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A Paracrystalline Array of Pseudorabies Virus Nucleocapsids  
in a HeLa Cell Nucleus

(By courtesy of Dr. W.S. Stevely)

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Messenger RNA Synthesis in HeLa Cells

Infected with Pseudorabies Virus

by

J. Keith Vass

A Dissertation Submitted to the

UNIVERSITY OF GLASGOW

for the degree of

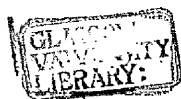
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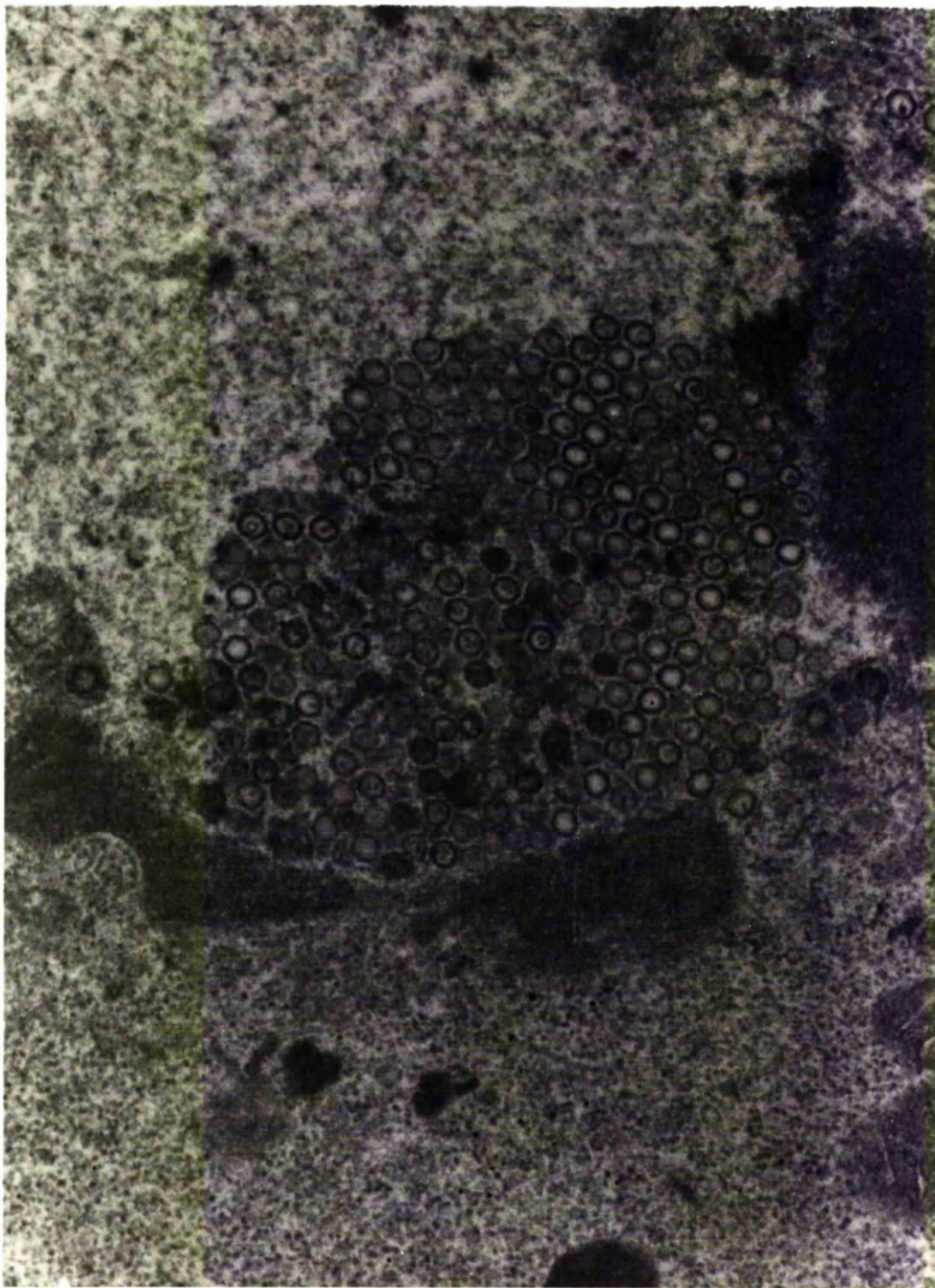
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Abbreviations

These are laid down in the Biochemical Journal Instructions to Authors (revised 1975) with the following additions:

PrV	pseudorabies virus (pig herpesvirus 1)
HSV	Herpes simplex virus
pfu	plaque forming unit
EDTA	ethylene diaminetetracetic acid (versene)
SDS	sodium dodecyl sulphate
PAGE	Polyacrylamide Gel Electrophoresis
HnRNA	heterogeneous nuclear RNA
mRNA	messenger RNA
rRNA	ribosomal RNA
poly-(A)	polyadenylic acid
RNP	ribonucleoprotein particle
pre-polysome pellet -	

that part of a cytoplasmic extract which sediments through a sucrose density gradient more rapidly than free polysomes.

### Summary

Polysomes isolated from HeLa cells infected with pig herpesvirus 1 (pseudorabies virus or PrV) were found to sediment more rapidly than those isolated from mock-infected cells. This result has been previously reported to occur in rabbit kidney cells, infected with PrV (Ben-Porat et al., 1971). These authors also reported that polysomes disaggregate after infection with PrV, and this was also noted in this study and the effect was quantitated, for the first time. The effects of the metabolic inhibitors actinomycin D and cordycepin were also investigated and both were found to increase the rate of polysome disaggregation in infected cells. These inhibitor studies could be interpreted in terms of the theory of Leibowitz & Penman (1971), who postulated that the disaggregation of cellular polysomes in HeLa cells infected with poliovirus was at least partly due to the inhibition of the synthesis of a small molecular weight cellular RNA species, with a translational control function.

Polyadenylated RNA was isolated from whole polysomal RNA by poly<sup>m</sup>(U) sepharose chromatography and shown to contain mRNA by its ability to stimulate the incorporation of labelled amino acids into protein in a cell free system. The polyadenylated RNA was characterised by polyacrylamide gel electrophoresis and found to be heterogeneous in size in both infected and mock-infected cells. Polysome size did not mirror the average size of mRNA - large mRNA species were found on small polysomes and small mRNA species were found in large polysomes. The most likely explanation of this was thought to be that the frequency of initiation of polypeptide synthesis was the rate limiting process in

protein synthesis, and that mRNAs with a high frequency of initiation would be associated with a larger number of ribosomes than those with a low initiation frequency. The PAGE profiles showed that mRNA in infected and mock-infected situations were of similar size; this together with the larger polysome size in infected cells indicates that more ribosomes may be found per unit length of viral mRNA than on the same length of cellular mRNA. It is possible that one mechanism whereby the switch from cellular to viral protein synthesis takes place is that viral mRNA promotes more efficient initiation than cellular mRNA.

Polysomal RNA synthesis was monitored in cells infected in the presence or absence of cycloheximide. This inhibitor of eukaryote protein synthesis was included to find if any species of viral RNA, detectable by PAGE, were only present if viral proteins had first been synthesised. Two species of polysomal RNA which normally appear around 3 - 4 hours post-infection, were still not synthesised by 5 hours post-infection in the presence of cycloheximide. Using molecular hybridisation techniques Rakusanova et al. (1971) found that only a subset of the viral RNA sequences normally present late in infection were synthesised in cells infected in the presence of cycloheximide. The specific effect of cycloheximide, reported in this thesis, confirms the results of Rakusanova et al. (1971) by another technique. This result shows that the production of viral mRNA is a controlled process and that some mRNA species do not appear in the cytoplasm until viral protein synthesis has begun. The first group of viral proteins probably affect the transcription or post-transcriptional processing of later classes of mRNA.

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## Introduction

### 1.1. General Introduction

Most mammalian cells synthesise many different species of messenger RNA. Some cells however produce large quantities of a single protein along with its mRNA and this has facilitated the purification of such RNA species from appropriate tissues. Control of transcription in eukaryotes is still poorly understood, but appears to be more complex than in bacteria. In at least some eukaryotic cells protein synthesis is controlled at the translational as well as at the transcriptional level, and for this reason the appearance or non-appearance of a protein does not necessarily indicate whether or not its messenger RNA has just been synthesised. The control of mRNA synthesis in virus infected cells is sometimes more easily studied than in uninfected cells as the transcription of host genes is often inhibited, a new set of viral mRNAs is synthesised and some synchronisation of events is achieved. Viral genomes are much smaller than that of the host and the number of proteins is correspondingly small. For these reasons it is likely that examination of transcription in virus infected cells could provide valuable insight into general mechanisms of control of RNA synthesis in eukaryotes.

Some viral infections also pose intriguing questions on translational control. Synthesis of host proteins is inhibited but this phenomenon cannot be explained by breakdown of host mRNA. How does the protein synthetic system discriminate between host and viral messengers? Not only do early viral mRNAs supplant host messengers in polysomes but they can in turn be replaced by late viral species. So it appears that

in infected as well as in normal cells control can be exercised both at transcriptional and translational levels.

## 1.2. The Aim of the Project

The aim of the project was to examine the control of mRNA synthesis in HeLa cells infected with Pig Herpesvirus 1 (Pseudorabies Virus). The distribution of mRNA species between light, heavy and membrane-bound polysomes was also to be investigated. The RNA species were to be characterised using polyacrylamide gel electrophoresis.

## 1.3. The Nature of Viruses

Viruses are completely parasitic entities and have an obligatory requirement for a susceptible host cell before they can replicate. They utilize the cellular systems for energy production and protein synthesis (Iwoff, 1957; Luria & Darnell, 1967). They possess only one type of nucleic acid, DNA or RNA, which is surrounded with a protein coat or capsid and, in some cases, this is in turn enclosed in a membrane, called the envelope.

## 1.4. The Herpesvirus Group

### 1.4.1. Classification

Virus classification is a subject open to a great deal of discussion and no single scheme has gained universal acceptance. However all classifications are based on various properties of viruses:

- (i) The host infected (animal, plant or bacterial)
- (ii) Clinical features of the disease (e.g. Burkitt lymphoma virus, tobacco mosaic virus and equine abortion virus)



- (iii) Intrinsic properties of the virus: these include the type of nucleic acid, symmetry of the nucleocapsid, presence or absence of an envelope, number of capsomeres (Tournier & Lwoff, 1966). Other characteristics which have been suggested are size, cellular-site of virus multiplication and maturation (Andrews, 1964) and molecular weight of nucleic acid (Melnick, 1973).

In 1970 the International Committee on Nomenclature of Viruses gave the following description of the herpesviruses:

(They) "Contain double-stranded DNA, molecular weight  $54 - 92 \times 10^6$ . G + C content 57 - 74%. Virus particle about 100 - 150 nm diameter, with a lipid containing membrane and therefore sensitive to lipid solvents. The DNA is about 7% of the particle weight. Bouyant density in CsCl 1.27 to 1.29 g/cm<sup>3</sup>. Development begins in the nucleus and is completed by the addition of membranes as the virus passes into the cytoplasm. Intranuclear bodies formed" (see Wildy, 1973).

In 1971 the International Committee for the Nomenclature of Viruses appointed a Herpesvirus Study Group to make recommendations concerning the nomenclature of these viruses. In the absence of sufficient information to establish an hierarchical system of classification, the following provisional system for the labelling of herpes viruses was proposed:

- (i) The label for each herpesvirus would be in an angledised form
- (ii) Each herpesvirus would be named after the taxonomic family to which its primary natural host belongs

- (iii) The herpesviruses within each group would be given arabic numbers. New herpesviruses will receive the next available.

In 1973, the time of the publication of this report (J. Gen. Virol., 20, 417-419), 39 herpesviruses were recognized. Table 1.4a compares the older names with the new for some of the more widely discussed herpesviruses.

Some suggestions for virus classification (Matthews, 1975) use the size of the genome as a parameter; this is inappropriate in the case of the herpesviruses until the variations in the molecular weights of their DNAs are understood. See table 1.4.b.

#### 1.4.2. Nucleic Acid

One of the first indications that the genome of herpesviruses is DNA was Kaplan and Ben-Porat's report (1961) that 5-fluorouracil blocked the formation of infectious pseudorabies virus. The block by this inhibitor of DNA synthesis was removed when thymidine was added to the medium. Herpesvirus DNA was subsequently shown to be a linear double-stranded molecule of molecular weight around  $100 \times 10^6$  (Russell & Crawford, 1963; Becker, Dym & Sarov, 1968; and Kieff, Bachenheimer & Roizman, 1971). There is a wide variety of sizes of herpesvirus DNA reported (see table 1.4.b), but the differences may, at least in part, be due to the techniques used.

The base composition of herpesvirus DNA varies widely between the different members of the group as is illustrated in Table 1.4.b. Pseudorabies virus has the highest G + C content, 73% (Kaplan & Ben-Porat, 1964) and canine herpesvirus 1 has the lowest reported, 33% (Plummer et al., 1969).

TABLE 1.4.a. (adapted from Herpesvirus Study Group, 1973)

A List of some of the herpes viruses

<u>Recommended Name</u>	<u>Trivial Name (with abbreviation)</u>
Human herpesvirus 1	Herpes simplex type 1 (HSV-1)
2	Herpes simplex type 2 (HSV-2)
3	Varicella-Zoster virus (V-Z)
4	Epstein-Barr virus (EBV)
5	Cytomegalovirus (CMV)
Pig herpesvirus 1	Pseudorabies virus (PrV)
2	Pig cytomegalovirus (CMV-pig)
Equid herpesvirus 1	Equine abortion virus
3	Coital-exanthema virus
Phasianid herpesvirus 2	Marek's disease virus
Ranid herpesvirus 1	Lucke virus
Catfish herpesvirus 1	Catfish herpesvirus
Bovid herpesvirus 1	Infectious bovine rhinotracheitis virus

TABLE 1.4.b. (Adapted from Gentry &amp; Randall, 1973)

Base Composition and Molecular Weights of Some Herpesvirus DNAs

Virus		G + C (moles %)	MW( $\times 10^6$ daltons)
Human herpesvirus 1	(HSV-1)	65 - 73	51 - 110
	2 (HSV-2)	66 - 72	88 - 95
	3 (V-Z)	46	-
	4 (EBV)	57 - 62	100
	5 (CMV)	57, 58	32
Pig herpesvirus	1 (PrV)	72 - 77	16, 22-70*
Equidherpesvirus	1 (EAV)	55 - 57	92
Bovid herpesvirus	1 (IBRV)	71 - 72	54
Phasianid herpesvirus	2 (MDV)	46 - 57	120, 130

\* 16 and 22 are the molecular weights of two fragments seen in one determination of M.W.

Herpesvirus DNAs have the unusual property of fragmenting when denatured in alkali. This was first observed for the DNA of Phasianid herpesvirus 2 (MDV) (Lee et al., 1971) and for Human herpesvirus 1 (HSV) (Kieff et al., 1971) and then for Human herpesvirus 4 (EBV) (Nonoyama & Pagano, 1972). The fragmentation could be due to the hydrolysis of phosphodiester bonds at the 3' end of ribonucleotides, reported to be covalently linked to HSV DNA (Biswal et al., 1974).

#### 1.4.3. Herpesvirus Structure

The architecture of herpesviruses has recently been reviewed by Watson (1973a) and by Roizman & Furlong (1974). Roizman and Furlong consider four main structures: (i) the core, (ii) the capsid, (iii) the tegument and (iv) the envelope; Watson has cast some doubt on the physical reality of the tegument.

##### 1.4.3.1. The Core

The core, located at the centre of the virus, appears as a structure of diameter 25 - 35 nm in thin sections viewed in the electron microscope (Morgan et al., 1959; Epstein, 1962; Siegart and Falke, 1966). This central structure has been shown to be sensitive to DNase but not to RNase or to proteolytic enzymes, indicating that the viral DNA is located in the core (Epstein, 1962; Zambarnard and Vatter, 1966; Chopra et al., 1970). Furlong et al. (1972) have studied the distribution of DNA in the core by selectively removing uranyl ions bound to the nucleic acid by treating stained preparations with EDTA. They concluded that the DNA is in the form of a toroid.

##### 1.4.3.2. The Capsid

The capsid has been intensively studied using the electron

microscope. In thin sections it appears as a fairly electron-dense hexagon or ring, separated from the core by an electron-translucent shell. The outer dimensions of the capsid generally vary between 85 and 110 nm (Stackpole and Mizell, 1968; Standberg and Carmichael, 1965; Wildy et al., 1960; Nazerian et al., 1971; Abodeely et al., 1970; and Nazerian and Witter, 1970). It is not yet known if the variation in size reported is a real phenomenon or due to the methods of sample preparation.

The capsid is made up of morphological subunits, known as capsomeres, which are visible in negatively stained preparations. Wildy et al. (1960) reported that the capsid is composed of 162 capsomeres, each 12.5 nm long and 8.0 - 9.0 nm in diameter, arranged as an icosahedron.

The region between the core and the capsid, the pericore, is not empty as the core remains in the centre of the capsid even after prolonged centrifugation and pelleting (Gibson and Roizman, 1972). In nucleocapsids (core + capsid) treated with the nonionic detergent NP40 for 30 minutes, the core loses its shape and can be seen lying next to the capsid (Abodeely et al., 1970) again suggesting that the pericore normally has some structure.

#### 1.4.3.3. The Tegument

The term tegument is suggested by Roizman and Furlong (1974) to describe the space between the capsid and envelope; this probably corresponds to the fibrous coat found around the capsid (Morgan et al., 1968; Wildy et al., 1960; Schwartz and Roizman, 1969). The tegument varies in thickness in different virions (Kazama and Schornstein, 1973; Nayak, 1971; Standberg and Carmichael, 1965; Stackpole and Mizell, 1968;

Nazerian and Witter, 1970). It must be noted that in at least two other recent reviews this structure is not considered (Watson, 1973a; Gentry and Randall, 1973) and its existence has still to be verified.

1.4.3.4. The Envelope is composed of lipids, glycoproteins and glycolipids, arranged in a bilayer structure, and surrounds the other virus structures (Morgan et al., 1954). Spikes project from the outer surface of the envelope (Wildy et al., 1960; Fong et al., 1973). The envelope is normally impermeable to negative stain (Watson, 1968) but seems to be more fragile than the cellular membrane (Cook and Stevens, 1970).

#### 1.4.4. The Infective Cycle

Watson (1973b), reviewing morphological studies on herpes-virus infection, cautions against a too facile interpretation of electron-micrographs. Drawing conclusions from electron-micrographs of samples taken sequentially throughout infection is difficult as herpesvirus infections are notoriously asynchronous (see Russell et al., 1964). Studies on attachment and penetration are usually carried out at a high multiplicity of infection and this approach implicitly assumes that all the particles interact with the cell in the same way.

##### 1.4.4.1. Attachment

Herpes simplex type-1 (HSV-1) is adsorbed at 4°C (Gostling and Bedson, 1956) but penetration only occurs after the cells are warmed (Farnham and Newton, 1959). Holmes and Watson (1963) found that less than 20% of particles adsorbed at 4°C were inside BHK 21 cells and most of these were in vesicles near the cell membrane. Enveloped particles adsorbed to the cells preferentially over naked nucleocapsids.

#### 1.4.4.2. Penetration

Penetration has been suggested to occur by one of two mechanisms: pinocytosis (Holmes and Watson, 1963; Epstein et al., 1964; Siegert and Falke, 1966), or by fusion of the cell membrane with the viral envelope (Morgan et al., 1968; McCracken and Clarke, 1971). Both these mechanisms may operate in different situations, depending on the cell line or the virus strain (Watson, 1973b).

Parental virion DNA is found associated with nucleocapsids in the cytoplasm (Hummeler et al., 1969) and these particles sometimes lack a surrounding vesicle or membrane. The fate of the capsid is uncertain but it is probably shed in the cytoplasm before the genome is transported to the nucleus. This is the start of the eclipse period.

#### 1.4.4.3. Eclipse

Virus particles cannot be identified in the cell during this phase and host chromosomes move to the nuclear membrane, a process known as margination. The nucleoli gradually disappear and this may be related to the inhibition of ribosomal RNA synthesis in infected cells. Along with rRNA, host DNA and protein synthesis are also inhibited (Roizman and Spear, 1969).

The length of the eclipse phase depends on the multiplicity of infection, which suggests that several virus particles can take part in the infective process. For pseudorabies eclipse lasts from 3 - 4 hours (Sydiskis, 1969).

#### 1.4.4.4. Assembly of Nucleocapsids

Following eclipse, isolated capsids are first seen in the



nucleus, later followed by paracrystalline arrays of aggregated particles (Morgan et al., 1959; Melnick et al., 1968; Schwartz and Roizman, 1969; Miyamoto, 1971). The most likely sequence of events is that the capsid is at least partially assembled and is then filled with DNA (Watson et al., 1964; McCracken and Clarke, 1971; Friedman et al., 1975). This is based on sequential studies during infection and on the effects of inhibitors of DNA synthesis.

#### 1.4.4.5. Envelopment of Nucleocapsids

Morgan et al. (1954) found enveloped herpes simplex virions in the cytoplasm of infected cells but only naked nucleocapsids in the nucleus and so suggested that envelopment took place in the cytoplasm. Darlington and Moss (1968) subsequently found large numbers of enveloped particles of HSV, PrV and EAV in vacuoles at the edge of the nucleus. Although a small proportion of enveloped virions have been seen in the nucleus in some studies (Reissig and Melnick, 1955; Stoker et al., 1959; Heine et al., 1971) the inner nuclear lamella is thought to be the normal site of envelopment (see Watson, 1973b), but other sites are believed to be involved sometimes.

