosomal associated RNA that is probably the viral messenger RNA coding for the core protein and envelope protein of this virus (Kennedy, 1972). However, SFV is a much simpler virus to NDV structurally, and much more is known biochemically about SFV replication than that of NDV. There is also the advantage of having a range of temperature sensitive mutants of SFV; the mutant used by Kennedy (1972) does not assemble the nucleocapsid which, having an $S_{20,w}$ value of 140S, would otherwise contaminate polyribosomes. Thus it could be predicted, almost at the beginning of experimental work on this

investigation, that without sufficient knowledge of NDV replication or the advantage of having well characterised temperature sensitive mutants of NDV, that unequivocal identification of NDV mRNAs would be difficult. This prediction has been borne out by experiments reported in this thesis relating to the association of virus specific RNA to polyribosomes of NDV infected chick embryo cells. It proved difficult to confirm the findings of Bratt and Robinson (1967) and Blair and Robinson (1970) that polyribosomal associated RNA contains 18S and 35S RNA of total vsi - RNA. This was probably because total polyribosomal associated RNA from NDV infected cells not only contained 18S and 35S RNA, but also a whole range of partially degraded 18S and 35S RNA molecules. However, the situation has become more hopeful with the finding that much of the degraded virus specific RNA associated with polyribosomes was released by heating to 37°C for 2 minutes in the presence of 0.5M potassium chloride, and could therefore be separated from polyribosomes with which intact 18S and 35S RNA molecules were associated. When puromycin was used to dissociate polyribosomes completely to give ribosomal subunits, transfer RNA and m RNA, it was found that virus specific RNA isolated from material sedimenting faster than ~ 30S contained probably all of the RNA species, i.e. 18S, 22S, 35S and 50S, normally found in total ND vsi - RNA. It is as yet unknown whether this virus specific RNA was associated with the ribosomal subunits or was present in viral messenger ribonucleoprotein complexes which sediment faster than the free RNA molecules. Further work is now in progress in this laboratory in an attempt to distinguish between these two possibilities. Whatever the outcome, it is almost certain that some, if not all, of the sub-genomic, complementary RNA species found in NDV infected cells will prove to be viral mRNAs.

Huang et al. (1971) showed NDV particles to contain an RNA-dependent RNA polymerase which used the viral genome as a template to synthesise complementary RNA. This RNA was initially found in association with the template RNA as a partially base-paired structure with a sedimentation coefficient of around 42S (Clavell and Bratt, 1971). These latter workers subsequently showed that on longer periods of incubation in the in vitro polymerase assay that single-stranded RNA molecules were released from the template RNA. Much of this single-stranded RNA had a sedimentation coefficient of approximately 16S, but there were also significant amounts of more slowly sedimenting RNA. Stone et al. (1971) have also found 16S RNA in the product extracted from the Sendai virus RNA polymerase assay mixture, but other workers have found predominately slowly sedimenting, single-stranded RNA in this system (Robinson, 1971b; Hutchinson and Mahy, 1972). Because of time considerations it was not possible to investigate extensively the products of the in vitro NDV RNA polymerase assay. However, large amounts of low molecular weight RNA ($\sim 2 \times 10^4$) were demonstrated plus small amounts of heterogeneous higher molecular weight RNA (2×10^4 - 10×10^6). Since "18S" RNA, "22S" RNA and 35S RNA, the usual components of ND vsi-RNA were not found, it seems probable that under the conditions of the in vitro assay that the RNA polymerase was restricted to the synthesis of small pieces of complementary RNA. It is suggested, however, that although NDV RNA polymerase may not synthesise complete copies of the viral mRNAs in vitro, that it is the enzyme responsible for their synthesis in vivo. Consistant with this was the demonstration that synthesis of 18S, 22S and 35S RNA was detectable in the presence of cycloheximide soon after paramyxovirus infection of chick cells (Robinson, 1971b; Clavell and Bratt, 1971). An attempt to confirm this observation was unsuccessful.

In the RNA polymerase *in vitro* assay different strains of NDV had different polymerase specific activities. It is not known whether different strains have different amounts of enzyme or whether enzyme activity is regulated to differing degrees among the strains. The latter seems the more likely with the finding that polymerase specific activity can be increased by storage at 37°C (see below). Whilst the polymerase specific activity may be characteristic for each NDV strain, neither does it correlate with virus virulence nor RNA synthesising ability *in vivo*. For example, the H strain RNA polymerase specific activity is particularly low, yet this strain is very virulent in chicks (Lomniczi et al., 1971).