#### 1.4.4.6. Release

Several mechanisms for the release of herpesviruses have been advanced. Morgan et al. (1959) proposed that reverse phagocytosis of the virus occurred; Schwartz and Roizman (1969a) suggested that particles travelled from the perinuclear space to the outside of the cell along endoplasmic reticulum channels connecting the two. Watson et al. (1964) suggest that in some cell lines release is at least partly due to cell lysis.

#### 1.4.5. Pseudorabies virus

The clinical manifestations of Pseudorabies virus (PrV) have recently been reviewed by McKercher (1973). PrV (Pig Herpesvirus 1) is a herpesvirus with typical physical characteristics of that group. It was one of the first viruses to be recognized (Aujeszky, 1902). The name pseudorabies comes from its clinical similarity to rabies. Pseudorabies occurs most frequently in swine, cattle, sheep, dogs and cats and to a limited extent in rats and mice (Gustafson, 1970). The natural route of infection is by nasal exposure to virus, or by ingestion (Gustafson, 1970). The virus multiplies in epithelial tissues in the nose, tonsils and pharynx and then spreads to the medulla by transport along nerve axons; on further multiplication the virus spreads throughout the brain (Gustafson, 1970; McCracken, McFerran and Dow, 1973; and Field and Hill, 1975).

Multiplication of the virus in the upper respiratory tract of swine causes leukocytes to be attracted to the infected area, pick up the virus and carry it to various organs, particularly the placenta. Infection of the placenta results in invasion of the foetus followed by abortion, or death in utero.

In pigs PrV mortality ranges from 100% in the newborn to almost nil in adults. In adult swine the most common effect of infection is a 50% abortion rate in affected sows (Gustafson, 1970). Some of the symptoms of PrV infection, depending on the age of the pigs, include fever, loss of appetite and vomiting. In swine which succumb further symptoms are: reluctance to move, muscular tremors, incoordination followed by coma and

then death. Itching is seldom seen in pigs, but is seen in the infections of cattle, cats and dogs and this has earned for the disease in these species the term "mad itch". In pigs the infection lasts between 48 and 72 hours in younger animals, but is prolonged in adolescents.

PrV has a wide cell culture host range, replicating in rabbit, swine, lamb, dog and monkey kidney; in HeLa cells; mouse fibroblasts; and in chicken embryos (Kaplan, 1969). Two types of cytopathic effect (CPE) are observed in cell culture: syncytium formation or granulation and clumping of cells with eventual lysis. Although both CPEs often occur in the same system one or the other predominates according to the virus strain and cell type.

#### 1.5. Effects of Herpesvirus Infection

Herpesvirus infection causes many biochemical and morphological changes in the host cell. The morphological effects such as margination of chromatin and disruption of nucleoli, which have been discussed earlier, are only a few of such effects on the host cell. Formation of giant cells by fusion of infected cells has been observed (Barski and Robineaux, 1959), intercellular filaments are disrupted, cell and nuclear membranes show altered morphology and microvilli are lost (Enlander et al., 1974). These morphological alterations are mirrored in biochemical changes, for example, virus-induced proteins have been found in infected-cell plasma-membranes (Kaaden and Dietschold, 1974).

##### 1.5.1. DNA Synthesis

Kaplan and Ben-Porat (1963) found that PrV infection causes a

gradual shift from host-cell to viral DNA synthesis. The inhibition of host DNA replication is practically complete by 7 - 8 h post infection. Similar results were obtained for the following herpesviruses: HSV (Roizman and Roane, 1964; Russell et al., 1964), EAV (O'Callaghan et al., 1968) and EBV (Gergeley et al., 1971; Nonoyama and Pagano, 1972).

Inhibition of cellular-DNA synthesis is thought to be dependent on post-infection protein synthesis (Ben-Porat and Kaplan, 1965). The mechanism involved in the switch-off of host-DNA replication has still to be elucidated. Virus-induced synthesis of histone-like proteins in PrV infected BHK-21 cells (Chantler and Stevely, 1973) could perhaps play a role in the process but many other possible explanations are still being advanced. A.T. Jamieson (personal communication) has noted that the pool sizes of deoxyribonucleoside triphosphates show profound changes on infection, the level of dTTP increases by 20 - 30 fold but dATP is greatly reduced. This may be due to the induction of a new ribonucleotide reductase and could be one reason for the inhibition of host-DNA synthesis.

Herpesviruses do not always inhibit cellular DNA synthesis. EBV and MDV, which are both thought to be oncogenic, confer upon leukocytes the ability to synthesise DNA and to multiply (Gerber and Hoyer, 1971; Lee, 1972). U.V. inactivated HSV-2 has also been shown to be capable of transforming a number of cell lines (see Duff and Rapp, 1975). The virus must be inactivated if the cells are to live long enough for transformation to be seen.

One virus type can have different effects on cellular metabolism. EBV does not transform cells when early viral antigens are synthesised,

instead cellular DNA replication is inhibited (Gergely et al., 1971).

Herpesviruses probably cause at least three kinds of infection: in the first, virus multiplication occurs; in the second the virus appears to be latent and gives rise to no gross effect; thirdly they can have an oncogenic effect and alter the normal cell controls.

### 1.5.2. RNA Synthesis

#### 1.5.2.1. Host-Specific RNA Synthesis

Kaplan and Ben-Porat (1967) found that RNA synthesis was inhibited in cells infected with PrV; this was also found to be true for HSV (Aurelian and Roizman, 1965; Hay et al., 1966; Flanagan, 1967). Hay et al. (1966) were first to find that rRNA precursor synthesis is undetectable by 7.5 hours post-infection with HSV. rRNA synthesis is more rapidly inhibited than 45S rRNA-precursor is (Wagner and Roizman, 1969). This indicates that the correct processing of rRNA precursor was inhibited in addition to the inhibition of its synthesis. Kaplan (1973) also found that the processing of 45S RNA was greatly inhibited in PrV infected cells. Methylation of 45S RNA is not affected although the processing of 45S RNA to rRNA is inhibited (Wagner and Roizman, 1969).

Roizman et al. (1970) and Rakusanova et al. (1972) found that cellular mRNA is processed abnormally in HSV and PrV infected cells, host messenger-like RNA species appear in the cytoplasm more rapidly than normal but do not appear to function as mRNA.

#### 1.5.2.2. Virus-Specific RNA

Wagner (1972) and Wagner et al. (1972) found that HSV-1

transcription was a controlled process; all the viral RNA transcribed before DNA replication begins (Early-viral RNA), continues to be synthesised late in infection, but at this time extra viral RNA sequences are transcribed (Swanstrom and Wagner, 1974; Murray et al., 1974). Not only is the "switch-on" of particular viral genes controlled but the rate of transcription also appears to vary during the infective cycle (Wagner et al., 1972; Frenkel and Roizman, 1972).

Honess and Roizman (1973, 1974, 1975) studied the control of HSV infection by following the synthesis of individual viral polypeptides. They concluded that there were at least 3 classes of mRNA, the synthesis of which is coordinately regulated and sequentially ordered. It would appear that these classes,  $\alpha$ ,  $\beta$  and  $\gamma$ , are similar to the immediate-early, early and late viral RNA classes, suggested by Rakusanova et al. (1971) to occur in PrV infected cells.

#### 1.5.2.3. Viral RNA in Cells Treated with Inhibitors of Protein Synthesis

Cycloheximide has been used extensively to study the control of virus-specific RNA in the absence of protein synthesis. Viral RNA is still transcribed even if protein synthesis is inhibited by cycloheximide from the time of infection (Rakusanova et al., 1971). However, when viral proteins are not present the viral RNA transcripts are only a subset of the species normally present before viral DNA replication begins. These RNA species which do not require prior virus protein synthesis for their transcription were called the "immediate-early" RNA. At least two other classes of herpesvirus RNA are thought to exist: early RNA which is synthesised only after some virus-induced proteins are present in the cell and late RNA which is transcribed only after viral DNA replication has begun. Frenkel et al. (1973) found no such restricted

transcription in HSV infected cells treated with cycloheximide but it was later shown that this was a difference between nuclear and polysomal RNA (Kozak and Roizman, 1974). Rakusanova et al. (1971) had isolated their RNA from polysomes but Frenkel et al. (1973) had extracted whole cell RNA for their report. The conclusions of Rakusanova et al. (1971) on the effect of cycloheximide on polysome-associated virus RNA were confirmed by Kozak and Roizman (1974). It appears that only immediate-early RNA is synthesised correctly when cycloheximide is present from the time of infection but other parts of the viral genome are expressed as RNA sequences which are however restricted to the nucleus.

#### 1.5.2.4. Viral RNA in Cells Treated with Inhibitors of DNA Synthesis

Late herpesvirus RNA is only synthesised after viral DNA replication has started. Inhibitors of DNA synthesis have been shown to prevent the appearance of late viral RNA in the polysomes. Early viral RNA however is unaffected and continues to be transcribed and to appear in the polysomes (Wagner et al., 1972; Murray et al., 1974; Swanstrom and Wagner, 1974; Swanstrom et al., 1975).

#### 1.5.2.5. Polyadenylation of Viral mRNA

Polyadenylic acid sequences have been found to be attached to at least some species of PrV mRNA (Rakusanova et al., 1972) and of HSV mRNA (Bachenheimer and Roizman, 1972; Silverstein et al., 1973). Polyadenylation is a post-transcriptional event (Bachenheimer et al., 1972).

#### 1.5.3. The Effect of Herpesvirus Infection on Polysomes

HEp-2 cell polysomes, characterised on sucrose gradients by a peak of 170S, are disaggregated during HSV infection and are replaced by virus-specific polysomes which sediment with a peak of about 270S

(Sydiskis and Roizman, 1966). PrV infection of rabbit kidney cells results in a similar effect (Ben-Porat et al., 1971). Proteins in PrV infected rabbit kidney cells have a lower lysine to leucine ratio than proteins in uninfected cells (Kaplan et al., 1970) and this fact has been used to determine if nascent polypeptides on polysomes are viral or cellular in origin. At 2.5 hours post-infection virus-specific mRNA predominated in the heavy polysomes while the light polysomes were still synthesising more cellular than viral proteins. By 5 hours after infection predominantly viral-like proteins are synthesised on both heavy and light polysomes (Ben-Porat et al., 1971).

The disaggregation of host polysomes may be caused by a highly efficient viral protein (Sydiskis and Roizman, 1967; Ben-Porat et al., 1971). Host polysome disaggregation occurs in cells infected with PrV in the presence of cycloheximide only after this inhibitor of protein synthesis has been removed for several minutes (Ben-Porat et al., 1971). HSV infection of permissive cells causes cellular polysomes to disaggregate and this effect cannot be reversed by analogues of amino acids; however the disaggregation found in non-permissive cells is abolished by adding amino acid analogues (Sydiskis and Roizman, 1967). This was interpreted as being due to a protein factor which functioned so efficiently in permissive cells that either a few unmodified molecules or the modified proteins themselves still disaggregated the polysomes. The factor would be less efficient in non-permissive cells and so disaggregation is much more sensitive to the amino acid analogues being incorporated into the protein.

The mechanism of host protein synthesis inhibition in



poliovirus-infected HeLa cells has been extensively studied. Viral protein synthesis seems to be a necessary prerequisite for this process (Penman and Summers, 1965). In this respect the situation is similar to the process in herpesvirus-infected cells. The rate of host-polysome disaggregation in poliovirus infected cells is increased in the presence of actinomycin D (Willems and Penman, 1966). This inhibition appears to be due to a block in the synthesis of a cellular RNA species with a half-life of less than 30 minutes (Leibowitz and Penman, 1971).

Indirect studies have shown that in this system it appears to be the initiation of polypeptide synthesis that is affected. Other RNA translational control factors have now been found in uninfected eukaryotic cells (Bester et al., 1975) and may prove to be of general importance.

A virus-specific factor of this kind might possibly discriminate between viral and cellular mRNA species. No such factor has yet been studied in herpesvirus-infected cells.

#### 1.5.4. Protein Synthesis

The overall rate of protein synthesis is not substantially altered in herpesvirus infected cells (Kaplan, 1973).

##### 1.5.4.1. Cell-Specific Proteins

Many animal viruses inhibit host protein synthesis and these include the picornaviruses, such as poliovirus (Shatkin, 1963; Salzman and Sebring, 1967), vaccinia (Shatkin, 1963), the arboviruses (Lust, 1966) and mengovirus (Franklin and Baltimore, 1962). Herpesviruses also inhibit cellular protein synthesis.

Cellular protein synthesis begins to be inhibited soon after PrV infection and the inhibition continues throughout the infection (Hamada and Kaplan, 1965; Kaplan et al., 1970). All cellular proteins appear to be equally affected and this must be largely due to the disaggregation of host polysomes discussed previously. Cellular mRNA does not appear to be degraded preferentially as host mRNA-like sequences are still found in the cytoplasm but they have not been shown to be functional mRNA molecules (Kaplan et al., 1970).

#### 1.5.4.2. Virus-Specific Proteins

It appears that virus-specific proteins in HSV infected HEp-2 cells form at least three groups (Honess and Roizman, 1973, 1974, 1975),  $\alpha$ ,  $\beta$  and  $\gamma$ . The synthesis of these groups is regulated coordinately and the classes appear sequentially. The  $\alpha$  group is synthesised early in infection and maximally at about 3 - 4 hours post-infection and then declines as  $\beta$  synthesis begins to predominate.  $\beta$  synthesis is maximal from 5 - 7 hours post-infection and it similarly declines while  $\gamma$  synthesis increases until at least 12 h after infection.

The  $\alpha$  polypeptides' mRNA is transcribed in the presence of cycloheximide or puromycin and so this process does not seem to require prior translation of virus-specific mRNA. One or more functional  $\alpha$  polypeptides is necessary to turn on the synthesis of the  $\beta$  groups' mRNA. A similar relationship was observed between  $\beta$  and  $\gamma$  synthesis (Honess and Roizman, 1974, 1975). Not only is the transcription of the viral mRNA controlled but translational control may also help to turn off  $\alpha$  polypeptide synthesis as this declines at a greater rate than would be expected from the calculated turnover of  $\alpha$  mRNA (Honess and Roizman, 1974, 1975).

The  $\alpha$  polypeptides appear to be largely non-structural in function,  $\beta$  polypeptides are a mixture of minor virion structural components and non-structural proteins.  $\gamma$  polypeptides are mainly major structural proteins (Hones and Roizman, 1974, 1975).

It is uncertain how far this scheme applies to other herpesviruses, particularly in view of the wide differences in the control of RNA synthesis between HSV-1 and HSV-2 (Frenkel et al., 1973); however broad similarities with the effect of cycloheximide on the control of PrV and HSV infection seem to be emerging. Rakusanova et al. (1971) found that if pseudorabies infection was carried out in the presence of cycloheximide only a subset of the normal viral transcripts was synthesised; after the inhibitor was removed the number of virus-induced proteins synthesised also appeared to be reduced. This was confirmed by Ben-Porat et al. (1975) who found that no virion structural antigens were synthesised immediately after cycloheximide was removed.

It must be stressed that studies of transcription and translation in cycloheximide treated cells must be considered with great caution as no functional virus particles are produced after the inhibitor is removed (Jean et al., 1974). Cycloheximide has a similar effect on vaccinia virus replication (Moss and Filler, 1970). Transcription may be altered in an unexpected manner in cycloheximide treated cells, for instance, the inhibitor increases the transcription of HSV-2 (Frenkel et al., 1973). It therefore cannot be assumed that only a simple effect is seen in cells treated with the inhibitor.

#### 1.6. Messenger RNA

The theory of messenger RNA (mRNA) formulated in 1961 by Jacob

and Monod has now been amply confirmed by many workers. mRNA is metabolically unstable, has a base composition similar to that of DNA and is heterogeneous in size.

The foundations for eukaryotic mRNA studies were laid during the isolation of the globin message. SDS treatment of rabbit reticulocyte polysomes was shown to result in the release of a 9S RNA species (Chantrenne et al., 1967). Polysomes are readily disrupted by mild RNase treatment and this was thought to be due to the cleavage of the mRNA chain which holds the ribosomes together, like beads on a string. Rabbit reticulocyte polysomes were sensitive to RNase treatment and the 9S RNA species disappeared as the polysomes were disrupted; this, along with the fact that globin is the main protein synthesised in rabbit reticulocytes, was taken as circumstantial evidence that the 9S RNA was the messenger species which specified globin.

More direct evidence for the identification of globin message soon followed: a similar 9S RNA species, isolated from mouse reticulocytes, when added to a lysate of rabbit reticulocyte cells, promoted the synthesis of mouse globin (Lockard and Lingrel, 1969; Lingrel et al., 1971). This component was also shown to direct the synthesis of globin when injected into frog oocytes (Lane et al., 1971). In this way it was demonstrated that the mRNA for the globin protein had been isolated.

A number of eukaryotic mRNA molecules have now been studied in a similar manner and these include: ovalbumin mRNA (Rhoads et al., 1971), immunoglobulin mRNA (Stavnezer and Huang, 1971), histone mRNA (Breindl and Gallwitz, 1973; Jacobs-Lorena et al., 1972), lens crystallin mRNA (Bloemendal et al., 1973) and myosin mRNA (Heywood and Nwagwu, 1969).

### 1.6.1. Isolation of Messenger RNA

#### 1.6.1.1. Polysomal RNA

It is usually impossible to isolate a species of mRNA solely on the basis of its size, but most of the procedures used to purify mRNA make use of this property. Globin mRNA was found to be present in the polysome fraction of reticulocytes and was not associated with single ribosomes (Gaskill and Kabat, 1971). Polysomes are now isolated as the first step in most mRNA purifications. Sometimes a subset of the polysomes is prepared, in which the required mRNA is enriched. For example immunoglobulin mRNA-containing polysomes were selectively immunoprecipitated by adding antibodies against immunoglobulins to mouse myeloma polysomes (Delovitch et al., 1972). Histones are mainly synthesised on small polysomes and histone mRNA is prepared from this subfraction of the total polysomes (Robbins and Borun, 1967).

#### 1.6.1.2. EDTA Release

EDTA treatment of reticulocyte polysomes releases the globin mRNA in a ribonucleoprotein structure that sediments at approximately 15S (Chantrenne et al., 1967). This technique is now used routinely to separate mRNA from large ribonucleoprotein particles which co-sediment with polysomes. The polysomes are disaggregated by the EDTA treatment and so their components sediment more slowly than normal, while the large ribonucleoprotein particles are unaffected and sediment at their normal rate.

#### 1.6.1.3. Poly-(A) Affinity

With the exception of histone mRNA, all mRNAs studied in detail at this time contain a region of polyadenylic acid residues.

Several methods of isolating poly-(A)-containing RNA have been developed. Polyadenylated RNA can be adsorbed to poly-(U) coupled to sepharose (Adesnik et al., 1972), oligo-(dT) coupled to cellulose (Nakazato and Edmonds, 1972), some cellulose preparations containing lignins (Sullivan and Roberts, 1973) and to cellulose nitrate membrane-filters (Brawerman et al., 1972). The mRNA can be eluted once the non-poly-(A) RNA has been washed away. Polyadenylated RNA is found in all eukaryotes but it is likely that many mRNA species are non-adenylated (Milcarek et al., 1974). In HeLa cells approximately 30% of the mRNA is believed to lack poly-(A).

1.6.1.4. Extraction of mRNA from polysomes or ribonucleoprotein particles is not a trivial operation. The conditions used for extracting rRNA employing cold phenol at neutral pH, were found to be unsuitable for mRNA. Cold phenol extractions of polysomes resulted in the DNA-like RNA being trapped at the interface along with the denatured protein (Georgiev and Mantieva, 1962). The phenomenon is due to poly-(A) binding to denatured proteins and this was used as a basis of separating poly-(A) RNA from non-poly-(A) RNA (Brawerman et al., 1972). The polyadenylated RNA was extracted from the interface by slightly increasing the pH. However it was found at about the same time that degradation of the RNA trapped at the interface occurred (Perry et al., 1972). If chloroform was present along with phenol the polyadenylated RNA was no longer trapped at the interface and viable mRNA could be extracted under a wide range of conditions (Perry et al., 1972). All the known factors influencing the physical separation of mRNA from proteins have recently been reviewed (Brawerman, 1974; Palmiter, 1974).

### 1.6.2. Synthesis of mRNA

At least three types of DNA-dependent RNA polymerases have been identified in eukaryotes (Roeder and Rutter, 1969). Polymerase I is located in the nucleolus and is responsible for the synthesis of rRNA (Blatti et al., 1970; Reeder and Roeder, 1972), it is insensitive to the fungal toxin,  $\alpha$  amanatin (Lindell et al., 1970; Kedinger et al., 1970). Polymerase II is inhibited by very low concentrations of  $\alpha$  amanatin (Roeder and Rutter, 1970a) and synthesises heterogeneous nuclear RNA (HnRNA) (Blatti et al., 1970; Zylber and Penman, 1971). Polymerase III polymerises low molecular weight RNA (Weinman and Roeder, 1974) and is inhibited by higher levels of  $\alpha$  amanatin than are required to inhibit polymerase II.

#### 1.6.2.1. Heterogeneous Nuclear RNA (HnRNA)

HnRNA is thought to be the precursor to cytoplasmic mRNA (see Weinberg, 1973). However the precursor-product relationship has still to be proved. mRNA is much smaller than HnRNA and this nuclear RNA has a very rapid turnover. Poly-(A) has been found attached to 20 - 40% of newly synthesised HnRNA (Sheldon et al., 1972; Greenberg and Perry, 1972).

### 1.6.3. Post-transcriptional Modification of mRNA

#### 1.6.3.1. Polyadenylation

An enzyme catalyzing the polymerisation of ATP molecules and forming an acid precipitable polyriboadenylic acid was first found in bacteria (August et al., 1962) and then in eukaryotes (Burdon, 1963). The enzyme is present in all subcellular organelles studied (Winters and Edmonds, 1973; Rose et al., 1975).

Kates first found poly-(A) to be associated with RNA which had been transcribed from vaccinia virions (Kates, 1970). Poly-(A) containing mRNA has been found in all eukaryotic cells studied: from lower organisms, such as yeast (McLaughlin et al., 1973) and cellular slime mould (Firtel et al., 1972), to higher organisms, such as mouse (Mendecki et al., 1972) and man (Edmonds et al., 1971). The initially observed association between poly-(A) and vaccinia RNA has been extended and many viral mRNAs are now known to be polyadenylated. These include: herpesviruses (Bachenheimer and Roizman, 1972; Rakusanova et al., 1972), adenovirus (Philipson et al., 1971), poliovirus, eastern equine encephalitis virus (Armstrong et al., 1972), sindbis virus (Johnston and Bose, 1972) and sendai virus (Marx et al., 1975).

The poly-(A) tract of the nuclear replicating DNA viruses, adenovirus and SV40, is added to the RNA molecules post-transcriptionally. The viral DNA lacks the poly-(dT) sequences capable of coding for the polyadenylic acid (Philipson et al., 1971; Weinberg et al., 1972). The poly-(A) of SV40 is identical in size to the poly-(A) associated with cellular mRNA in host cells (Weinberg et al., 1972), circumstantial evidence that cellular and viral polyadenylation are both carried out by the cellular system. No poly-(dT) sequences have been found in cellular DNA (Southern, 1970), a further indication that poly-(A) is not coded for by the host DNA.

3' deoxyadenosine (cordycepin) is reported to inhibit polyadenylation (Truman and Frederikson, 1969) but to have no effect on HnRNA synthesis (Penman et al., 1970). When polyadenylation is inhibited by cordycepin, transport of mRNA into the cytoplasm is severely reduced (Adesnik et al., 1972).



The poly-(A) sequence is located at the 3' terminus of mRNA (Mendecki et al., 1972) and is longest when newly synthesised (Greenberg and Perry, 1972; Sheiness and Darnell, 1973). Poly-(A) is possibly shortened during translation as both mRNA turnover and poly-(A) shortening are inhibited when translation is halted (Sheiness et al., 1975).

The function of poly-(A) is not known; it does not appear to be concerned with translation (Bard et al., 1974; Munoz and Darnell, 1974) although it has been reported that a translation initiation factor binds to poly-(A) (Hellerman and Shafritz, 1975) and the RNA translation control factor found by Bester et al. (1975) contains an oligo-(U) region which is postulated to attach to the poly-(A). Numerous other suggestions have been advanced for a function of poly-(A) but none have yet been shown to be correct.

#### 1.6.3.2. Methylation of Messenger RNA

Mouse L cell and Novikoff hepatoma cell mRNAs are methylated in internal positions (Perry and Kelley, 1974; Desrosiers et al., 1974). Messenger RNAs from many sources have been shown to contain a 7-methyl-guanosine residue at the 5' terminus: HeLa cell (Furuichi et al., 1975a), vesicular stomatis virus (Abraham et al., 1975), cytoplasmic polyhedrosis virus (Furuichi, 1974), reovirus (Shatkin, 1974; Furuichi et al., 1975b) and vaccinia (Wei and Moss, 1974). Unmethylated mRNA from reovirus or vesicular stomatitis virus is translated less efficiently than methylated mRNA by a cell-free wheat germ extract. Unmethylated mRNA is translated and methylated by the wheat germ system in the presence of S-adenosyl methionine (Both et al., 1975). It is thought that the 5' methylated sequences have a dual purpose, firstly they aid translation of mRNA and

secondly they may make the mRNA less easily degradable. It is known that mRNA is degraded from the 5' end (Tocchini-Valenti and Mattochia, 1968) and this lends support to the 7-methyl guanosine acting as a protecting group.

#### 1.6.3.3. Other Modifications of Messenger RNA

Some viral RNAs are aminoacylated; these include some plant viruses (Kohl and Hall, 1974) and mengovirus (Salmon and Littauer, 1974). Host-cell specific enzymes carry out these modifications so the modifications may also be found on cellular mRNA.

#### 1.6.4. Stability of mRNA

Several attempts have been made to find the lifetime of mRNA molecules. Actinomycin D has been used to inhibit RNA synthesis and the inhibition of protein synthesis was monitored (Penman et al., 1963). However, Singer and Penman (1972) found that polyadenylated RNA was still present in almost undiminished quantities after protein synthesis was greatly reduced. Using different techniques, half lives for mRNA of between 6 hours and 2 - 3 days have been reported (Singer and Penman, 1973; Murphy and Attardi, 1973).

## Chapter 2

### The Effect of Pseudorabies Infection on HeLa Polysomes

#### 2.1. Introduction

The size distribution of polysomes is readily studied by velocity sedimentation through sucrose density gradients. After centrifugation under appropriate conditions the largest polysomes are found near the foot of the gradient and the smallest ("disomes") about one-third of the way down, just separated from the ribosomes and ribosomal subunits (see fig 2.1).

This technique has been used to study the effect of HSV and PrV infection on the cellular polysome distribution (Sydiskis and Roizman, 1966, 1967, 1968; Ben-Porat et al., 1971). Both viruses caused two changes in the polysome profiles of infected cells: host polysomes disaggregated, resulting in an increased number of free ribosomes and ribosomal subunits; the remaining polysomes were larger in size than those in the uninfected cell and this was illustrated by the peak of polysomes sedimenting at an increased rate. In HSV infected cells these changes occur as virus mRNA replaces cellular messengers on the polysomes (Sydiskis and Roizman, 1966; Spear and Roizman, 1968). Ben-Porat et al. (1971) found this effect also took place in PrV infected rabbit kidney cells and claimed that even by 2.5 hours after infection the bulk of cellular mRNA was associated with two or less ribosomes. This conclusion was based on the amino acid composition of the nascent polypeptides, and on hybridisation of [ $^3\text{H}$ ] labelled polysomal RNA to cellular <sup>or</sup> viral DNA. In the labelling conditions employed a large part of the tritiated RNA would be rRNA, and so this cellular non-mRNA species would hybridise to

Fig 2.1

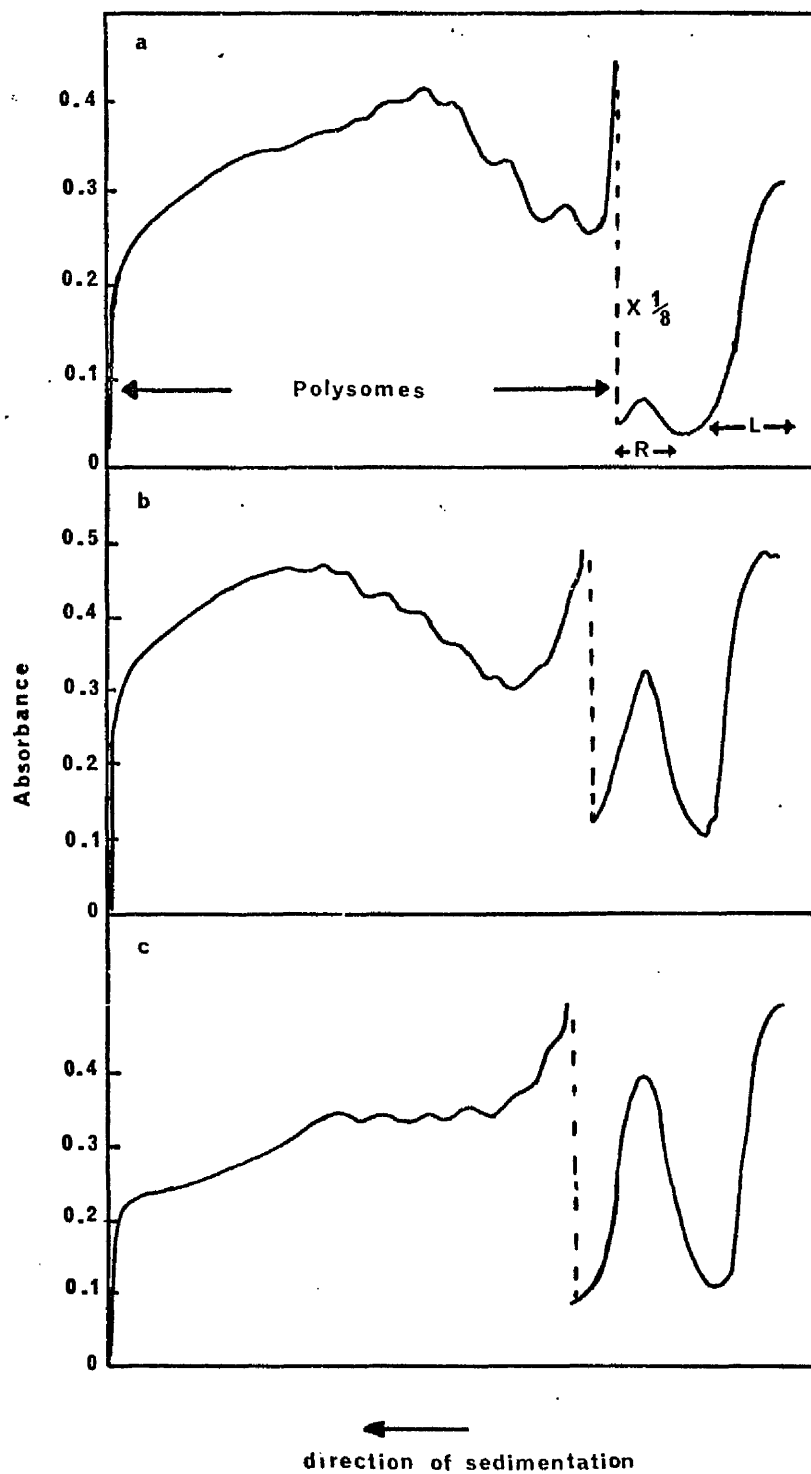
Polysome Profiles in Mock-Infected and Infected Cells

Exponentially growing HeLa cells were mock-infected or infected with 20 pfu/cell PrV. The cells were harvested by scraping and cytoplasmic extracts layered onto 15 - 30% (w/v) sucrose gradients; these were centrifuged for 2 hours at 27,000 r.p.m. in the SW27 rotor. The gradients were then collected by pumping from the bottom of the centrifuge tube, through a Gilford 2000 recording spectrophotometer, which was used to monitor the optical density at 260 nm.

The regions of polysomes, ribosomes + ribosomal subunits (R) and low molecular weight material (L) are indicated.

The cytoplasmic extracts were prepared from cells harvested after:

- a) Mock-infection
- b) 3 h Post-infection
- c) 5 h Post-infection



cellular DNA, effectively overestimating the amount of rabbit kidney mRNA remaining in the polysomes.

The ratio of lysine to leucine in polysome-bound nascent polypeptides in infected cells quickly becomes very similar to that ratio found in virus-induced proteins (Ben-Porat et al., 1971). This finding supports rapid replacement of cellular with viral mRNA on the polyribosomes. In HSV infected cells Roizman et al. (1970) claim that by 4 hours after infection the polysomes are exclusively synthesising viral proteins.

When I first began this project I tried to prepare polysomes from baby hamster kidney cells (BHK-21), which at that time were used for all work on PrV in our laboratory. I was, however, unable to isolate polysomes in reasonable yields from these cells, except on one occasion. I tried many different methods to prepare BHK polysomes, all without success. I then decided to use HeLa cells and from that time was able to isolate polysomes routinely.

Polysomes have been isolated in this project for three principal reasons:-

- (i) as the first stage in the isolation of mRNA
- (ii) examination of the polysome profiles indicates if gross degradation of mRNA is taking place, at an early stage of isolation
- (iii) the effect on polysomes of PrV infection of HeLa cells can be compared with previous studies of polysomes in infected cells and the effect on polysomes of various drugs used in this project can be studied.

(i) and (ii) will be discussed in later chapters and (iii) in the following sections.

## 2.2. The Increase in Polysome Size in Infected Cells

Pseudorabies infection of rabbit kidney cells causes the polysomes to sediment more rapidly (Ben-Porat et al., 1971). This increase in sedimentation rate was also found for polysomes in PrV infected HeLa cells in this project (see fig 2.1). However although the effect is easily seen by examination of the polysome profiles it is difficult to quantitate. Polysomes with the maximum modal value appeared anywhere between 3 and 5 hours post infection and this variation is typical of the differences found between polysome profiles from week to week. Not only did the effects of PrV on the polysomes vary but the uninfected cell polysomes also showed considerable variation. This is perhaps connected to known changes in the rate of growth of HeLa cells in tissue culture.

After passing through a peak of polysome size there followed a decrease in the ratio of large to small polysomes (see fig 2.1). This effect was not always very noticeable.

## 2.3. Disaggregation of Polysomes in Infected Cells

As infection proceeds fewer ribosomes are associated with mRNA. In sucrose gradients this is seen as an increase in the amount of ribosomes and ribosomal subunits at the top of the polysome region (see fig 2.1). This effect was also noted earlier by Sydiskis and Roizman (1966, 1967, 1968) and by Ben-Porat et al. (1971). This increased proportion of monosomes over polysomes in infected cells was seen in all experiments throughout the course of this project (see fig 2.4 and table 2.5).

#### 2.4. Time Course of Polysome Disaggregation

Although a number of studies have been carried out on polysome disaggregation in herpesvirus infected cells, the results have never been quantitated in any way. Instead a series of polysome profiles have been shown. In this study I decided to attempt to quantitate the disaggregating process, if only in an empirical fashion. The number of polysomes decreases in infected cells while the number of ribosomes and ribosomal subunits ("monosomes") increases, so I thought that a ratio of the amount of RNA in polysomes over the amount of RNA in "monosomes" would provide a sensitive index to polysome disaggregation. Initially I calculated the ratio from the whole polysome fraction but this changed as a variable quantity of the heaviest polysomes were pelleted in different centrifuge tubes. The ratio varied considerably among identical samples in different tubes. I then decided to base the calculation on the RNA content of lighter polysomes which would not be pelleted even with wide variations in the centrifugation conditions employed. The empirically chosen subset was of polysomes containing between 2 and 8 ribosomes on the mRNA.

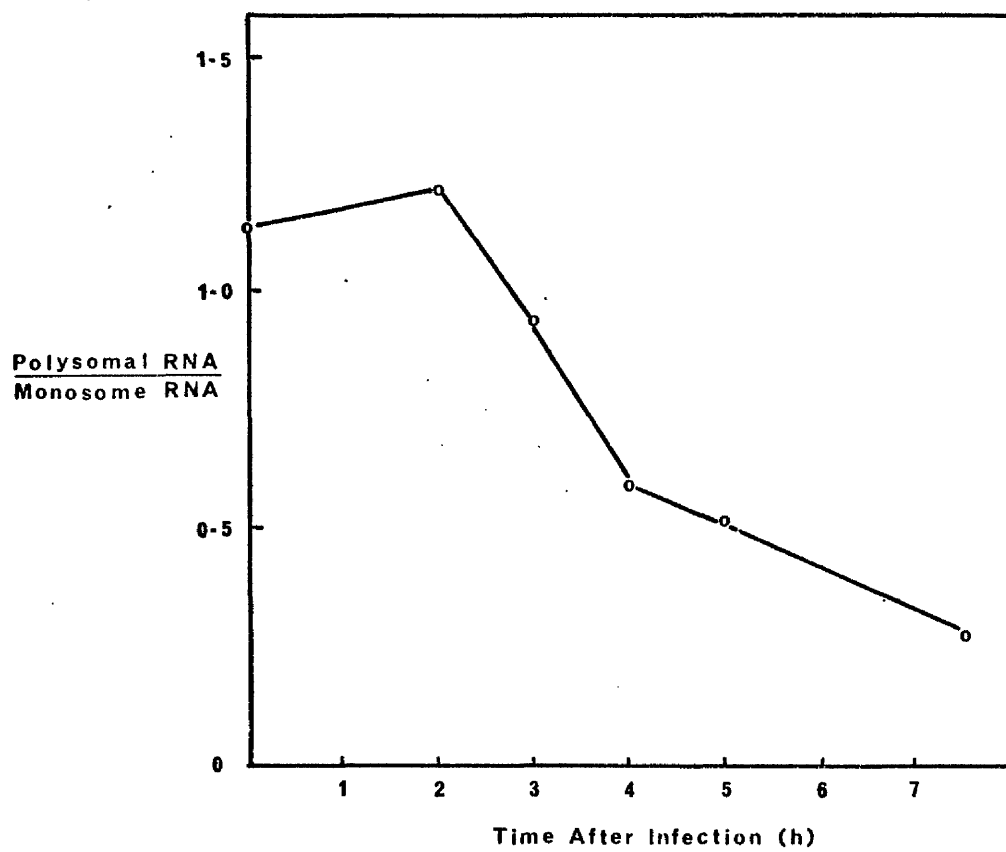
Fig 2.4 shows the ratio of RNA in polysomes (2 - 8 subunits) to the RNA in "monosomes" found at various times after infection. A decrease in this ratio indicates that polysome disaggregation is occurring. At early times after infection (2 h) there is an increase in the (polysomal RNA/"monosome" RNA) ratio followed by a rapid disaggregation which slows down between 4 and 5 hours post infection. The initial increase in polysomal RNA was always found at very early times after infection (see table 2.7 also), and could be due to two effects:

- (i) Both virus and cellular mRNA being associated with polysomes



Fig 2.4      Time Course of Polysome Disaggregation in PrV Infected  
                 HeLa Cells

The ratio of Polysomal RNA/Monosome RNA was calculated from optical density curves (260 nm) obtained from the recording spectrophotometer, as shown in Fig 2.1. The polysome area taken was the area under the curve due to polysomes with 2 - 8 subunits.



before the host polysomes are disaggregated by a virus-coded mechanism.

- (ii) A decrease in the initiation rate of HeLa protein synthesis may occur with the result that the largest cellular polysomes would decrease in size and move into the 2 - 8 subunit region.

It is not known what part each of these effects play in the resultant increase in polysomes with 2 - 9 subunits. This effect has not previously been reported.

The continued polysome disaggregation between 2 and  $7\frac{1}{2}$  hours post-infection is also likely to be a combination of two effects:

- (i) Virus specific polysomes sediment more rapidly than host polysomes (Ben-Porat et al., 1971) at least up to 3 - 4 hours post-infection (see fig 2.1). This would result in a shift of some polysomes from the 2 - 8 subunit region to the 9+ subunit region causing fewer polysomes in the measured area.
- (ii) The loss of cellular mRNA from the polysomes may be less rapid than suggested by Ben-Porat et al. (1971) and may still be continuing at  $7\frac{1}{2}$  hours after infection.

## 2.5. Polysomes in Actinomycin D-Treated Cells

Actinomycin D was used in an attempt to specifically label mRNA in infected cells. However mRNA synthesis in infected cells was found to be strongly inhibited (see fig 2.5) and for this reason actinomycin D was only used in a limited number of experiments. Actinomycin D preferentially inhibits rRNA synthesis in uninfected HeLa cells (Perry, 1963). The reason for the strong inhibition of virus mRNA

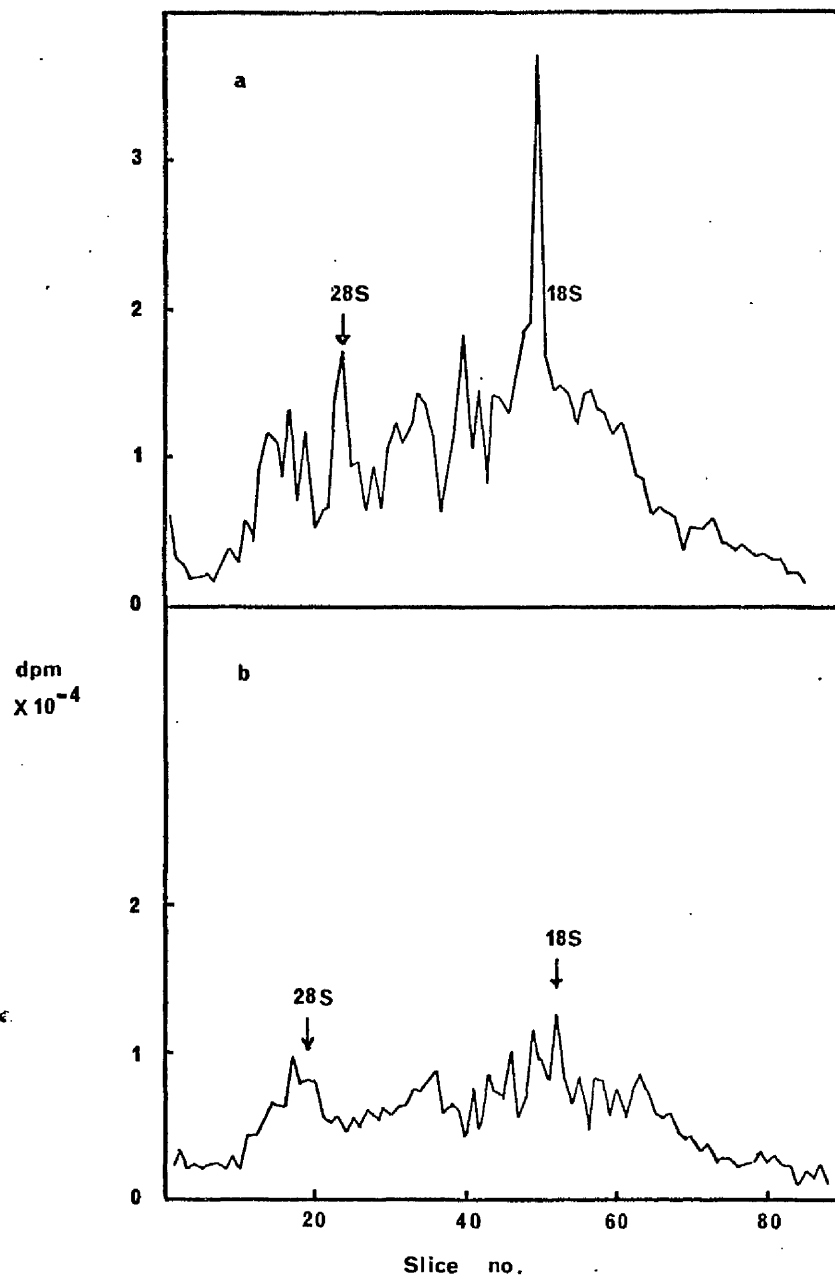
Fig 2.5.