Like many other enzymes, NDV RNA-dependent RNA polymerase was unstable. Work done in this laboratory (Sheaff et al., 1972) showed that heating NDV at 56°C caused loss of polymerase activity at the same rate as loss of infectivity suggesting that polymerase activity was necessary for infectivity. It was also found that both RNA polymerase activity and infectivity were unstable at 4°C as reported in this thesis. Similar loss of VSV RNA polymerase activity during storage at 4°C (and -20°C, -70°C) of purified VSV has also been observed by Bishop (1971). The reason for this is most probably due to internal configurational changes within the virus particles which progressively decrease the activity of the RNA polymerase rather than denaturation of the enzyme which is the process occurring at 56°C. Consistent with this was the finding that after storage of purified NDV strains at 4°C resulting in considerable loss of polymerase activity, that polymerase activity could be increased again by incubation at 37°C for 48 hours. During 37°C incubation infectivity fell while polymerase activity rose. The rate of decay of polymerase activity at 4°C was approximately the same in all NDV strains tested, but "re-activation" of polymerase activity by incubating at 37°C for 48h was not. Two NDV strains, H and F, after incubation at 4°C and then at 37°C, were shown

to have polymerase activities greater than those assayed immediately after purification. This information coupled with the variation in initial polymerase activities found in different NDV strains, suggests the presence of control factors for NDV RNA polymerase. These control factors might determine the characteristic polymerase level of each NDV strain and be affected by incubation at 37°C so that an elevated polymerase activity results. The rise in polymerase activity, accompanied by a fall in infectivity, suggests that the products of the RNA polymerase after incubation at 37°C might be different from normal. This is now under investigation in this laboratory.

The action of Triton N101 on NDV was rapid and limited. Examination of electron micrographs and determination of the sedimentation coefficients showed that the sub-viral particles produced by the action of Triton N101 were heterogeneous in their morphology. They ranged from completely stripped particles to apparently intact virus particles. It was also shown that no more protein or [^{32}P]-label (phospholipid) could be removed from virus particles after 30 minutes' incubation at 32°C.

Protein polyacrylamide gel electrophoresis showed that all NDV glycoproteins were substantially removed by Triton N101. An analogous situation was observed by György et al. (1971) for rabies virus. Treatment of rabies virus with the non-ionic detergent Non-idet P-40 (very similar to Triton N101) resulted in solubilisation of viral lipids and preferential release of the envelope glycoprotein. Only one of the NDV glycoproteins (VP3) was a major component of untreated virus, and this has been shown to be associated with virus haemagglutinating activity (Haslam et al., 1969, Evans and Kingsbury, 1969; Iinuma et al., 1971). The other two glycoproteins, the smaller of which has not been described before, can only be present in minor amount, for only small differences were observed in the peak areas on Coomassie blue stained gels of either VP4 (15%) or VP7 (1.5%), their positions of electro-

phoretic mobility, after removal of the glycoproteins by detergent treatment. Therefore, the action of Triton N101 in activating NDV RNA polymerase probably consisted of removal of the viral envelope, comprising the protein surface projections (spikes) and a phospholipid layer. This yielded sub-viral particles that were permeable to the RNA polymerase substrates and co-factors, thus revealing enzyme activity. In agreement with this interpretation, it was found that the isolated sub-viral particles produced by Triton N101 treatment could be assayed for polymerase activity without further addition of Triton N101. Since polymerase activity was shown by particles which contained no surface proteins, it is suggested that the viral envelope plays no part in determining RNA polymerase activity.

Since the sub-viral particles produced by Triton N101 treatment had polymerase activity, they clearly contained the RNA polymerase enzyme complex. However, they proteins which make up the RNA polymerase enzyme remain unidentified. NDV nucleocapsids did not have RNA polymerase activity. This could be due to loss of enzyme or enzyme co-factors during purification, denaturation of the enzyme by deoxycholate or incomplete removal of this, or aggregation of nucleocapsids to form impermeable clumps.

The overall picture of NDV RNA synthesis is growing, but is still far from complete. It would appear that the viral genome enters the cytoplasmic matrix in a nucleoprotein complex. This nucleoprotein complex contains an RNA-dependent RNA polymerase capable of transcribing sub-genomic, complementary RNA molecules which serve as the viral messenger RNAs. Replication of the viral genome probably requires new protein synthesis as this is inhibited in the presence of cycloheximide (Robinson, 1971b; Clavell and Bratt, 1971; Kaverin and Varich, 1971). Much of the evidence for this scheme of viral RNA synthesis, although positive, remains circumstantial and the control of viral RNA synthesis and its possible relationship with virulence is not understood. It is hoped that

future work will provide more direct evidence to enhance further the understanding of the mechanism and control of RNA synthesis by this very interesting virus.

The role of NDV RNA-dependent RNA polymerase in the induction of interferon formation by NDV.

It has been known for some time that myxoviruses, irradiated with ultraviolet light, are good inducers of interferon formation (Burke and Isaacs, 1958; Ho and Breinig, 1965; Cantell and Paucker, 1963; Wagner, 1964; Youngner et al., 1966). However, several lines of evidence suggest that the unirradiated myxoviruses are intrinsically capable of inducing interferon formation. A large number of unirradiated NDV strains produce interferon in chick cells if the cells have been pre-treated with interferon (Lomniczi and Burke, 1971), while the H strain, at low multiplicities of infection, will produce low titres of interferon even without interferon pre-treatment (Lomniczi and Burke, 1971), and unirradiated NDV will produce interferon in L cells (Wagner, 1964; Youngner et al., 1966). If infective virus is therefore capable of inducing interferon in certain conditions, why does it not do so in all? Gandhi and Burke (1970) suggested that unirradiated myxoviruses failed to produce interferon in chick cells because infection led to an inhibition of cellular RNA and protein synthesis, and hence of interferon production. They found that when virus was ultraviolet irradiated it lost its capacity to depress host cell RNA and protein synthesis, and that interferon was not formed until virus was sufficiently irradiated to have no effect on these functions. L cells, however, infected with NDV produced interferon in spite of the fact that the inhibition of cellular RNA and protein synthesis was more marked than in chick cells (Thacore and Youngner, 1970; Gandhi and Burke, unpublished work) suggesting that this inhibitory effect of virus infection on cellular RNA and protein synthesis was not responsible for the control of interferon production. This was confirmed by the results

obtained with lentogenic (avirulent) strains of NDV which, although they did not inhibit cellular RNA and protein synthesis, did not produce interferon (Lomniczi and Burke, 1971). Therefore, it can be concluded that generally interferon formation is only induced by unirradiated virus after infection of L cells or chick cells pre-treated with interferon. These results suggested that infective virus has the intrinsic capacity to induce interferon, but that interferon production is blocked by a product of virus replication, which is probably a protein (Colby, 1971; Lomniczi and Burke, 1971; Clavell and Bratt, 1971; Meager and Burke, 1972). An investigation carried out by Dr. E.T. Sheaff in this laboratory showed that NDV heated at 56°C for a few minutes became an interferon inducer. There are two possible explanations for this:- (i) infective virus is intrinsically unable to induce interferon formation, but that heating leads to the formation of an active inducer, (ii) infective virus is intrinsically capable of inducing interferon formation, but does not do so because a product of virus infection inhibits induction or synthesis of interferon, and this viral product is dependent on a heat labile function of the virus particle. If the first explanation were correct, a mixture of unheated and heat inactivated virus should produce interferon as if it contained only heat inactivated virus. If the second explanation were correct then the infective (unheated) virus should inhibit interferon formation by the inactivated virus, and the mixture would behave as if it contained only infective virus. It was conclusively shown by Dr. E.T. Sheaff that by using mixtures of unheated virus and heat inactivated virus that interferon formation was inhibited. This indicates that the second explanation, i.e. the production of an interferon inhibitor by infective virus during the replicative cycle, is correct. Therefore, it was suggested that treatment of NDV with ultraviolet light or heat at 56°C, both of which yield virus capable of inducing interferon formation, destroys the ability of the virus to produce the interferon inhibitor.

It was further suggested that this inhibitor was not produced, produced in reduced amount, or its production delayed in mouse L cells and in chick cells pre-treated with interferon. Failure to produce the inhibitor in sufficient amount thus allows interferon production to proceed (Sheaff et al., 1972).

The capacity of ultra-violet irradiated non-infective viruses to produce interferon is restricted to the ortho- and para-myxoviruses and reoviruses (Burke and Isaacs, 1958; Cantell and Paucker, 1963; Long and Burke, 1971). All three groups contain RNA-dependent RNA polymerases in the virus particle (Chow and Simpson, 1971; Huang et al., 1971; Shatkin and Sipe, 1968). Therefore, it is possible that the RNA polymerase has a role in interferon induction, and work reported in this thesis strengthens this idea.