The Effect of Actinomycin D on Polysomal RNA

Synthesis in Infected Cells

2 bottles containing  $10^8$  HeLa cells were infected with PrV (20 pfu/cell) and 0.04  $\mu\text{g/ml}$  of actinomycin D was added to one bottle, 3 h post-infection. Both cultures were labelled with 1.25 mCi  $[5,6-^3\text{H}]$  Uridine from 3-4 h post-infection. Polysomes were isolated, as described in the Methods section, and the RNA was extracted with a mixture of phenol and chloroform. The polysomal RNA was subjected to poly-(U) sepharose chromatography and the polyadenylated RNA was analysed by PAGE. rRNA which was present as a slight contaminant of the poly-(A) RNA is identified in the diagram as 28S or 18S RNA. rRNA was present in equal amounts in both samples. The gels were divided into 1 mm slices and the radioactivity in these was determined (see Methods section).

- a) No actinomycin D
- b) actinomycin D added



synthesis is probably due to the fact that actinomycin D inhibits RNA synthesis by binding to deoxyguanosine (Gellert et al., 1965) and both PrV DNA and the rRNA genes in HeLa cells have a high G + C content (Kaplan and Ben-Porat, 1964; Soeiro et al., 1966).

It was also initially hoped that actinomycin D might inhibit some viral mRNA species and leave others relatively unaffected. This effect might occur if some regions of the viral genome were particularly GC rich. No such specific effect was noted and all mRNA sizes showed about the same degree of inhibition (fig 2.5).

In poliovirus infected HeLa cells actinomycin D increases the rate of polysome disaggregation normally found during infection (Willems and Penman, 1966). This effect is thought to be due to actinomycin inhibiting the synthesis of a small molecular weight cellular RNA with a half-life of less than 30 minutes (Leibowitz and Penman, 1971). This RNA species found in HeLa cells is thought to play a role in the initiation of cellular polypeptide synthesis. Actinomycin D was also found to increase the rate of disaggregation of polysomes in PrV infected HeLa cells (see table 2.5). Actinomycin D has a similar effect on uninfected HeLa cells. The disaggregation, however, is very much slower than is observed in PrV infected cells, for recognizable polysomes are still present after 9 hours treatment of uninfected cells (N.W. Fraser, personal communication).

Table 2.5. (Polysomal RNA/Monosome RNA) Ratios in Infected and Mock-infected Cells in the Presence and Absence of Actinomycin D

Expt. No.	Length of Infection	Length of Actinomycin Treatment Prior to Harvest	$\frac{\text{(Polysomal RNA)}}{\text{(Monosome RNA)}}$
1	0 h	-	2.2
	3 h	-	1.1
	5 h	-	0.76
2	0 h	-	2.0
	0 h	3 h	1.6
	4 h	-	1.25
	4 h	3 h	0.83
3	0 h	-	1.26
	2 h	-	1.1
	2 h	1 h	0.29
	3 h	-	0.72
	3 h	1 h	0.57
	6 h	-	0.70

The ratio of (polysomal RNA/monosome RNA) was calculated in the same way as for Fig 2.4.

## 2.6. Polysome Profiles in Infected and Mock-Infected Cells Treated with Cordycepin

Cordycepin (3' deoxyadenosine) is a specific inhibitor for the post-transcriptional addition of polyadenylic acid to mRNA and HnRNA (Penman et al., 1970). However some doubt has recently been cast on this with a report that cordycepin is much less specific than had previously been reported (Maale et al., 1975).

If the viral proteins causing the switch-off of host protein synthesis are coded for by poly-(A) mRNA cordycepin should abolish the polysome disaggregation due to PrV infection if it is present at the time of synthesis of the appropriate messengers. Cordycepin itself causes polysome disaggregation in uninfected HeLa cells (see table 2.6) but no extra disaggregation occurs in infected cells in which the inhibitor is present from the time of infection. However if infection is allowed to proceed for 1 hour before cordycepin is added extra disaggregation does occur. By 2 hours post-infection the effect of cordycepin is less marked.

It may therefore be the case that cordycepin inhibits the synthesis of Penman's RNA species which functions as an initiator of protein synthesis, so causing polysome disaggregation. The greater disaggregation of polysomes found when cordycepin was only added 1 hour after PrV infection is likely to be due to immediate-early or early viral mRNA being synthesised in this time and then being translated into the viral proteins which are thought to be responsible for polysome



Table 2.6. (Polysomal RNA/Monosome RNA) Ratios in Infected  
and Mock-Infected Cells Treated in the Presence  
or Absence of Cordycepin

<u>Expt.</u> <u>No.</u>	<u>Length of</u> <u>Infection</u>	<u>Length of Cordycepin</u> <u>Treatment prior to</u> <u>Harvest</u>	<u>(Polysomal RNA)</u> <u>(Monosomal RNA)</u>
1	0 h	-	0.60
	0 h	2 h	0.45
	0 h	5 h	0.46
	2 h	2 h	0.46
	5 h	-	0.40
	5 h	5 h	0.41
2	0 h	-	0.62
	0 h	5 h	0.33
	5 h	-	0.53
	5 h	5 h	0.36
	5 h	4½ h	0.35
	5 h	4 h	0.25
	5 h	3 h	0.41

disaggregation (Rakusanova et al., 1971). It is possible that a dual effect is seen when cordycepin was added 1 hour after infection, the adenosine analogue may inhibit the Penman RNA factor and the normal virus disaggregation is also likely to be occurring at the same time. However when cordycepin is added 2 hours after infection a substantial amount of virus mRNA is likely to be present in the polysomes and these may be less sensitive to the effect of cordycepin than the cellular polysomes are in infected cells.

## 2.7. The Effect of Cycloheximide on Polysomes in Infected Cells

Cycloheximide is an inhibitor of polypeptide chain elongation (Fukuhara, 1965; Clarke-Walker and Linnane, 1966). When present from the time of herpesvirus infection it inhibits all viral protein synthesis, but allows some viral mRNA synthesis to continue (Rakusanova et al., 1971; Kozak and Roizman, 1974). This is the subset of PrV called "immediate-early" mRNA by Kaplan and his co-workers (see Kaplan, 1973) and  $\alpha$  mRNA in HSV infected cells (Honess and Roizman, 1974).

When cycloheximide inhibition is released for 20 min prior to harvesting, protein synthesis restarts and the viral mRNA species present in the cytoplasm move into the polysome fraction (Rakusanova et al., 1971). In this project it was found that after  $4\frac{1}{2}$  h cycloheximide treatment from the time of infection, followed by 20 min in the absence of the inhibitor, the polysome profile was similar to that obtained from untreated cells early in infection (Fig 2.7).

Fig. 2.7.

Length of Infection	Time of Cyclohex. Treatment	(Polysomes) (Monosomes)
0 h	-	0.85
1.5 h	-	1.10
5 h	-	0.25
5 h	0-4 h 40 min	1.10

## 2.8. Variation Between Experiments

The polysome profiles in infected and uninfected cells were found to vary from experiment to experiment. This can be seen in table 2.5 by comparing the polysome/monosome ratios in control cells in experiments 1 - 3. This is probably only one aspect of many variations found in tissue culture cells from week to week. On a few occasions I found that there were very few polysomes present in either infected or mock-infected cells while at other times, using the same conditions, solutions and techniques, very good yields were obtained.

## 2.9. Conclusions

Polysome disaggregation reported to occur in PrV infected RK cells and in HSV infected HEp-2 cells also occurs in PrV infected HeLa cells. The effects of the metabolic inhibitors cordycepin and cycloheximide are consistent with a viral-induced protein being required for cellular-polysome disaggregation. It is possible that actinomycin D inhibits the synthesis of an RNA species which acts as an initiator of cellular protein synthesis.

### Chapter 3

#### Isolation of Polyadenylated RNA from Pseudorabies

##### Virus Infected HeLa Cells

### 3.1. Introduction

Polyadenylic acid is associated with both PrV and HSV - specific cytoplasmic RNA (Rakusanova et al., 1972; Bachenheimer & Roizman, 1972; Silverstein et al., 1973). I decided to use this property as a basis for isolating viral mRNA species. Silverstein et al. (1973) reported that there were two classes of viral mRNA: an abundant class, containing poly-(A) and a scarce class, lacking poly-(A). If polyadenylated-mRNA were isolated from infected cells some time after infection I hoped to select a subset of the viral specific RNA. However the situation is more complex than was initially believed by Silverstein et al. (1973). No class of HSV mRNA is thought to completely lack poly-(A), but the extent of adenylation is believed to vary between different mRNA classes and also with the age of the mRNA (unpublished observations by Bachenheimer & Roizman, see Roizman & Furlong, 1974).

In this work mRNA was extracted from isolated polysomes for 4 main reasons:

- (i) Using molecular hybridisation techniques Kozak & Roizman (1971) showed that all virus-specified RNA sequences present in the cytoplasm of infected cells were also located in polysomal RNA.
- (ii) Cellular RNA sequences are still present in the cytoplasm of PrV infected cells even late in infection (Ben-Porat et al., 1971) but

they are located mainly in the post-polysome supernatant fraction. So by isolating the polysome fraction the cellular mRNAs should be substantially removed.

- (iii) Very low yields of polysomes, accompanied by an increased proportion of small polysomes, indicates that mRNA is being degraded. mRNA in polysomes is more sensitive to RNase breakdown than rRNA, so high polysome yields indicate that the bulk of mRNA species are not degraded.
- (iv) In infected cells a considerable part of the rRNA is in the form of ribosomal subunits - not associated with polysomes - so the polysomal mRNA has less accompanying rRNA than messengers extracted from the whole cytoplasm.

Three methods of isolating polyadenylated RNA have been investigated in this project. The method first tried was by differential phenol extraction, initially at pH 7.6 then at pH 9.0; this is the method described by Brawerman et al. (1972). The pH 7.6 extraction is reported to allow most of the ribosomal RNA to remain in the aqueous phase while polyadenylated RNA is trapped at the interface along with denatured protein. This RNA fraction is then extracted into a pH 9.0 buffer, enriched in poly-(A) containing sequences.

The second method used was oligo-(dT) cellulose chromatography. This method depends on polyadenylic acid binding to the oligo-(dT) sequences which are covalently attached to the cellulose matrix.

Thirdly poly-(U) sepharose chromatography, which operates by the same principle as the oligo-(dT) cellulose, was employed.

The RNA, fractionated by these methods was characterised by two techniques, polyacrylamide gel electrophoresis and sucrose density gradient sedimentation.

#### Extraction

Phenol-chloroform was used to extract RNA which was to be fractionated on oligo-(dT) cellulose or poly-(U) sepharose; chloroform was not present during differential phenol extractions. SDS was present in every extraction method used. Chloroform was used along with phenol as it is reported to prevent the degradation of RNA which occurs under certain conditions when phenol alone is used (see Brawerman, 1974; Perry et al., 1972). Several precautions were routinely observed to minimise nucleolytic breakdown. Dextran sulphate was used as an RNase inhibitor in a large part of the work, however it was found that RNA protected in this way markedly inhibited amino acid incorporation into protein in a cell-free wheat germ translation system. This inhibition could be abolished by repeated washings of the RNA with 2 molar sodium acetate (Palmiter, 1974). Unfortunately this resulted in a poor yield of RNA. All solutions were routinely treated with diethyl-pyrocabonate to destroy any RNase present, this was thought to be particularly important in sucrose gradients as Analar sucrose was used and this may contain some RNase. All solutions were autoclaved, both to sterilise and to destroy any remaining diethyl pyrocabonate. RNA prepared with solutions treated with diethyl pyrocabonate, but without dextran sulphate, was found to stimulate in vitro translation.

### 3.2.1. Phenol Extraction of RNA at pH 7.6 and pH 9.0

Brawerman et al. (1972) reported that sequential phenol extractions of cytoplasmic RNA at pH 7.6 then pH 9.0 resulted in the polyadenylated RNA being enriched in the pH 9 extract. It was decided to find if this technique could be used to extract poly-(A) RNA from infected cells. However I found that the pH 9.0 extract contained a large proportion of small molecular weight RNA. This was probably the result of the nucleolytic degradation reported to occur when this technique is used (Perry et al., 1972).

In agreement with the report of Brawerman et al. (1972) I found that rRNA was largely confined to the pH 7.6 extract but that heterogeneously sedimenting material was present in the pH 9.0 extract (see fig 3.2.1). However as the RNA appeared to be at least partially degraded I decided to try to find a suitable alternative method.

### 3.2.2. Oligo-(dT) Cellulose Chromatography

Oligo-(dT) cellulose has been reported to bind the poly-(A) tracts attached to many mRNA molecules (Aviv & Leder, 1972). The complementary homopolymers hybridise to each other and so the mRNA is retained while non-adenylated RNA, which does not bind to oligo-(dT), should be eluted. By lowering the salt concentration of the buffer the poly-(A) : oligo-(U) hybrids can be dissociated and the mRNA molecules are eluted.

When I tried to isolate mRNA by oligo-(dT) cellulose chromatography I found that rRNA was also bound and subsequently eluted along with the putative mRNA (see fig 3.2.2). No minor RNA species

Fig 3.2.1.

Differential Phenol Extraction of Cytoplasmic  
RNA from HeLa Cells

About  $10^8$  cells were incubated in medium containing 200  $\mu$ Ci 5,6- $^3$ H uridine for 3 h prior to harvest.

Whole cytoplasmic RNA was extracted with phenol, first at pH 7.6 and then at pH 9.0, according to the method of Brawerman et al. (1972) and was analysed by centrifugation through a 15-30% sucrose gradient in LETS buffer.

- a) pH 7.6 Extract
- b) pH 9.0 Extract



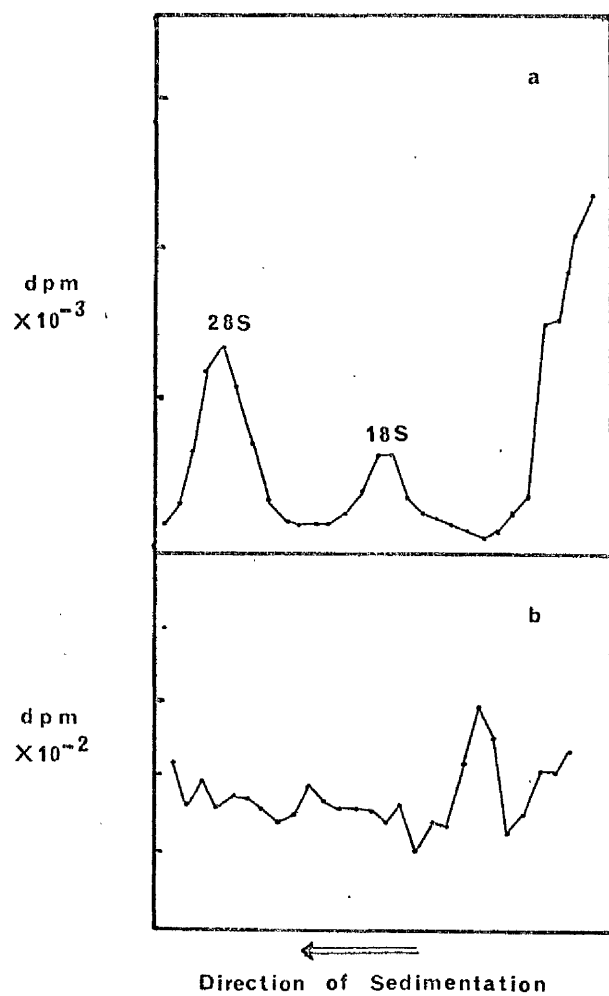


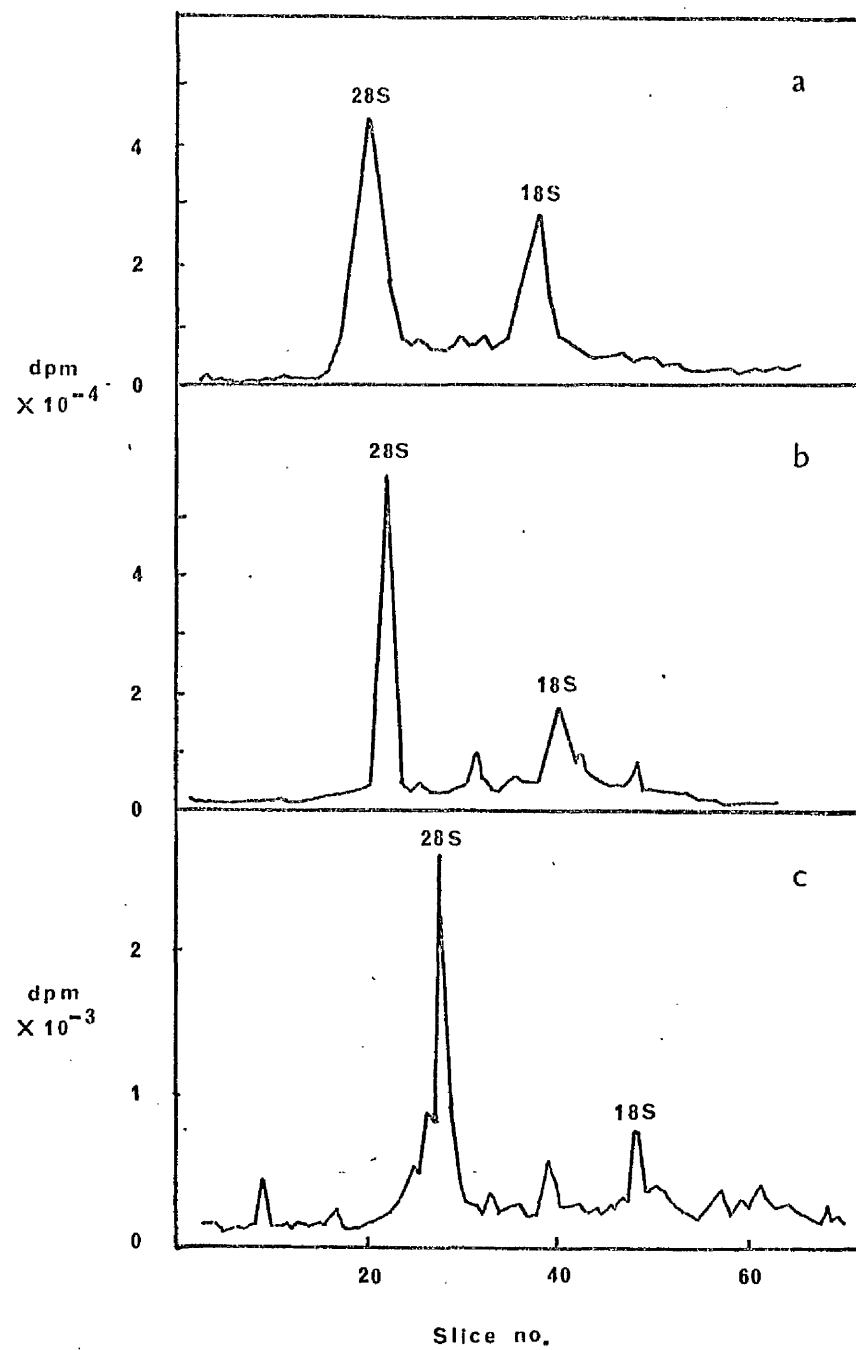
Fig 3.2.2.

Oligo-(dT) Cellulose Chromatography of HeLa Cell

Cytoplasmic RNA

$10^8$  HeLa cells were incubated along with 1.25 mCi 5,6- $^3\text{H}$  -Uridine for 5 h prior to harvest. One third of the total cytoplasmic RNA was subjected to PAGE without further treatment. The remaining  $\frac{2}{3}$  was applied to a column of oligo-(dT) cellulose and the bound and unbound fractions were also analysed by PAGE. The gels were sliced and the radioactivity in each slice determined.

- a) Whole cytoplasmic RNA
- b) Unbound fraction of cytoplasmic RNA
- c) Bound fraction of cytoplasmic RNA



bound strongly to the column and the bulk of these species appeared in the unbound fraction. After elution of the bound RNA virtually no RNA was still bound to the oligo-(dT) cellulose.

### 3.2.3. Poly-(U) Sepharose Chromatography

When RNA is added to a column of poly-(U) sepharose, under

Polyadenylated RNA was separated from non-adenylated RNA by poly-(U) sepharose chromatography by the method of Lindberg, U. and Persson, T. (1972) Eur. J. Biochem. 31, 246-254. These workers showed that under their conditions polyadenylated RNA was bound to the poly-(U) sepharose whereas non-adenylated RNA was not.

poly-(A) : poly-(U) hybrids, in this case a buffer containing 90% formamide.

Both the unbound and bound RNA fractions are subsequently examined by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS (Loening, 1969). The gels are first scanned at 260 nm using an ultraviolet recording spectrophotometer and are then cut into 1 mm slices for determination of radioactivity. rRNA was found to be present in vast excess in the unbound fraction but is also present, although to a much lesser extent, in the bound fraction (see fig 3.2.3). This figure also illustrates the increase in relative amounts of other RNA species, in comparison with rRNA, between the unbound and bound poly-(U) sepharose fractions. The contamination of poly-adenylated RNA with rRNA was often less than shown in this figure. This figure

Fig 3.2.3.

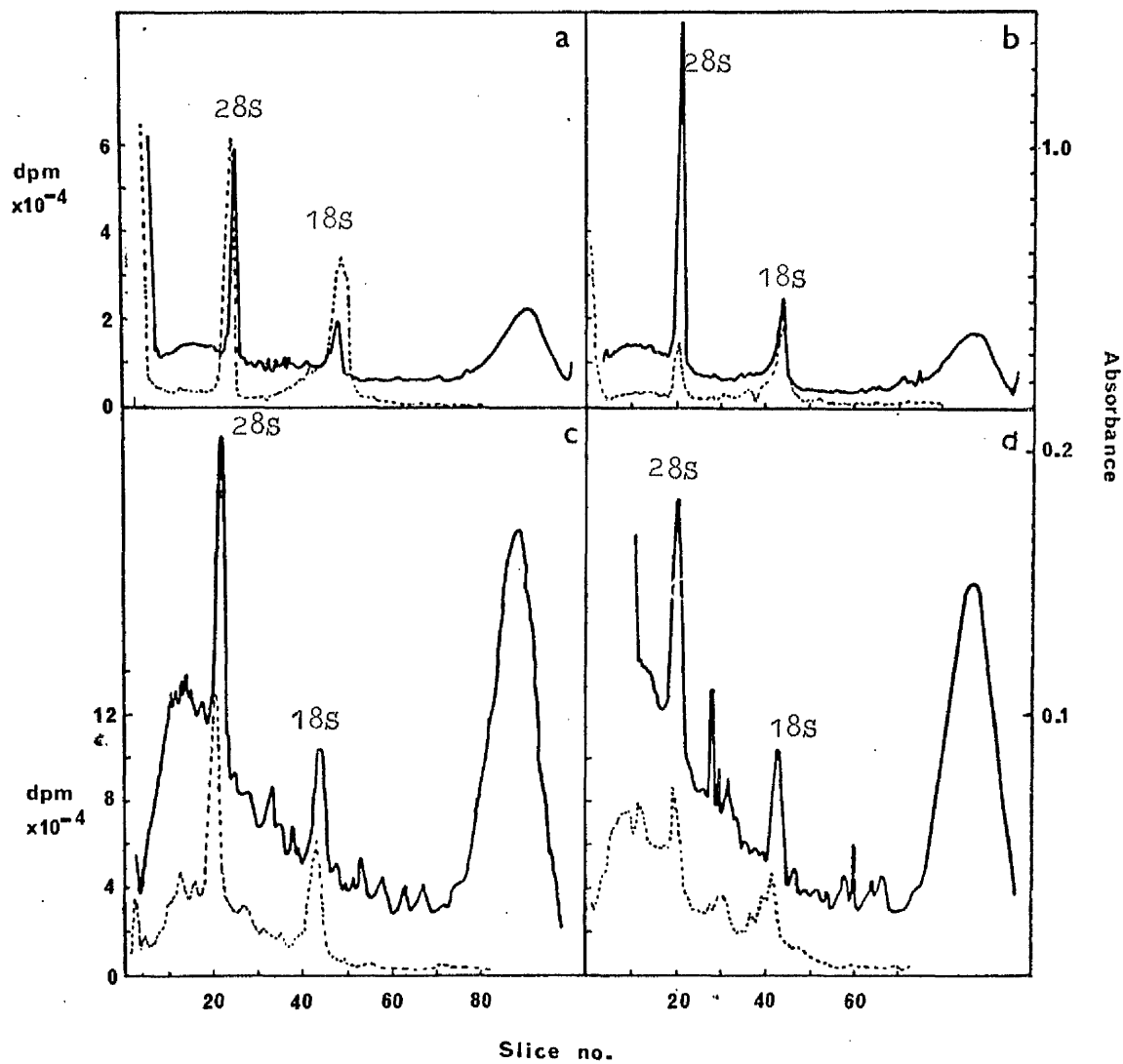
PAGE of Polysomal RNA from Infected and Mock-infected

Cells

The RNA was labelled by incubating approximately  $10^8$  infected or mock-infected cells in the presence of 1.25 mCi [5,6- $^3\text{H}$ ] -uridine, in 20 ml medium, for 1-3 h after infection or mock-infection. Polysomal RNA was isolated and submitted to poly-(U) sepharose chromatography. Both the unbound and bound RNA fractions were analysed by PAGE.

The solid line is the optical density trace and the dashed line shows the radioactivity in the gel slices. The large peak shown in the absorbance trace at about slice no. 80 is due to the marker dye, bromophenol blue.

- a) mock-infected, unbound fraction
- b) infected, unbound fraction
- c) mock-infected, bound fraction
- d) infected, bound fraction



also shows the inhibition of rRNA synthesis in herpesvirus infected cells, first noted by Hay et al. (1966).

### 3.3. Discussion

Differential phenol extractions of RNA resulted in a substantial enrichment of non-rRNA in the alkaline extract but there appeared to be a preponderance of low molecular weight RNA which made me suspect that degradation of this RNA was occurring as reported by Perry et al. (1972). For this reason this method was only used for very few experiments at the beginning of the project.

Oligo-(dT) cellulose seemed to raise two problems, the minor species of RNA were only weakly bound but rRNA was bound so that ribosomal species contaminated the putative mRNAs.

Poly-(U) sepharose was most efficient at binding the minor species of RNA and rRNA was only weakly bound to the column, although this was found to be variable. rRNA was therefore separated from some putative mRNAs to a reasonable extent and so this method was used in preference to the others.

## Chapter 4

### Identification of Polysomal RNA Bound to Poly-(U)

#### Sepharose as Messenger RNA

##### 4.1. Introduction

If PAGE analysis of RNA shows that a species of RNA in the bound fraction of poly-(U) sepharose is enriched compared to rRNA this is insufficient evidence that the species is mRNA. At least two criticisms could be raised against such a claim:

- (a) the RNA might not be a functional messenger but merely present in a RNP which is co-sedimenting with the polysomes
- (b) the RNA might be a breakdown product of rRNA which is bound preferentially to the poly-(U) sepharose.

Perry et al. (1973) have shown that ribonucleoprotein particles (RNPs) in the cytoplasm are of such a size that they co-sediment with polysomes. The RNA extracted from these polysomes will then contain RNA originating from the RNPs along with polysome-associated mRNA. These RNA species could then be falsely assumed to be mRNA.

It is also possible that any species of RNA found on a polyacrylamide gel could be either a breakdown product or an aggregate of rRNA. Such aggregates or degradation products of rRNA might also bind preferentially to poly-(U) sepharose and so be confused with polyadenylated RNA.



To investigate these possibilities four types of experiments were carried out:

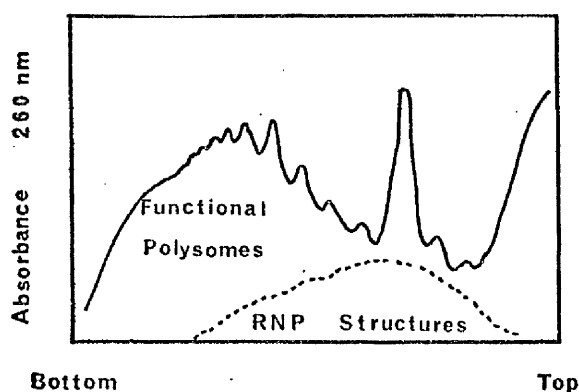
- (i) RNA was prepared from EDTA dissociated polysomes from which rapidly sedimenting RNPs had been removed.
- (ii) The effect of cordycepin on rRNA and putative mRNA was investigated.
- (iii) The effect of actinomycin D on rRNA and mRNA was also investigated.
- (iv) Poly-(U) sepharose-bound RNA extracted from whole polysomes was added to a cell-free wheat germ system to find if it directed the translation of any proteins.

Many species of polyadenylated RNA are found in polysomes in infected cells and some species are not readily identifiable going from one polyacrylamide gel to the next. I therefore decided to concentrate this study on RNA species which migrated between 18S and 28S rRNAs. There were two reasons for this decision:

- (i) rRNA was normally present in sufficient amounts in the samples bound to poly-(U) sepharose for these species to be detected when scanning the gels in the recording spectrophotometer. The ribosomal RNA species therefore provide useful internal markers.
- (ii) The migration rates of RNAs varied from gel to gel making comparison of the faster moving species very difficult, these variations had only a minor effect on the distance moved by the large species of between 18 and 28S.

#### 4.2. EDTA Treatment of Polysomes

Perry et al. (1973) suggested that RNPs co-sediment with polysomes, purely on the basis of the two structures having similar sedimentation properties and not because of any physical association between them. The diagram below (adapted from Perry et al., 1973) illustrates the co-sedimentation of RNPs with polysomes found in mouse L-cells.

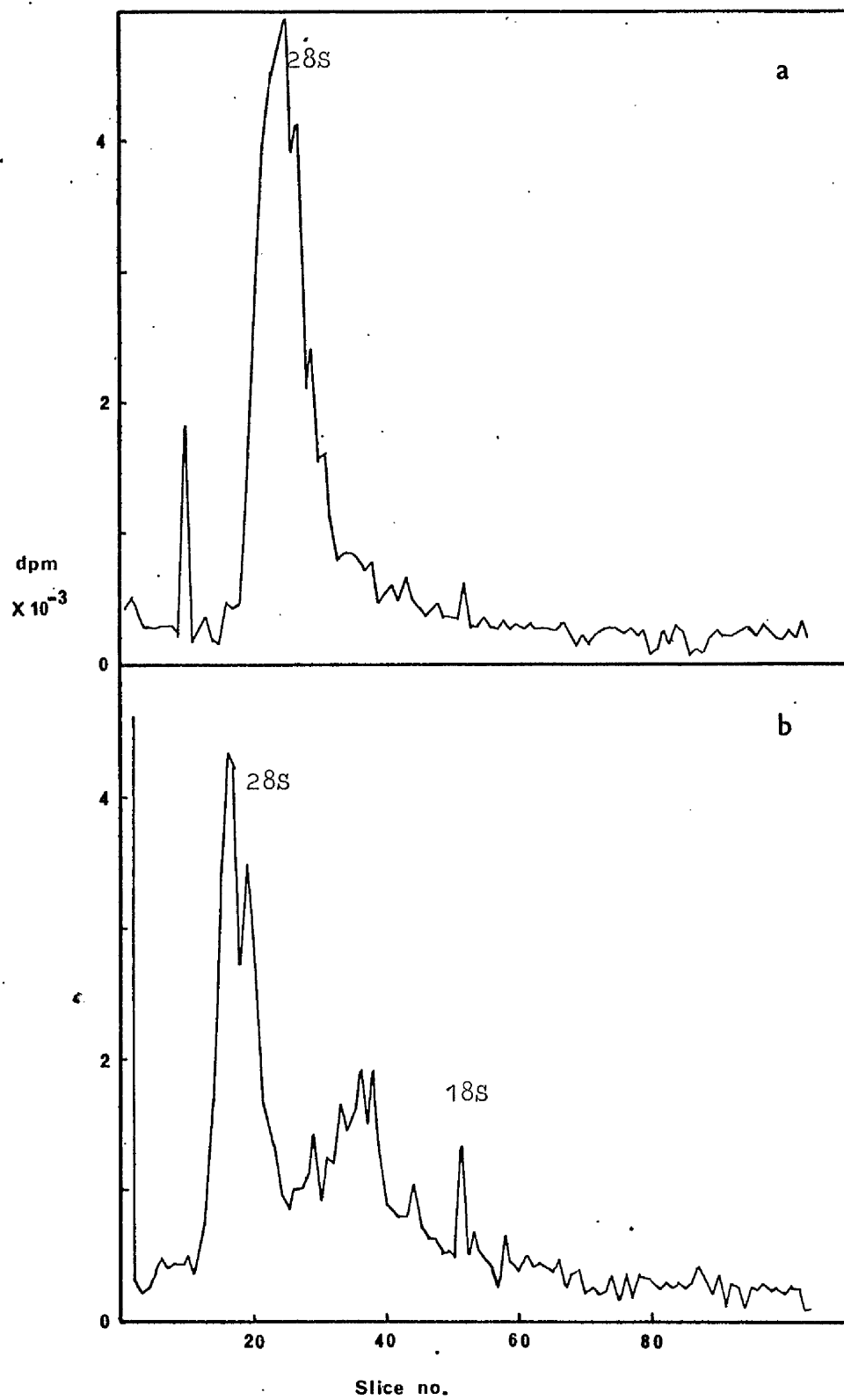


Chantrenne et al. (1967) found that while polysomes are disaggregated in the presence of EDTA, RNPs are not affected by this treatment. If polysomes are isolated in the normal way, treated with EDTA, and then recentrifuged through a sucrose density gradient the RNPs will sediment at the normal rate but the polysomal RNA will sediment at a speed determined by the individual components, such as the ribosomal subunits, and so travel down the gradient much more slowly than normal. If centrifugation is prolonged the RNPs can largely be pelleted leaving the dissociated polysome structures on the gradient. This was carried out and the diagram below shows a typical

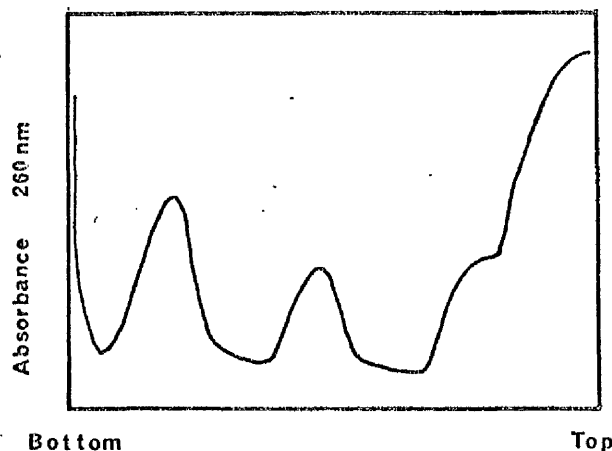
Fig 4.2.

EDTA Treatment of Polysomes, Followed by PAGE of  
the RNA Extracted from RNPs of between 50-70S

Cells were mock-infected (a) or infected (b) and labelled with 1 mCi  $[5,6-^3\text{H}]$  Uridine for 1-5 h after infection or mock-infection. Polysomes were isolated and pelleted and then treated with EDTA (TESEN) to dissociate the polysomes and release the mRNA in ribonucleoprotein particles (RNPs). The EDTA treated polysomes were then centrifuged through 15-30% sucrose gradients in TESEN buffer so that the large ribosomal subunits were near the foot of the gradient. Various fractions were taken from the gradient and the RNA was extracted using phenol/chloroform. The RNA was then analysed using PAGE. The RNA in this figure was extracted from RNPs which sedimented with S-values between about 50 and 70S.



result of the procedure as carried out in this project.



The EDTA treated polysomes were centrifuged through a 15 - 30% sucrose gradient so that the 60S ribosomal subunit was near the bottom of the tube. All large RNPs would have been pelleted under these conditions. The putative mRNA species of between 18 and 28S were found to be located in RNPs which sedimented between approximately 50 - 70S (see fig 4.2). This was the first step towards identifying these species as mRNA as they are dissociated from polysomes by EDTA treatment. However this result does not exclude the possibility that the rRNA species being studied are derived from rRNA breakdown or aggregation.

#### 4.3. The Effect of Cordycepin on Cytoplasmic RNA Synthesis

To investigate the possibility that the peaks of radioactivity found on polyacrylamide gels between 18 and 28S RNA were derived from rRNA I decided to label RNA in the presence of cordycepin (3' deoxy-adenosine). Cordycepin was reported to specifically inhibit post-transcriptional adenylation of HnRNA while leaving the synthesis of

Effect of Cordycepin on Ribosomal RNA Synthesis  
in Mock-Infected and Infected Cells

Cells were mock-infected (i) or infected (ii) in 25 ml pooled media which was replaced by 25 ml fresh media after 1 h and cordycepin was also added to the required concentration at this time. Labelling with 200  $\mu$ Ci [5,6- $^3$ H] Uridine was carried out from 3-6 h after infection or mock-infection.

Whole cytoplasmic RNA was extracted with phenol at pH 7.6 by the method of Brawerman et al. (1972) and was analysed by centrifugation through a 15-30% sucrose gradient in LETS buffer.

Cordycepin was added to the following concentrations:

- (a) None
- (b) 5  $\mu$ g/ml
- (c) 10  $\mu$ g/ml
- (d) 20  $\mu$ g/ml
- (e) 25  $\mu$ g/ml
- (f) 40  $\mu$ g/ml

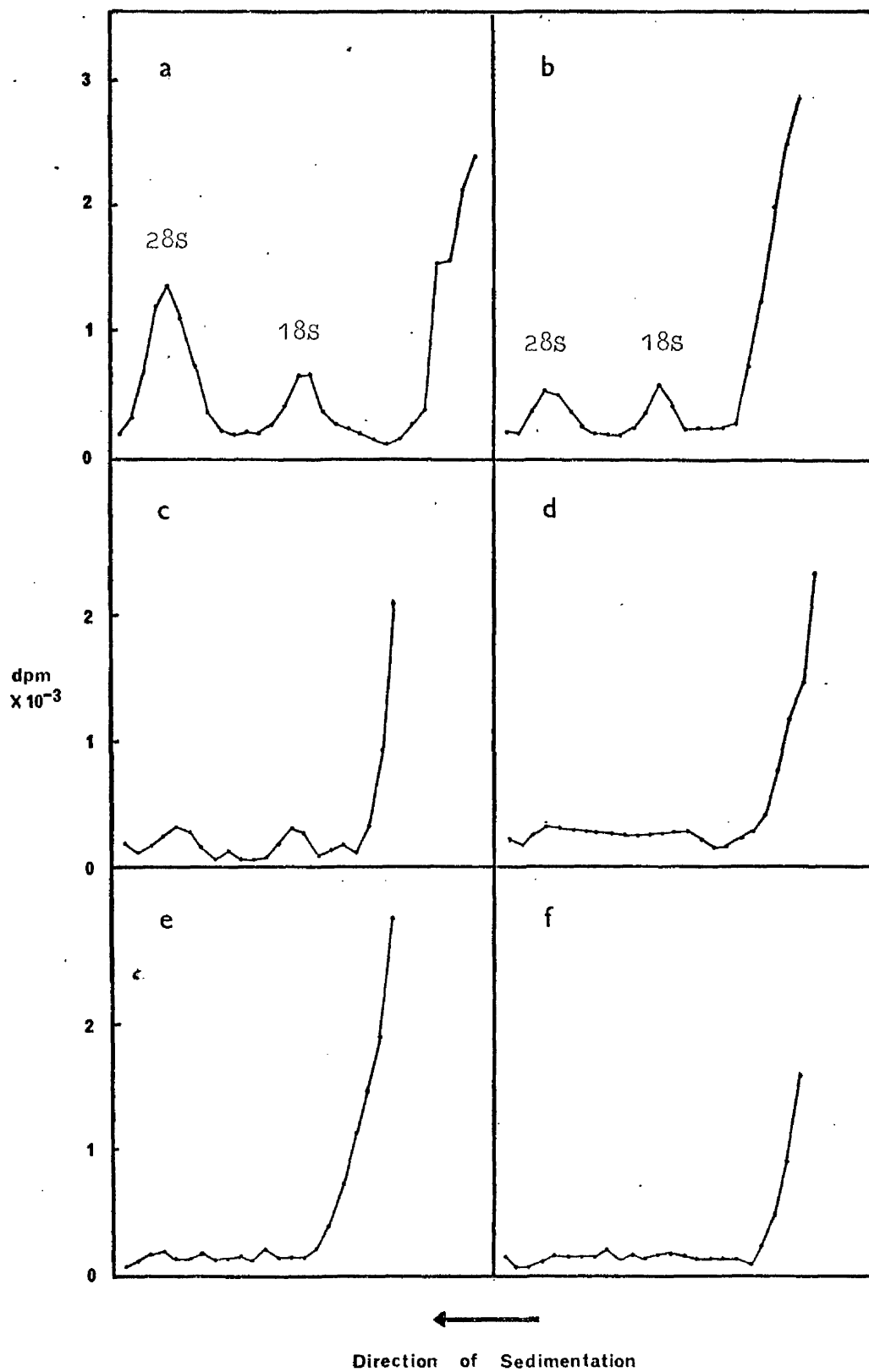


Fig 4.3.a (i)