NDV infectivity and RNA polymerase activity are both lost on UV irradiation of the virus as has been reported in this thesis. However, the rate of loss of RNA polymerase activity is ten times slower than the rate of loss of infectivity. Why is this? The initial effect of UV irradiation on RNA viruses is to cause formation of uracil hydrate and possibly uracil dimers (Tao et al., 1969; Carpenter and Kleczkowski, 1969; Remson et al., 1971). In the case of viruses like NDV, which carry their own RNA polymerase, these lesions are likely to block the progress of the polymerase (Micalke and Bremer, 1969), thereby preventing complete transcription of the RNA. As it is in all probability the newly synthesised complementary RNA molecules made by the RNA polymerase that are translated in the polyribosomes (Bratt and Robinson, 1967; Blair and Robinson, 1970), viral protein synthesis is inhibited and no infective virus is produced. Thus, there may be some viral RNA synthesis without subsequent translation. In agreement with this Clavell and Bratt (1971) showed that the single-stranded RNA products made in the <u>in vitro</u> RNA polymerase assay became progressively smaller as the UV-dose was increased. Therefore if

viral protein synthesis and replication are inhibited when cells are infected with UV-irradiated virus, then the formation of the interferon inhibitor will also be inhibited and interferon production ensue. However, the question as to what the inducer of interferon formation is in UV-irradiated NDV infected cells still remains. There are three possibilities. First, interferon induction requires the formation of double-stranded RNA. It is known that in both NDV and the similar vesicular stomatitis virus (VSV) infection the newly synthesised RNA molecules are associated with the template RNA of the virus in a partially base-paired structure (Huppert et al., 1969, 1970; Zhdanov and Kingsbury, 1969; Zhdanov et al., 1970; Bratt and Robinson, 1971; Schincariol and Howatson, 1970), and a partially double-stranded structure has been isolated from the in vitro RNA polymerase system (Huang et al., 1971; Clavell and Bratt, 1971; Bishop and Roy, 1971a,b). This double-stranded structure is associated with a sub-viral particle, and on longer periods of incubation newly synthesised single-stranded RNA is released from the particle (Bishop and Roy, 1971a,b; Clavell and Bratt, 1971). However, the precise amount of natural base-pairing in this transcriptive complex is unknown as SDS-phenol extraction of RNA artifactually induces further base-pairing by removal of protein from the complex (Oberg and Philipson, 1971; Clavell and Bratt, 1971). Nor is it known whether such transcriptive complexes exist in vivo. However, Bukrinskaya et al. (1969) found using Sendai virus infected Ehrlich tumour cells, partially deproteinised nucleocapsids on which newly synthesised RNA was being made in association with polyribosomes. This evidence, plus the demonstration of some ribonuclease resistant RNA in NDV infected cells soon after infection (2.5 h p.i.) (Huppert et al., 1970; Zhdanov et al., 1970), indicate that some virus specific, base-paired RNA exists in infected cells whether or not the transcriptive complexes in vitro or in vivo are the same. UV-irradiated virus has less ability to make both single-stranded and double-stranded RNA in vitro

(Clavell and Bratt, 1971), and this almost certainly applies to the in vivo situation (Clavell and Bratt, 1971; Huppert et al., 1969, 1970). The question that remains to be answered is whether this small amount of base-paired RNA associated with a transcriptive complex is the inducer of interferon? It is not yet possible to answer this question because of the lack of experimental techniques required to investigate such a problem. Nor does it seem likely that this first possibility, i.e. the formation of double-stranded RNA, can be distinguished from a second possibility which is as follows:- interferon induction is a consequence of limited viral RNA synthesis, which may also, but not necessarily, lead to the formation of double-stranded RNA structures. This may arise if the association of a host cell factor with the RNA polymerase is necessary for viral messenger RNA synthesis, and this association or the subsequent viral RNA synthesis triggers the sequence of events leading to the derepression of the interferon gene. Huang and Baltimore (1971) have described such a host cell factor in cytoplasmic extracts of uninfected Chinese hamster ovary cells which enhances VSV RNA polymerase activity. In cells infected with UV-irradiated virus, transcription by the polymerase of complete mRNAs probably does not occur, but limited RNA synthesis, i.e. synthesis of small pieces of complementary RNA, is possible.

A third possibility is that interferon is induced by the undegraded NDV RNA. However, this is not now tenable with the demonstration that NDV inactivated with β-propiolactone (BPL) to an extent that it can no longer induce interferon formation contained undegraded virus RNA. Summarising, it is thought that there are two alternative mechanisms by which UV-irradiated virus induces interferon: (i) formation of double-stranded RNA; (ii) limited viral RNA synthesis and the association of a host cell factor with the virus RNA polymerase.