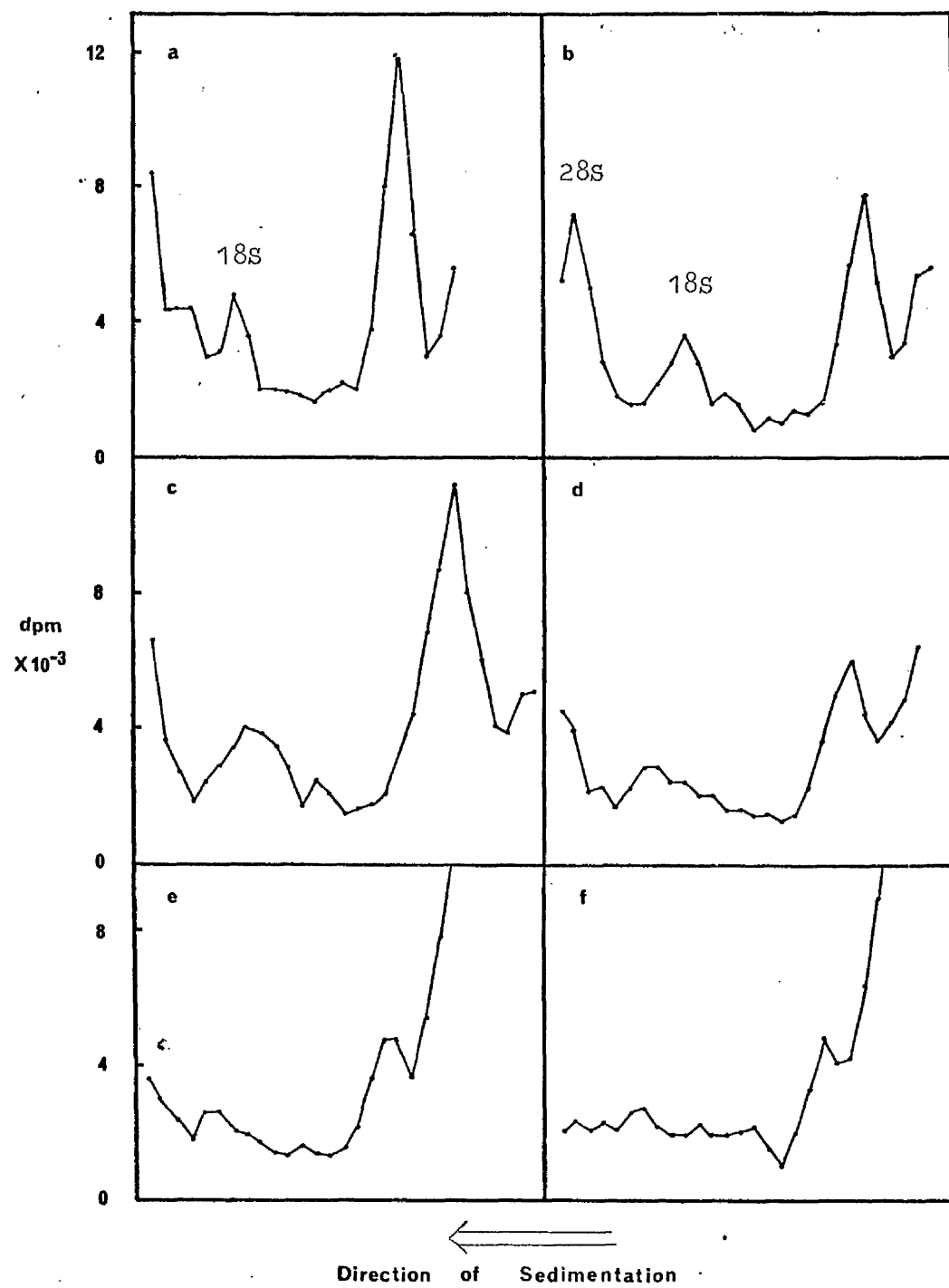


Fig 4.3.a (ii)



Fig 4.3.b

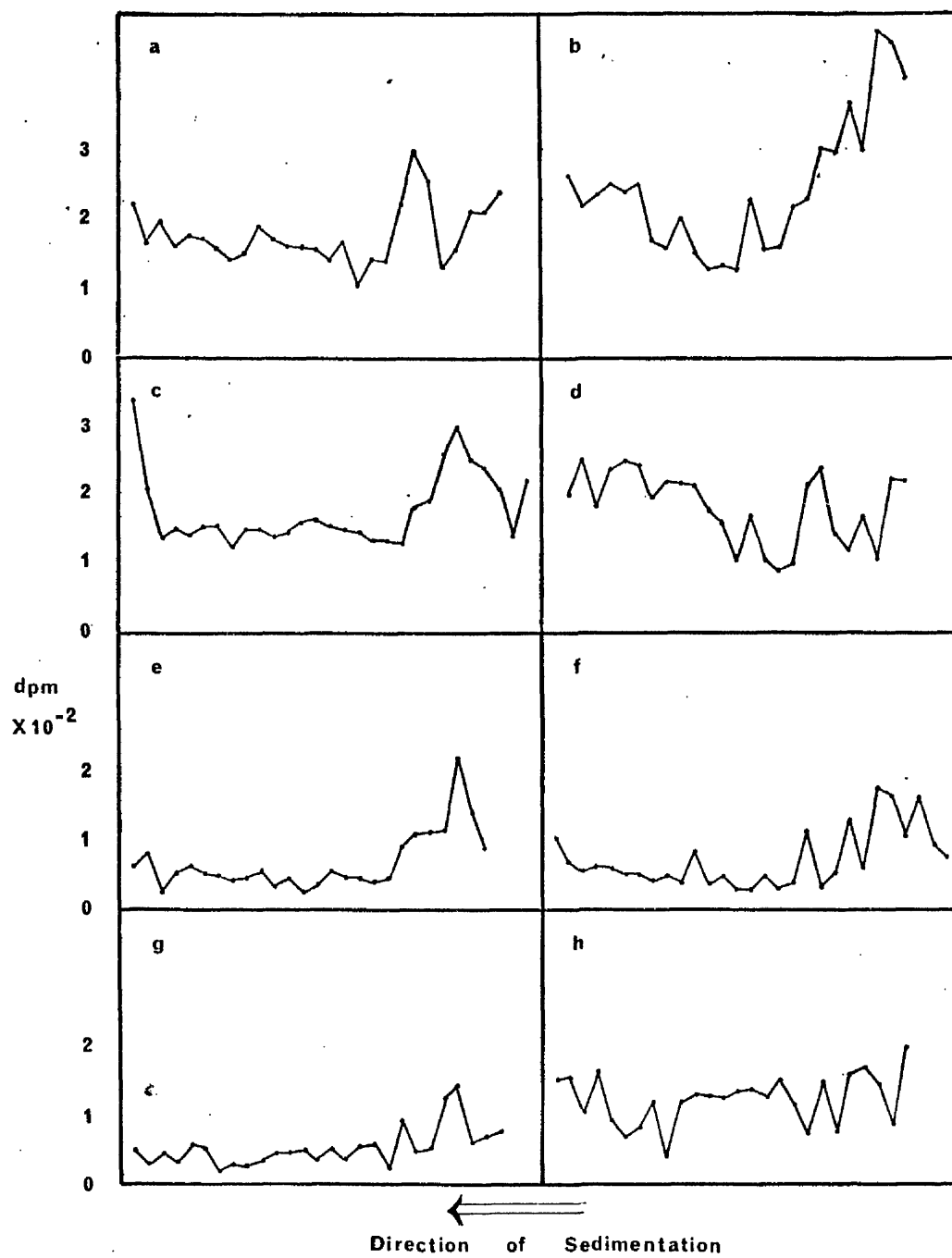
Effect of Cordycepin on Non-Ribosomal RNA  
Synthesis in Mock-Infected and Infected Cells

Cells were mock-infected (a, c, e, g) or infected (b, d, f, h) in 25 ml pooled media which was replaced by 25 ml fresh media after 1 h and cordycepin was added to the required concentration at this time. Labelling with 200  $\mu$ Ci [5,6- $^3$ H] Uridine was carried out from 3-6 h after infection or mock-infection.

Whole cytoplasmic RNA was extracted with phenol at pH 7.6 by the method of Brawerman et al. (1972) and the residual RNA found at the interface along with denatured protein was then extracted at pH 9.0. The pH 9.0 extract, which should be enriched in polyadenylated RNA was then analysed by centrifugation through a 15-30% sucrose gradient in LETS buffer.

Cordycepin was added to the following concentrations:

- (a, b) None
- (c, d) 5  $\mu$ g/ml
- (e, f) 20  $\mu$ g/ml
- (g, h) 25  $\mu$ g/ml



HnRNA itself relatively unaffected (Penman et al., 1970). rRNA synthesis was also thought to be less sensitive than mRNA to cordycepin. Putative mRNA species would therefore be expected to be preferentially inhibited while rRNA synthesis would not. If the RNA species thought to be messengers were insensitive to cordycepin, this might suggest that they could be derived from rRNA, but if they were preferentially inhibited then this would show that they have a different origin.

The experiments with cordycepin were carried out mainly in the early part of the project when I was extracting RNA at pH 7.6 and pH 9.0 (see section 3.2.1). Figs 4.3 a and b show the effects of various concentrations of cordycepin on the synthesis of whole cytoplasmic RNA extracted at pH 7.6 and pH 9.0. Fig 4.3.a shows the effect on the pH 7.6 extract which consist largely of rRNA. The synthesis of 18S rRNA was practically completely inhibited by the time the concentration of cordycepin was raised to 20 µg/ml. The synthesis of RNA extracted at pH 9.0 does not appear to be more sensitive to cordycepin than is the synthesis of rRNA (fig 4.3.6). However rRNA synthesis was so strongly inhibited by cordycepin that this approach to proving that the supposed mRNAs were not derived in some way from rRNA was not feasible.

#### 4.4. Sensitivity of Polysomal RNA Synthesis to Actinomycin D

As discussed in section 2.5 in uninfected HeLa cells actinomycin D specifically inhibits rRNA synthesis leaving mRNA relatively unaffected. If this is also true in infected cells it should be possible to tell if RNA species are derived from rRNA or from some other source. At the concentration of actinomycin D used (0.04 µg/ml) rRNA was found to be markedly inhibited in both infected and mock-infected

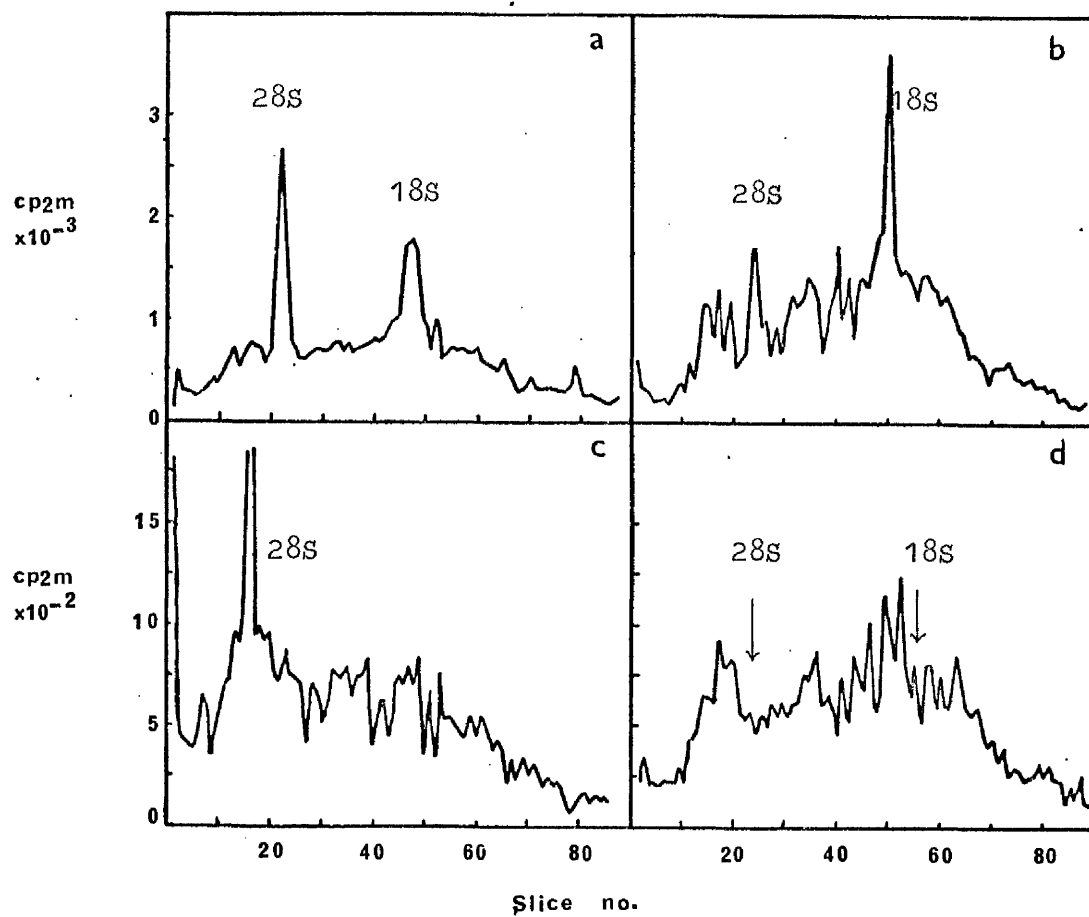
Fig 4.4.

Effect of Actinomycin D on the Appearance of  
Polyadenylated RNA in the Pre-Polysome Pellet Fraction

Cells were mock-infected (a, c) or infected (b, d) in 25 ml pooled media which was replaced with fresh media after 1 h, 0.04 µg/ml actinomycin D was also added to (c) and (d) at this time. 1.25 mCi [5,6-<sup>3</sup>H] Uridine was present between 1 and 4 h after mock-infection or infection after which the cells were harvested. Polysomes were prepared in the normal way and RNA was extracted from the pre-polysome pellet using phenol/chloroform. The RNA was subjected to poly-(U) sepharose chromatography and the bound RNA was then analysed using PAGE.

a) mock-infected	c) infected
b) mock-infected	d) infected
+	+
actinomycin D	actinomycin D.

The amounts of rRNA on all four gels was almost identical as the absorbance profiles were practically indistinguishable.



cells. This effect is particularly pronounced in infected cells as it seems to operate in addition to the inhibition of rRNA synthesis due to the virus.

The species of RNA thought to be mRNA were considerably less affected by actinomycin D than rRNA was. This shows that they are synthesised by some relatively actinomycin insensitive process and that they are not derived from rRNA.

As actinomycin D acts by binding to G residues I had initially hoped that some regions of the PrV DNA might be GC rich and the synthesis of mRNA coded by these regions should be preferentially reduced in the presence of the inhibitor. However this did not prove to be the case and all mRNAs were inhibited to about the same extent (see fig 4.4. ). This result is in agreement with the partial denaturation mapping experiments of Reischig et al. (1975) who found that high GC regions are randomly distributed along the genome.

#### 4.5. Translation of RNA Bound to Poly-(U) Sepharose

Polysomal RNA which had been bound to poly-(U) sepharose was added to an in vitro wheat-germ translation system. It was hoped that recognizable polypeptides would be synthesized which would later be characterised on polyacrylamide gels and it was hoped also by immunoprecipitation with antibodies prepared against PrV nucleocapsid.

Initially it was found that the samples of RNA bound to poly-(U) sepharose inhibited the endogenous incorporation of labelled amino acids into acid precipitable products by the wheat-germ system. This may have been due to the RNA being contaminated with EDTA or dextran sulphate both of which inhibited the system and were present in the preparative procedure. Dextran sulphate was then omitted and the final RNA sample washed with 2 M sodium acetate. The RNA extracts prepared in this way stimulated wheat-germ amino acid incorporation into polypeptides, the stimulation was however variable. Figure 4.5.a shows the incorporation of [ $^3\text{H}$ ] leucine into acid precipitable material in the wheat-germ system, with added polyadenylated RNA from infected and mock-infected cell polysomes. The optimum concentration of RNA from infected cells was 10  $\mu\text{g}/\text{ml}$ . This concentration was used in a larger scale preparation and the translation products were analysed by PAGE. Figure 4.5.b shows the result of this procedure. The conclusion that can be drawn from this is that the polyadenylated RNA from infected cells stimulated the incorporation of labelled amino acids into discrete species of polypeptides. We hoped to demonstrate that these proteins were virus specific by serological methods. To this end it was attempted to prepare antibodies against purified main capsid

Fig 4.5.a

Protein Synthesis in a Wheat Germ Extract with  
RNA Added to Various Concentrations

3 bottles of about  $10^8$  cells were infected with PrV and 3 bottles were mock-infected. 5 h after infection both sets of cells were harvested and polyadenylated polysomal RNA was prepared. This RNA was added to the wheat germ translation system (as described in the Methods Section) to the concentrations shown. The incorporation of [ $^3\text{H}$ ] leucine into acid precipitable counts was determined.

- ▲ Infected Cell RNA
- Mock-Infected Cell RNA



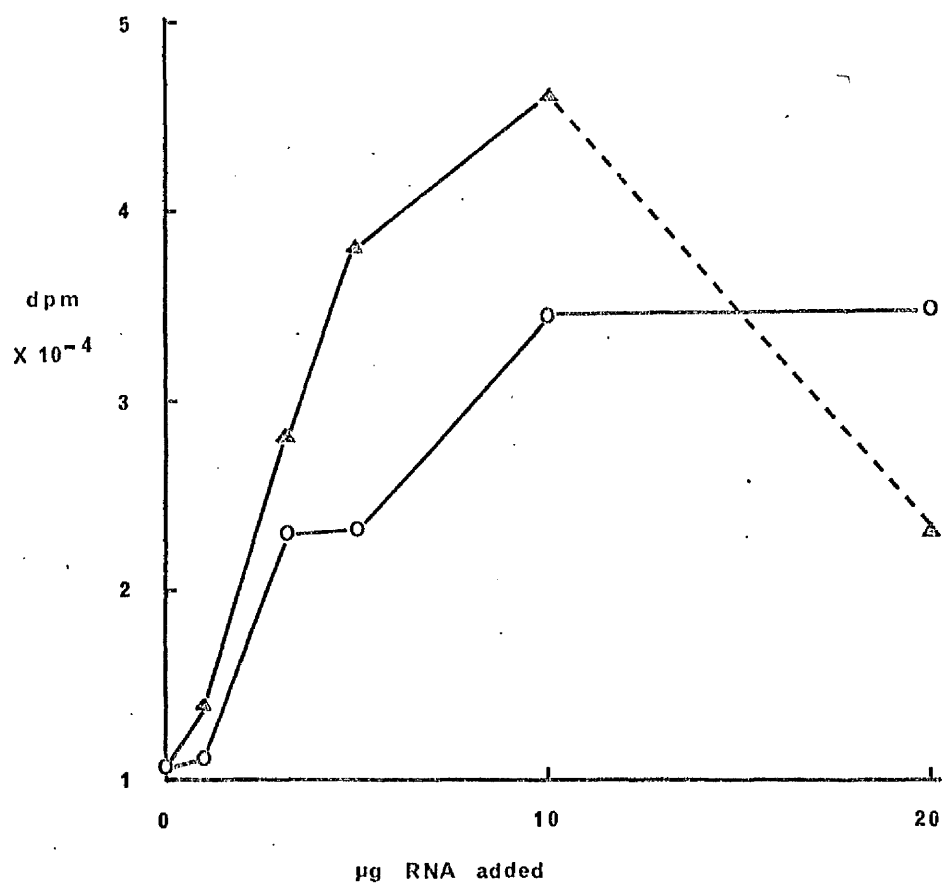


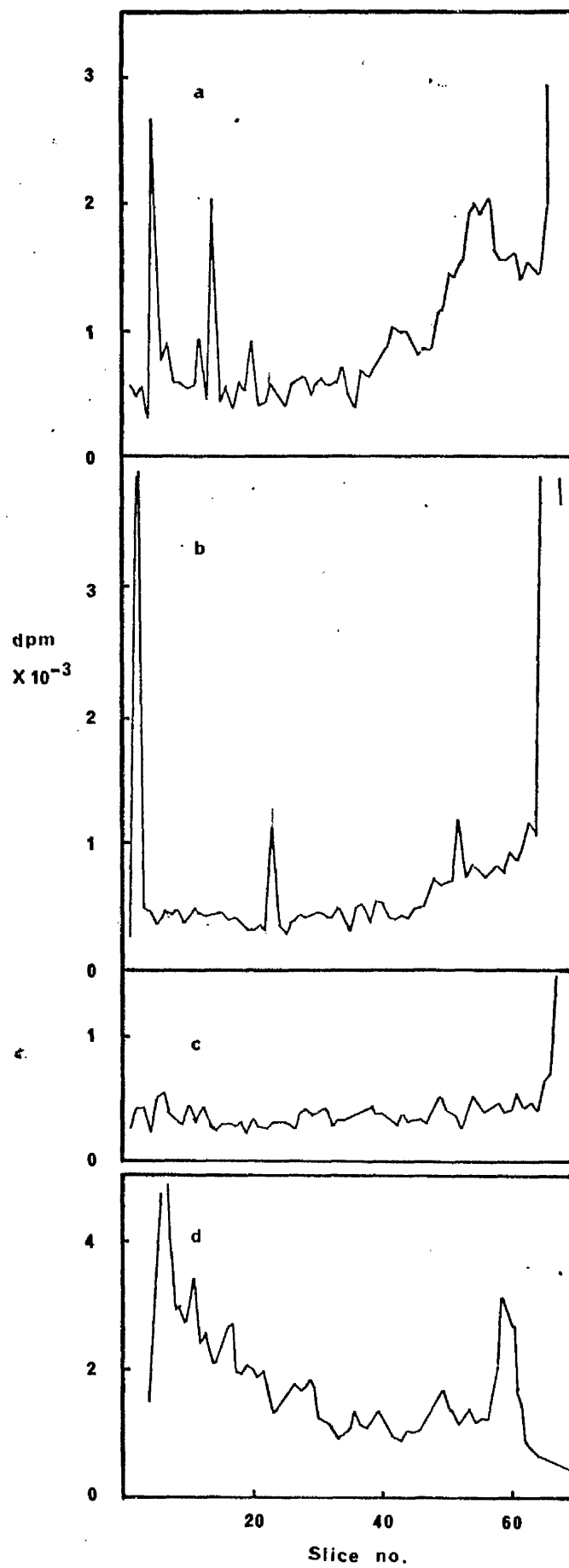
Fig 4.5.b

PAGE of Proteins Synthesised in Wheat-Germ

Extracts with RNA added

a, b and c show the radioactivity profiles of proteins synthesised in the cell-free wheat germ system with or without added RNA; d is the profile cytoplasmic proteins extracted from cells labelled 1 - 5 h post infection. Incubations were carried out as described in the methods section with added RNA from the following sources: (a) Poly-(U) sepharose bound polysomal RNA extracted from cells 5 h PI, (b) RNA prepared in the same way from mock-infected cells, (c) no added RNA.