It is now possible to ask why interferon inducing capacity is lost on long exposure to UV light. Presumably this is because of the loss of ability to form the inducer. It has been demonstrated that the rate of loss of interferon inducing capacity and rate of loss of RNA polymerase activity were similar suggesting that they were connected. If double-stranded RNA is the interferon inducer, then it can be envisaged that when RNA polymerase activity has fallen below a certain level not enough of this double-stranded RNA is made to induce interferon formation. The breakdown of NDV RNA on UV-irradiation of virus particles may also be a contributing cause to the loss of both RNA polymerase activity and interferon inducing capacity.

The breakdown of NDV RNA on UV-irradiation of NDV particles is a novel and puzzling phenomenon. Work reported in this thesis has indicated that the involvement of any nucleolytic process was unlikely. Further, the demonstration that in two other RNA viruses, TMV and SFV, that although extraction of the virus RNA was reduced, possibly because of a UV-induced photochemical reaction causing covalent bonding of protein and RNA, breakdown of virus RNA probably did not occur, makes any explanation of this breakdown difficult. It is suggested that breakdown results from a photochemical reaction catalysed by UV light and some amino acid residue of the RNP, such as tryptophan. Helene and Charlier (1971) have shown that tryptophan or 5-hydroxytryptophan will photosensitize the splitting of pyrimidine dimers at certain wavelengths. Determination of whether or not pyrimidine dimers are formed in NDV RNA when virus particles are UV irradiated, and whether these dimers are photosensitized by amino acid residues to split and hence give rise to polynucleotide chain breaks, is likely to be difficult.

It is likely that β-propiolactone destroys virus infectivity and RNA polymerase in a similar way to UV-light. That is, by modification of the nucleotide bases in the NDV RNA so that viral mRNA synthesis by the polymerase is inhibited and no infectious virus produced. It has been shown that BPL reacted with guanosine monophosphate (GMP) under the conditions used to inactivate NDV to give 7-carboxyethyl guanine and 7-carboxyethyl guanosine (J. Tyrrell, personal communication). Roberts and Warwick (1963) have reported a similar finding using yeast RNA. Therefore, inactivation of NDV by BPL is probably caused by modification of GMP residues in NDV RNA. With increasing BPL concentrations these modifications lead to:- (i) progressive loss of infectivity and interferon inhibitor production resulting from the formation of transcription terminating lesions in NDV RNA and reduced viral mRNA synthesis; (ii) production of interferon inducers, e.g. double-stranded RNA; (iii) further loss of polymerase activity as a consequence of over-modification of NDV RNA, resulting in the loss of ability to produce interferon inducers. The demonstration that NDV RNA does not breakdown with increasing concentrations of BPL indicates that this does not contribute to the loss of interferon inducing capacity in this case (cf. UV-irradiation).

Ultraviolet irradiation and BPL treatment of NDV are not the only ways in which virus can be converted to an interferon inducer. It is known that heat at 56°C for short times (a few minutes), storage at 37°C for long times (days), or treatment with acid pH buffer (e.g. pH 2.5) potentiates the interferon inducing capacity of NDV (Kohno and Kohase, 1969; Kohno et al., 1969). The work of Sheaff et al. (1972) also relates to such treatments of NDV, and is relevant to this discussion. Reiterating, they found that purified NDV heated at 56°C lost infectivity and RNA polymerase activity at the same rate, and became capable of inducing interferon formation. This led them to suggest firstly, that the effect of heat at 56°C was on the RNA polymerase protein complex rather than on the virus RNA (contrast the effects of UV and

BPL), and secondly, that heat destroyed the capacity to produce the inhibitor of interferon formation faster than it destroyed the capacity to induce interferon. Since it was also found that virus which had no polymerase activity had also lost its capacity to produce interferon, it was further suggested that interferon induction required limited polymerase activity, while both infectivity and production of the interferon inhibitor required complete transcription of NDV RNA. The results obtained with virus treated at pH 2.5 were difficult to explain since it was shown that virus did not lose much infectivity yet became able to induce interferon. However, interferon yields were low and it was suggested that as there was some loss of polymerase activity this was responsible for the loss of infectivity and led to a decreased rate of interferon inhibitor production thus allowing limited interferon formation.

As reported in this thesis, incubation at 37°C of purified NDV strains caused a rise in polymerase activity accompanied by a fall in infectivity, suggesting that the products of the RNA polymerase were different from normal. Therefore, it was predicted that, as in the case of virus incubated at 56°C, mRNA synthesis became faulty leading to decreased production of interferon inhibitor, and the ability of all NDV strains to induce interferon formation. However, of five strains tested, only two strains, Herts 33 and H, became interferon inducers. It is interesting that in one case, Herts 33, infectivity was very unstable both at 4°C and 37°C, and in the other case, H, RNA polymerase was easily the most "re-activated" at 37°C. Therefore, it is suggested that the Herts 33 strain after 37°C incubation, because of low infectivity, is unlikely to make much interferon inhibitor, and low titres of interferon are produced due to faulty transcription and increased production of interferon inducer, while the H strain produces a decreased amount of inhibitor and large amounts of inducer to produce high titres of

interferon. This emphasises the point of this interferon inhibitor - inducer hypothesis; that here is a system where the process of virus infection can lead to two opposing sequences of events, replication and interferon production, and these are delicately balanced. Normally when virus infects cells it is able to replicate without interferon formation because a product of replication blocks this. However, any perturbation of the virus potentiates interferon production at the expense of replication and interferon inhibitor synthesis. Perturbation of the virus can be caused in two ways:- (i) treatment of the virus with an agent, e.g. UV light, which is likely to destroy viral functions, e.g. RNA polymerase activity; (ii) treatment of the cells to be infected with an agent, e.g. interferon, that is likely to inhibit virus replication, e.g. by inhibiting viral RNA synthesis (Richman et al., 1970; Thacore and Youngner, 1972).

SUMMARY

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It was shown by sucrose density gradient centrifugation that NDV particles contain two RNA species, a single high molecular weight RNA species and heterogeneous low molecular weight RNA. The high molecular weight RNA species, which is the viral genome, had a sedimentation coefficient of approximately 50S, and a molecular weight of $\sim 5 \times 10^6$ as determined by RNA polyacrylamide gel electrophoresis. The heterogeneous low molecular weight RNA, which was not examined, was probably comprised of degraded 50S RNA and contaminating cellular RNA, and had a sedimentation coefficient of 4S. Virus specific RNA isolated from NDV infected cells was shown by sucrose density gradient centrifugation to contain 4 main components, 50S, 35S, 22S and 18S RNAs. Resolution of the component RNA species of Newcastle Disease virus specific intracellular RNA (ND vsi-RNA) using sucrose gradients was poor, and it was later shown that this could be much improved using RNA polyacrylamide gel electrophoresis. Both the 22S and 18S size class RNAs of ND vsi-RNA were demonstrated to be heterogeneous by using polyacrylamide gel electrophoresis. The molecular weights of all the distinguishable RNA species contained in ND vsi-RNA were determined by calibration of the polyacrylamide gels with a range of RNA molecules of well characterised molecular weights, i.e. HeLa cell nucleolar RNA.

A correlation between virus virulence and ND-vsi RNA synthesis, particularly of the 18S size class of RNA, was observed. The explanation of this correlation is not yet possible. The sub-genomic RNA species of ND vsi-RNA were found in association with chick polyribosomes implicating them as viral messengers.

The general properties of NDV RNA-dependent RNA polymerase were investigated and found in the main to be in agreement with published results (Huang et al., 1971). This enzyme, contained in NDV particles, is probably responsible for the synthesis of sub-genomic, complementary RNA species found in NDV infected

cells, although it was demonstrated that it did not synthesise these species *in vitro*. It was found that different NDV strains had different polymerase specific activities, but no correlation with virus virulence was apparent. It was also shown that RNA polymerase activity of several NDV strains was unstable at 4°C and 37°C. Polymerase activity was reduced at 4°C, but increased by incubation at 37°C. It was shown that polymerase activity was retained after removal of the viral envelope, comprising virus glycoproteins and phospholipid, suggesting that the viral envelope plays no part in determining polymerase activity. The protein(s) contained in the virus particles responsible for polymerase activity was not identified.

NDV RNA polymerase activity and infectivity were shown to be destroyed by ultra-violet irradiation and the virucide, β-propiolactone. NDV inactivated by ultra-violet irradiation or β-propiolactone was a good inducer of interferon in chick cells. The retention of some RNA polymerase activity in inactivated NDV particles suggested that this enzyme might have a role in the induction of interferon formation. Further investigations using NDV inactivated in various ways, e.g. heat at 56°C, has strengthened this idea (Sheaff et al., 1972).

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