The proteins were analysed by PAGE and sliced and counted as described in the methods section.



protein. This has still to be carried out successfully and so far no immunoprecipitation of the main capsid protein has been obtained with sera from treated animals.

#### 4.6. Summary

RNA species, migrating between 18S and 28S rRNAs on polyacrylamide gels, were found to be associated with polysomes and were not derived from EDTA insensitive ribonucleoprotein particles. This provides some evidence that these RNAs are messengers.

The RNA species under consideration are also relatively resistant to actinomycin D, unlike rRNA and so their synthesis appears to be insensitive to this inhibitor of rRNA synthesis. This finding shows that these RNA species are not derived from rRNA aggregation or degradation.

When polyadenylated RNA from infected cell polysomes was added to a cell free wheat germ extract a similar PAGE profile of proteins is found to that obtained with infected cell cytoplasmic proteins. This shows that mRNA is present, but does not show definitively that the messenger is virus specific.

Heavy  
Polysomes



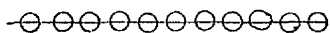
(i)

Light  
Polysomes



(ii)

(a)



(i)



(ii)

(b)



(iii)



(iv)

## Chapter 5

### Distribution of Polyadenylated RNA

#### 5.1. Introduction

The distribution of mRNA molecules of different size between large and small polysomes can provide indirect information on translation. If elongation of polypeptides is the rate limiting process, polysome size should be directly proportional to mRNA length. This would result in only small mRNAs being found in small polysomes and large mRNAs would be restricted to large polysomes. This scheme is shown in the schematic diagram on the facing page (scheme a). Alternatively if initiation frequency determined the rate of synthesis of polypeptides the distance between ribosomes on polysomes would tend to be greater than if elongation were the limiting process (scheme b). Diagram (b i and ii) show mRNA molecules with approximately equal initiation frequency and in this situation the messenger length determines the relative distribution of the two species. However when the initiation frequencies are widely different (b iii and iv) the relative distribution of messengers between heavy and light polysomes may bear no relation to the lengths of the mRNAs.

A number of reports say it is often the frequency of initiation that determines the rate of synthesis of proteins and this is thought to be true of both viral and cellular systems (Saborio et al., 1974; Nuss et al., 1975).

## 5.2. Distribution of Polyadenylated RNA

It was decided to examine the distribution of polyadenylated RNA among heavy and light polysomes and the pre-polysome pellet and I, hoped that some viral RNA species would be found predominantly in one or other of these fractions. In this way I thought it might be possible to study subsets of viral messengers. I looked at the distribution both early and late in infection but found that insufficient radioactivity was incorporated into RNA at early times to give a good estimation of the distribution of amounts of different sizes of RNA.

I found that late in infection the bulk of newly synthesised RNA was located in the pre-polysome pellet. In infected cells the size distribution of polyadenylated RNA was very similar in all three fractions (see figs 5.2. a, b). In contrast to the inhibition of rRNA synthesis in infected cells polyadenylated RNA was synthesised at a similar rate in infected and mock-infected cells (fig 5.2.a).

Figures 5.2.a and b show that polyadenylated RNA is heterogeneous with respect to size in both infected and mock-infected cells. Individual species of cellular mRNA cannot be identified by PAGE and so it is impossible to tell from these results whether host mRNA is in fact no longer synthesised and found in polysomes. The reported loss of cellular mRNA from polysomes depends on hybridisation data which is not clear cut and confirmation by some other method is required.

In both infected and mock-infected cells polysome size does

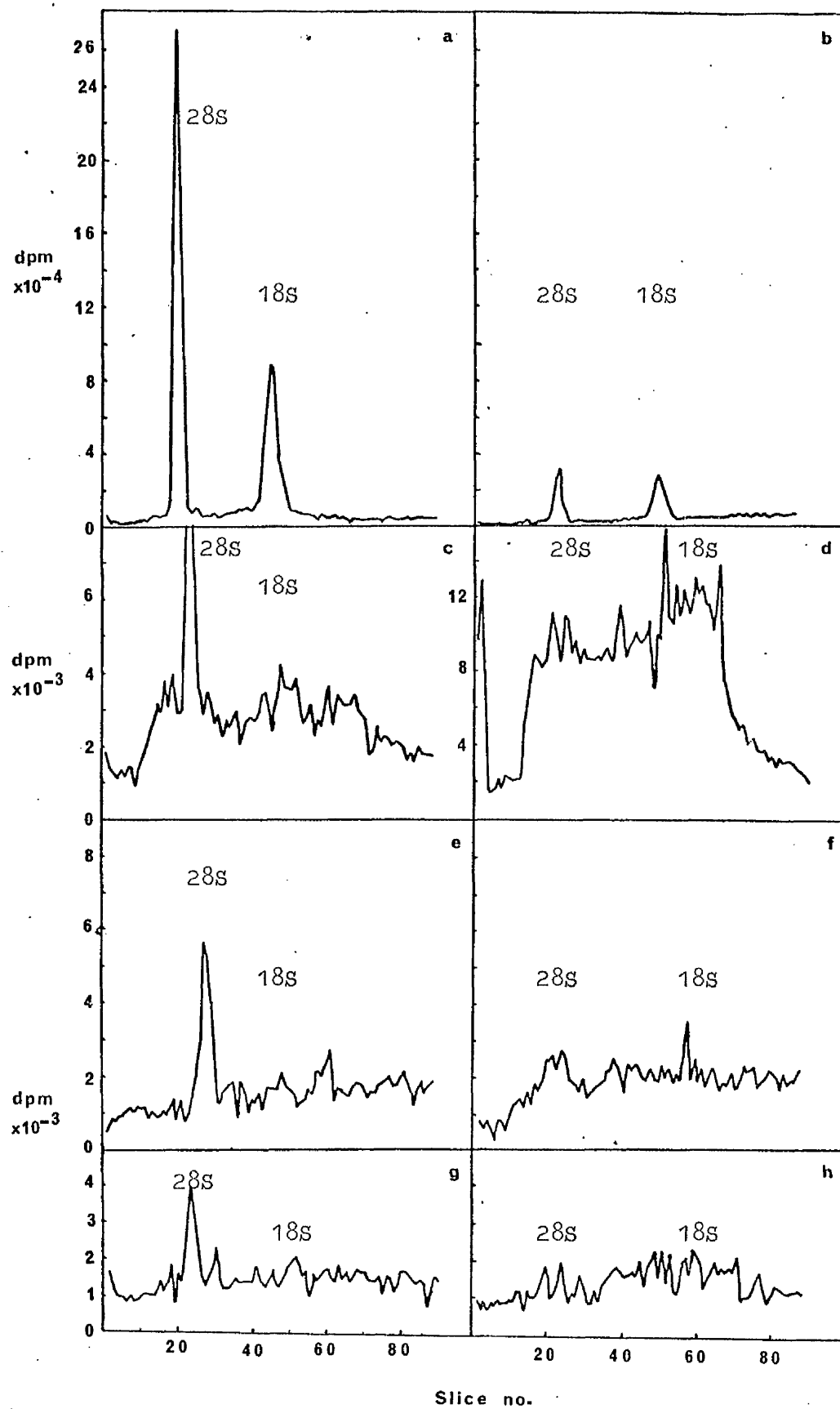
Fig 5.2.a

Distribution of Polyadenylated RNA in Polysomes

RNA in mock-infected and infected cells was labelled for 1 - 8 h after infection with [ $^3\text{H}$ ] uridine. Polysomes were isolated and divided into three fractions:- the pre-polysome pellet, heavy polysomes and light polysomes. The RNA, extracted from these fractions, was then subjected to poly-(U) sepharose chromatography and the following samples were analysed by PAGE.

- |    |               |    |                                      |
|----|---------------|----|--------------------------------------|
| a) | mock-infected | b) | infected Unbound Heavy Polysomal RNA |
| c) | " "           | d) | " Bound pre-polysome pellet RNA      |
| e) | " "           | f) | " Bound Heavy-polysome RNA           |
| g) | " "           | h) | " Bound Light-polysome RNA           |





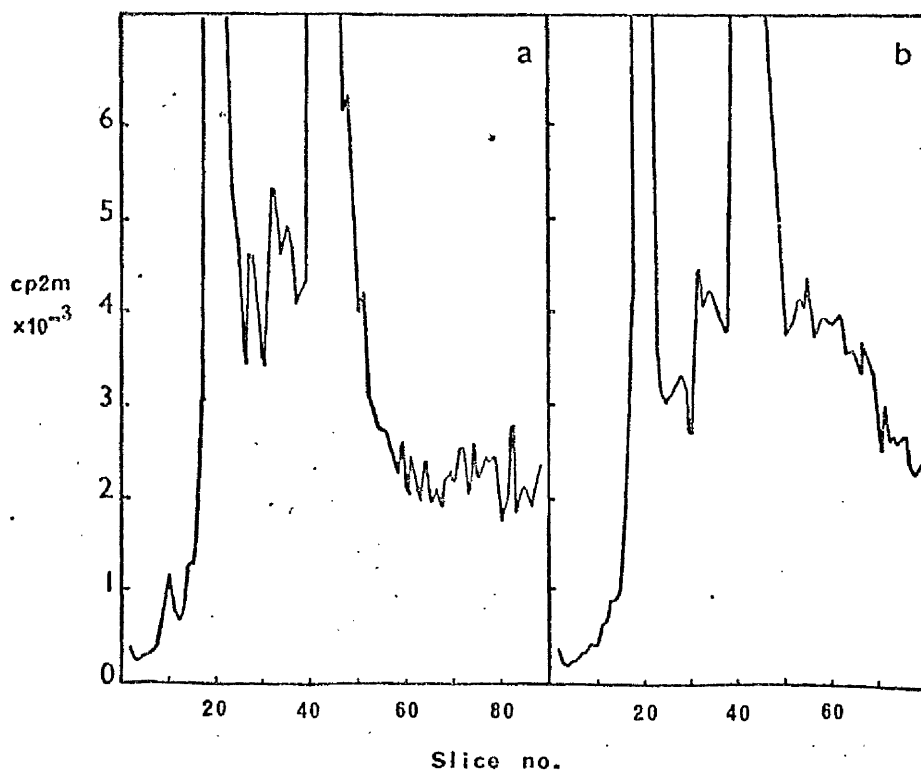
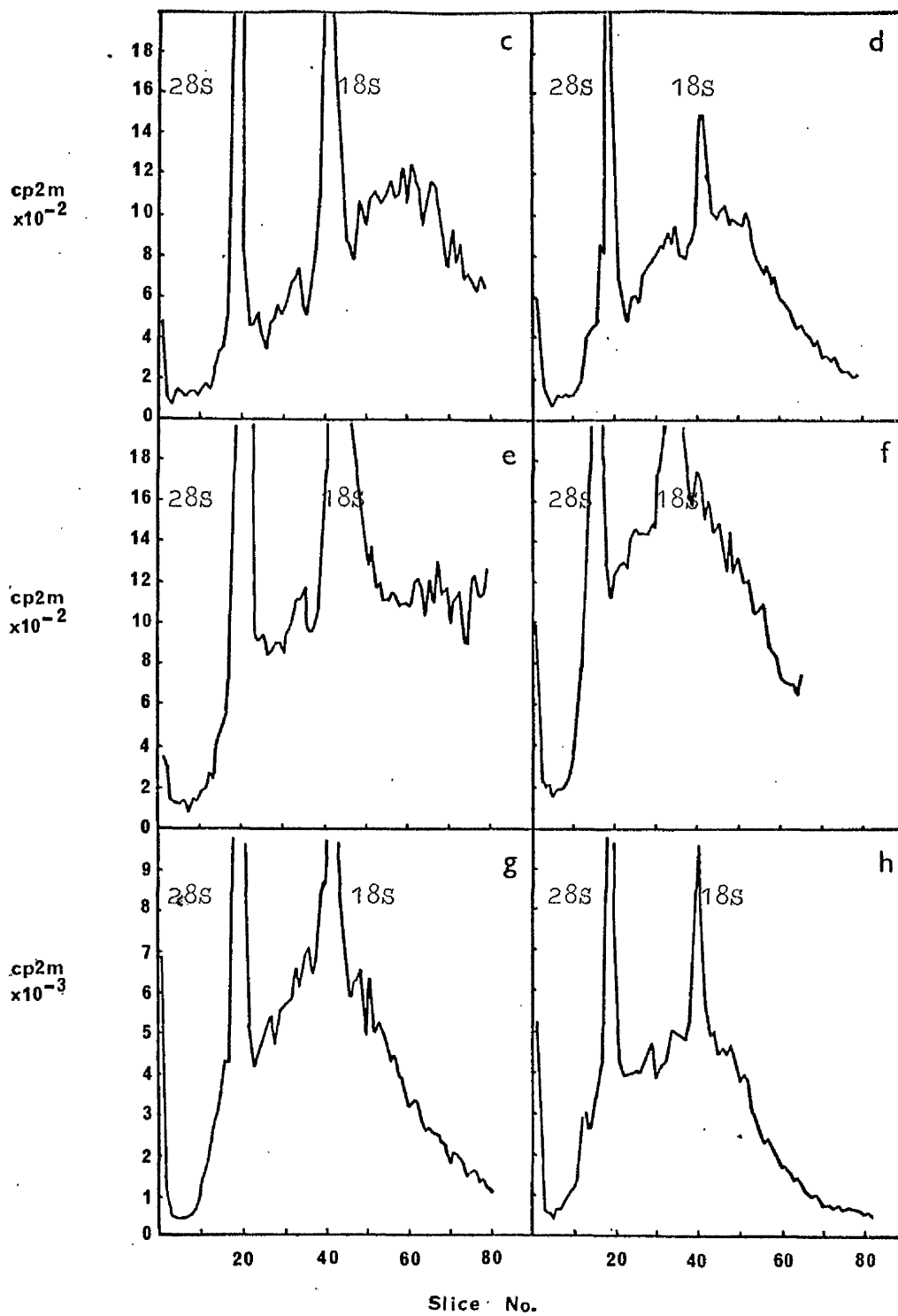


Fig 5.2.b Distribution of RNA in heavy and light polysomes  
and in pre-polysome pellets

RNA was labelled in mock-infected and in infected cells with 1.25 mCi  $[5,6-^3\text{H}]$  Uridine from 1-7 $\frac{1}{2}$  h after infection or mock-infection. Polysomes were isolated and divided into 3 fractions, heavy and light polysomes and the pre-polysome pellet. The RNA was extracted and fractionated using poly-(U) sepharose, and then analysed by PAGE.

- |  |   |                    |
|--|---|--------------------|
| a) Mock-infected heavy polysomal RNA     | ) | Not bound to       |
| b) Infected " " "                        | ) | Poly-(U) Sepharose |
| c) Mock-infected " " "                   | ) | Bound to Poly-(U)  |
| d) Infected " " "                        | ) | Sepharose          |
| e) Mock-infected Light " "               | ) | "                  |
| f) Infected " " "                        | ) |                    |
| g) Mock-infected Pre-polysome pellet RNA | ) |                    |
| h) Infected " " " "                      | ) | "                  |



not appear to be closely related to the length of polyadenylated RNA. There is a similar distribution of size of mRNA in heavy and light polysomes (see figs 5.2.a, b) and this is the expected result if the frequency of initiation determines the rate of translation.

There is a smaller proportion of low molecular weight polyadenylated RNA in the pre-polysome pellet than in heavy or light polysomes (see fig 5.2.b). This could be due to high molecular weight mRNA promoting a high frequency of initiation of translation and hence being found in very large polysomes which then sediment to the foot of the gradient. Alternatively large mRNAs may more commonly be located on membrane bound polysomes which will sediment to the pre-polysome pellet.

Figure 5.2.b illustrates a further important experimental fact - the same type of RNA profiles are found in the bound and unbound fractions except that rRNA is present in vast excess in the unbound fraction. Samples not subjected to poly-(U) sepharose chromatography also give similar polyacrylamide gel profiles. This shows that the PAGE migration of polyadenylated RNA is not affected by the chromatographic process or the buffers involved.

### 5.3. Effect of Cycloheximide on RNA Distribution

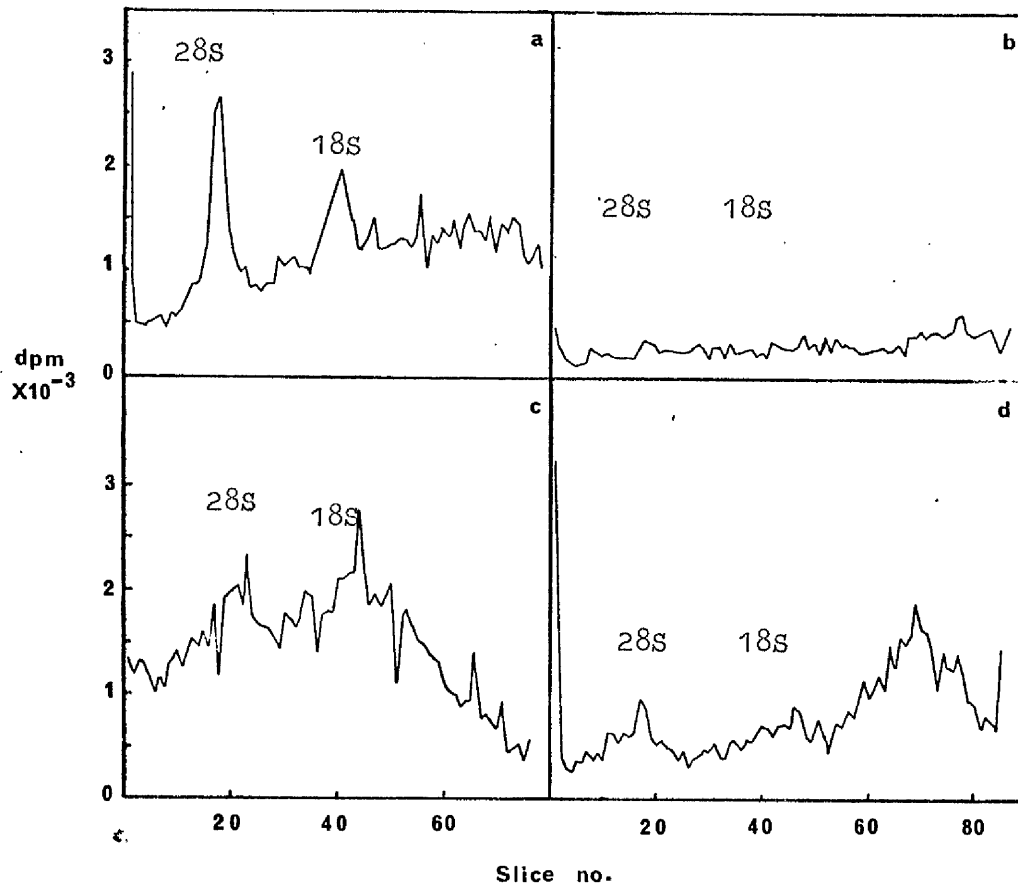
Cycloheximide inhibits protein synthesis by preventing the elongation of nascent polypeptides (Fukuhara, 1965; Clarke-Walker & Linnane, 1966). When cells are treated with the inhibitor in low concentrations the rate of elongation of polypeptides becomes the rate limiting step to protein synthesis (Nuss et al., 1975). Cycloheximide

Fig 5.3.

Effect of Cycloheximide on RNA Distribution in  
Light Polysomes and Post Polysome Supernatant  
Fractions in Infected Cells

$10^8$  cells were infected in the presence or absence of cycloheximide (50  $\mu\text{g/ml}$ ) and the RNA was labelled with 1.25 mCi  $[5,6\text{-}^3\text{H}]$  uridine for 1 - 6 h post infection. The cycloheximide was removed with 3 changes of fresh media and the cells were incubated for a further 20 min. Polysomes were isolated and RNA was extracted from light polysomes and the post-polysome supernatant fraction.

- a) post-polysome supernatant
- b) post-polysome supernatant + cycloheximide
- c) light polysomes
- d) light polysomes + cycloheximide.



was used to inhibit protein synthesis in PrV infected cells by Rakusanova et al. (1971). It was found that synthesis of viral cytoplasmic RNA was restricted if no viral proteins were synthesised in the infected cell, that is only a subset of the viral sequences normally appearing were present in the treated cells. These workers further noted that viral mRNAs became associated with polysomes if the cycloheximide was removed from the cells. This method was also used in this project. It is likely that a small amount of cycloheximide remained in the cells after the bulk of the inhibitor had been removed. In the cycloheximide treated cells only low molecular weight mRNA is found in light polysomes (fig 5.3). This indicates that the rate of elongation has probably become the rate limiting process in polypeptide synthesis and that all the larger molecular weight messengers will now be associated with a large number of ribosomes and so will only be found in heavier polysomes. This is in marked contrast to the size distribution of mRNA between heavy and light polysomes in cells which have not been treated with cycloheximide. So this different distribution in treated and untreated cells suggests that the elongation rate is not normally the factor that determines the number of ribosomes on a messenger RNA.

Some polyadenylated RNA species, synthesised between 1 and 6 hours after infection, are normally present in the post polysome supernatant fraction. These species are no longer present in this supernatant fraction in cells treated with cycloheximide (see fig 5.3) and may have become associated with polysomes by virtue of cycloheximide causing a build up of ribosomes on what could be mRNAs with a very low initiation frequency.

#### 5.4. Nascent Polypeptides

Viral polysomes are known to be larger than the cellular structures and it was initially thought that this might be correlated to the length of the messengers. If such a relationship holds true then heavy polysomes would be expected to contain a much higher proportion of large molecular weight nascent polypeptides than light polysomes. No such relationship was found, in fact at  $2\frac{1}{2}$  h post-infection the heavy polysomes seem to contain a higher proportion of low molecular weight nascent polypeptides than is found in the light polysome fraction. This is in agreement with the size distribution of mRNA species between heavy and light polysomes discussed in the previous section (5.3). At  $2\frac{1}{2}$  h post infection there is a very high proportion of low molecular weight nascent polypeptides on heavy polysomes. It may therefore be that the rate of elongation of polypeptides is beginning to limit the rate of translation and causing a build up of ribosomes at the beginning of the mRNA. This may be the reason that viral polysomes are heavier than those in uninfected cells.

#### 5.5. Discussion

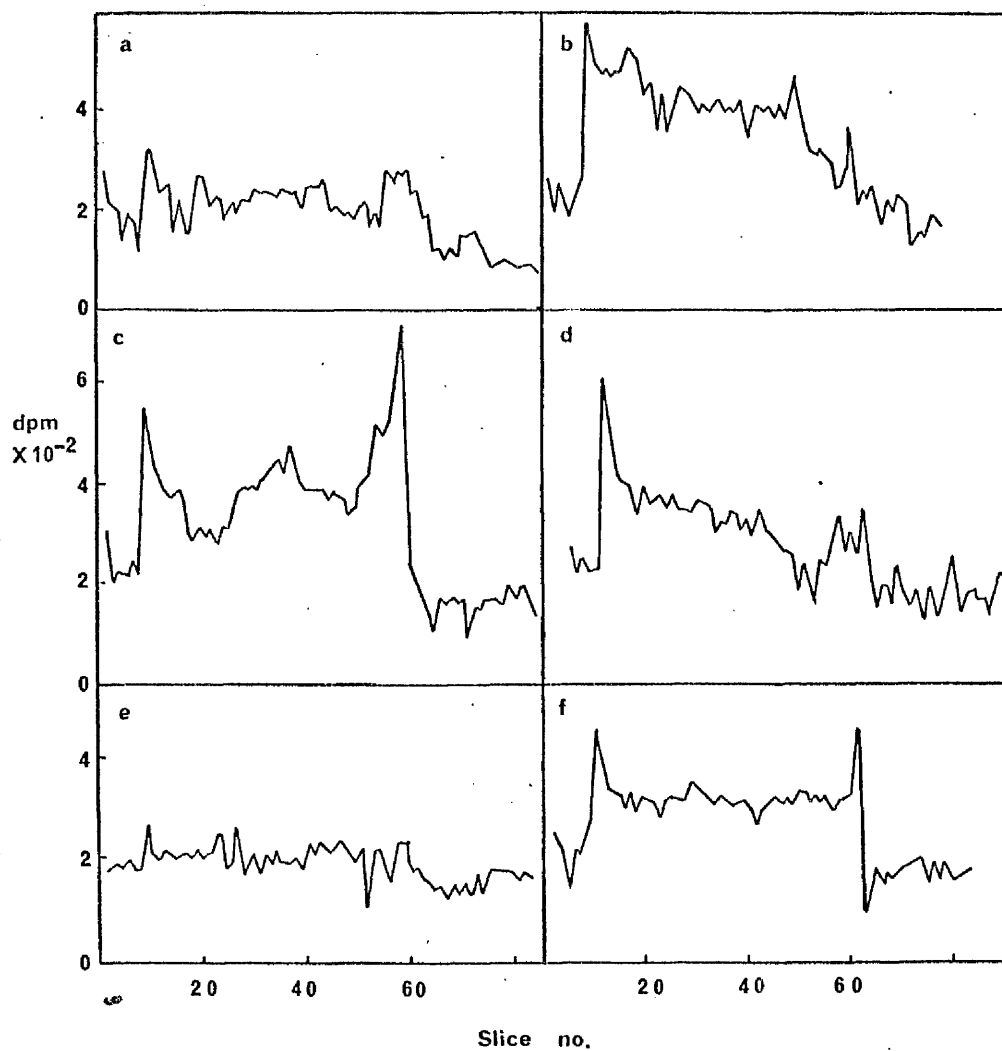
The size of mRNAs was found not to determine the size of polysome on which they were found: small mRNAs were found in large polysomes and large mRNAs on small polysomes. The size distribution of nascent polypeptides was also found not to be directly proportional to polysome size. Both these observations suggest that in both infected and mock-infected HeLa cells the rate determining process in protein synthesis is the frequency of initiation. If elongation were to determine the rate of polypeptide synthesis then small mRNAs would



1 bottle of  $10^8$  cells was mock-infected and 2 bottles infected with 20 p.f.u. PrV/cell. At 1 h post-infection fresh media, containing only  $\frac{1}{5}$  of the normal concentration of amino acids, was added. The cells were labelled at  $2\frac{1}{2}$  h after mock-infection,  $2\frac{1}{2}$  h post-infection or 5 h post-infection for 5 min with  $[^3\text{H}]$  mixed amino acids and harvested at the end of the labelling period. Polysomes were isolated on sucrose gradients in the usual way and then the heavy and light polysomes were pelleted separately and the pellets were treated with SDS and  $\beta$ -mercaptoethanol and the labelled nascent polypeptides were analysed by PAGE.

a) Heavy Polysomes from Mock-infected Cells

b) Light	"	"	"	"	"
c) Heavy	"	"	Infected cells	$2\frac{1}{2}$ h PI	
d) Light	"	"	"	"	$2\frac{1}{2}$ h PI
e) Heavy	"	"	"	"	5 h PI
f) Light	"	"	"	"	5 h PI



be expected to be found predominantly on small polysomes. This was found to be the case in the presence of low concentrations of cycloheximide which is thought to act by inhibiting polypeptide elongation. The difference in mRNA size distribution between heavy and light polysomes in the presence or absence of cycloheximide is further evidence that the frequency of initiation and not the rate of elongation normally determines the rate of protein synthesis.

2 $\frac{1}{2}$  hours after infection there is a larger proportion of low molecular weight polypeptides in heavy polysomes than in the heavy polysomes of uninfected cells. This may be due to viral mRNA giving rise to a higher frequency of initiation of polypeptide synthesis and so causing a build up of ribosomes near the initiation site on the mRNA with a correspondingly larger proportion of low molecular weight nascent polypeptides. If this proves to be the case it may be one mechanism whereby protein synthesis is switched from cellular to viral mRNA.

## Chapter 6

### Temporal Control of the Synthesis of Polysomal RNA in Infected Cells

#### 6.1. Introduction

Cycloheximide has been extensively used to study the sequential control of viral RNA synthesis in both PrV and HSV infected cells. Some hybridisation studies separated the RNA synthesised into classes (Rakusanova et al., 1971; Kozak & Roizman, 1974). Two definitions of RNA classes have been used:

- (i) RNA is divided into abundant and scarce classes, according to the molar concentrations of the species (Wagner et al., 1972; Frenkel et al., 1973; Frenkel & Roizman, 1972; Swanstrom et al., 1974)
- (ii) the time of appearance of RNA sequences has been used to class the species as immediate-early, early or late RNA (Rakusanova et al., 1971; Swanstrom et al., 1975).

The terms "immediate-early", "early" and "late" have been used principally in Kaplan's laboratory (see Kaplan, 1973) but similar terms have been employed by other workers in the herpesvirus field. Immediate-early is defined to be synthesised in the absence of any prior viral protein synthesis and it is this class of RNA that would be synthesised in cells treated with inhibitors of protein synthesis from the time of infection. By molecular hybridisation techniques Rakusanova et al. (1971) showed that if rabbit kidney cells are infected with PrV in the presence of cycloheximide then this resulted in a restricted transcription of

polysomal RNA. Kozak and Roizman (1974) confirmed this finding in HSV infected HEP-2 cells. Restricted synthesis of viral polysomal RNA also occurs in cells treated with Tricodermin (Swanstrom et al., 1975), another inhibitor of eukaryotic protein synthesis (Wei et al., 1974).

In addition to the molecular hybridisation work, Honess and Roizman (1973, 1974, 1975) have studied the control of protein synthesis in infected cells. These workers characterised proteins from infected cells by PAGE. They compared the amounts of individual proteins at different times after infection and found that some proteins are synthesised earlier after infection than others (Honess & Roizman, 1973). They then studied the synthesis of proteins after treating cells with cycloheximide from the time of infection and then washing the inhibitor away to allow protein synthesis to restart. They noted that only a subset of the viral proteins found late in infection was synthesised a short time after removal of the cycloheximide. This subset of proteins was termed the  $\alpha$  proteins. The  $\alpha$  protein synthesis declined after a time and was replaced by  $\beta$  protein synthesis which was subsequently supplanted by  $\gamma$  proteins. Honess and Roizman (1974) further showed that in order for  $\beta$  protein synthesis to take over from  $\alpha$  proteins new RNA synthesis was required after the removal of cycloheximide. This conclusion was indicated by their finding that actinomycin D, added at the time of removal of cycloheximide, so inhibiting RNA synthesis, also prevented the appearance of  $\beta$  proteins. However these elegant studies do not give any direct evidence on the control of viral RNA synthesis, and the hybridization studies, although direct, do not reveal any information with respect to individual species of RNA.

In order to try to supplement these conclusions I decided to see if cycloheximide specifically inhibited the synthesis of any individual species of polysomal RNA which was detectable using PAGE. It was also decided to try to see if any such effect was able to be related to variations in the PAGE profiles at different times after infection.

## 6.2. The Effect of Cycloheximide on Polyadenylated RNA Synthesis in Infected Cells

Cells were infected in the presence of cycloheximide so that the synthesis of viral RNA could be studied in the absence of viral protein synthesis. In order to isolate viral mRNA from polysomes of cycloheximide treated cells the procedure of Rakusanova et al. (1971) was followed: cycloheximide was removed from the cells so that viral mRNA could move on to the polysomes as protein synthesis was restarted.

Polyadenylated RNA was isolated from heavy and light polysomes and also from the pre-polysome pellet. The polyadenylated RNA, isolated from these fractions, was then analysed by PAGE. Differences were most striking between RNA extracted from the pre-polysome pellets in treated and untreated cells. When cells were infected in the presence of cycloheximide two RNA species (A & B in fig 6.2) present in the pre-polysome pellet of untreated infected cells were no longer found in that fraction (compare e and f in fig 6.2). The same differences also seemed to occur in the RNA extracted from heavy polysomes in the presence and absence of the inhibitor, but this result is less clear due to the lower levels of radioactivity found in this fraction.

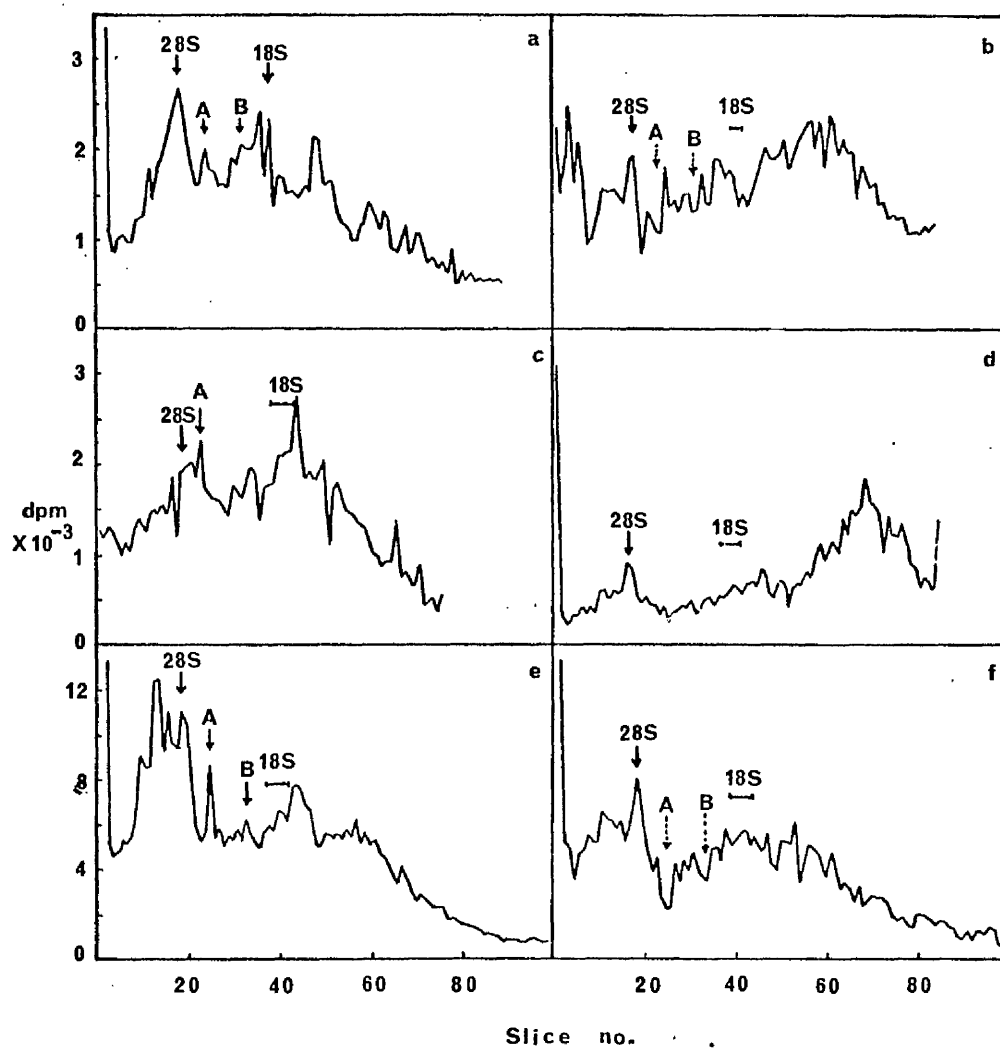
Fig 6.2.

Synthesis of Polyadenylated Messenger RNA in PrV  
Infected Cells in the Presence and Absence of  
Cycloheximide.

HeLa cells were infected in the presence or absence of 50 µg/ml cycloheximide and incubated with 2.5 mCi [5,6-<sup>3</sup>H] uridine from 1 - 5 h post infection. The medium, containing uridine with or without cycloheximide, was removed and the cells were washed twice with 20 ml fresh medium and then incubated for a further 20 min in fresh medium before harvest. After harvesting cytoplasmic extracts were prepared and polysomes were then isolated. RNA was extracted from the pre-polysome pellet, light and heavy polysomes.

a) - cyclohex	b) + cyclohex	- heavy polysome RNA
c) "	d) "	- light polysome RNA
e) "	f) "	- pre-polysome pellet
		RNA

(In order to facilitate comparison between gel profiles a transparent copy of this diagram is enclosed at the end of the thesis, inside the back board).





Two possible explanations of these differences were thought to be likely:

- (i) The RNA species (designated A & B) may not be synthesised properly and so fail to reach the cytoplasm.
- (ii) A and B may be present in the cytoplasm but may fail to associate with polysomes after the removal of cycloheximide.

### 6.3. Effect of Cycloheximide on Polyadenylated RNA in the Post-Polysome Supernatant

In order to distinguish between the possible explanations, outlined above, for the non-appearance of A and B in polysomes in cycloheximide treated infected cells, polyadenylated RNA was prepared from the post-polysome supernatant. This subcellular fraction contained all cytoplasmic structures which sedimented less rapidly than light polysomes. So if A and B were present in the cytoplasm but not found in the pre-polysome supernatant, heavy or light polysomes they will be present in the post-polysome supernatant. Fig 6.3 shows that this is not the case so A and B are not present in the cytoplasm of cells which have been infected in the presence of cycloheximide.

Having identified two RNA species which seem to require prior viral protein synthesis before they can appear in the polysomes it then seemed likely that these RNA species would not be synthesised very early in infection. It was decided to test this prediction.

### 6.4. Polysomal RNA synthesis at Three Times after Infection

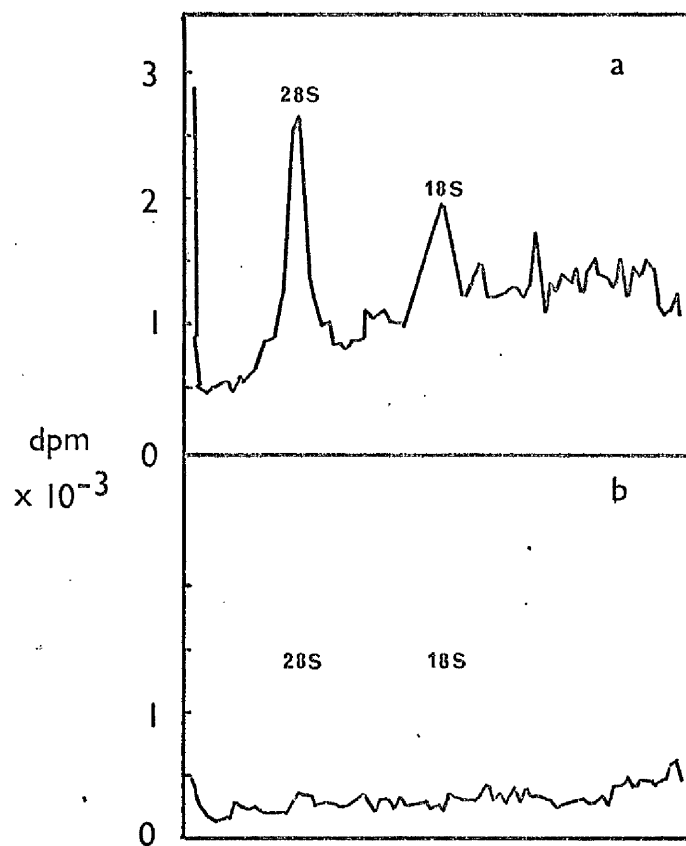
Figure 6.4 shows the PAGE profile of polyadenylated RNA

Fig 6.3.

Effect of Cycloheximide on Polyadenylate RNA  
in the Post-Polysome Supernatant

$10^8$  HeLa cells were infected in the presence or absence of 50  $\mu\text{g/ml}$  cycloheximide and incubated with 2.5 mCi  $[5,6\text{-}^3\text{H}]$  uridine from 1 - 5 h post infection. The cycloheximide was removed by washing the cells twice with 20 ml fresh medium and the cells were incubated for a further 20 min before harvest. After harvesting, cytoplasmic extracts were prepared and polysomes were separated from more slowly sedimenting material on sucrose gradients. The post-polysome supernatant RNA was extracted with phenol/chloroform and this RNA was then subjected to poly-(U) sepharose chromatography. The polyadenylated RNA was then analysed by PAGE.

- a) no cycloheximide
- b) cycloheximide treated.



isolated from the pre-polysome pellet fraction from cells at 3 times after infection. In cells labelled from  $\frac{1}{2}$  h -  $1\frac{1}{2}$  h post-infection neither A nor B appears to be present in significant amounts, by  $1\frac{1}{2}$  - 3 h post infection a small amount of A seems to be appearing but B still seems to be absent. However between  $3\frac{1}{2}$  and 5 h post infection both A and B seem to be synthesised in insignificant quantities. These differences were also noted in the heavy polysomal RNA fractions but were less easily followed as the amount of radioactivity incorporated was lower than in the pre-polysome pellet RNA.

#### 6.5. Discussion

The results obtained in these experiments are consistent with the model proposed for control of herpesvirus RNA synthesis advanced by Rakusanova et al. (1971) and by Honess and Roizman (1974). Some viral RNA species are thought only to be transcribed after prior viral protein synthesis has occurred. Molecular hybridisation studies have shown this requirement for viral protein synthesis, before the virus genome is fully expressed, only in terms of RNA classes and not individual species of RNA. Studies on the control of viral protein synthesis (Honess & Roizman, 1973, 1974, 1975; Powell & Courtney, 1975) have only given circumstantial evidence on the control of individual species of mRNA. It is possible to conclude when a mRNA species for a particular protein is active in promoting <sup>translation</sup> ~~mRNA~~, but this does not show <sup>when</sup> ~~that~~ the mRNA is present or absent in the cytoplasm. For this reason it is very interesting to show that at least two unidentified RNA species seem to require prior viral protein synthesis before they appear in the cytoplasm. However I feel that the cycloheximide studies should not be

Fig 6.4.

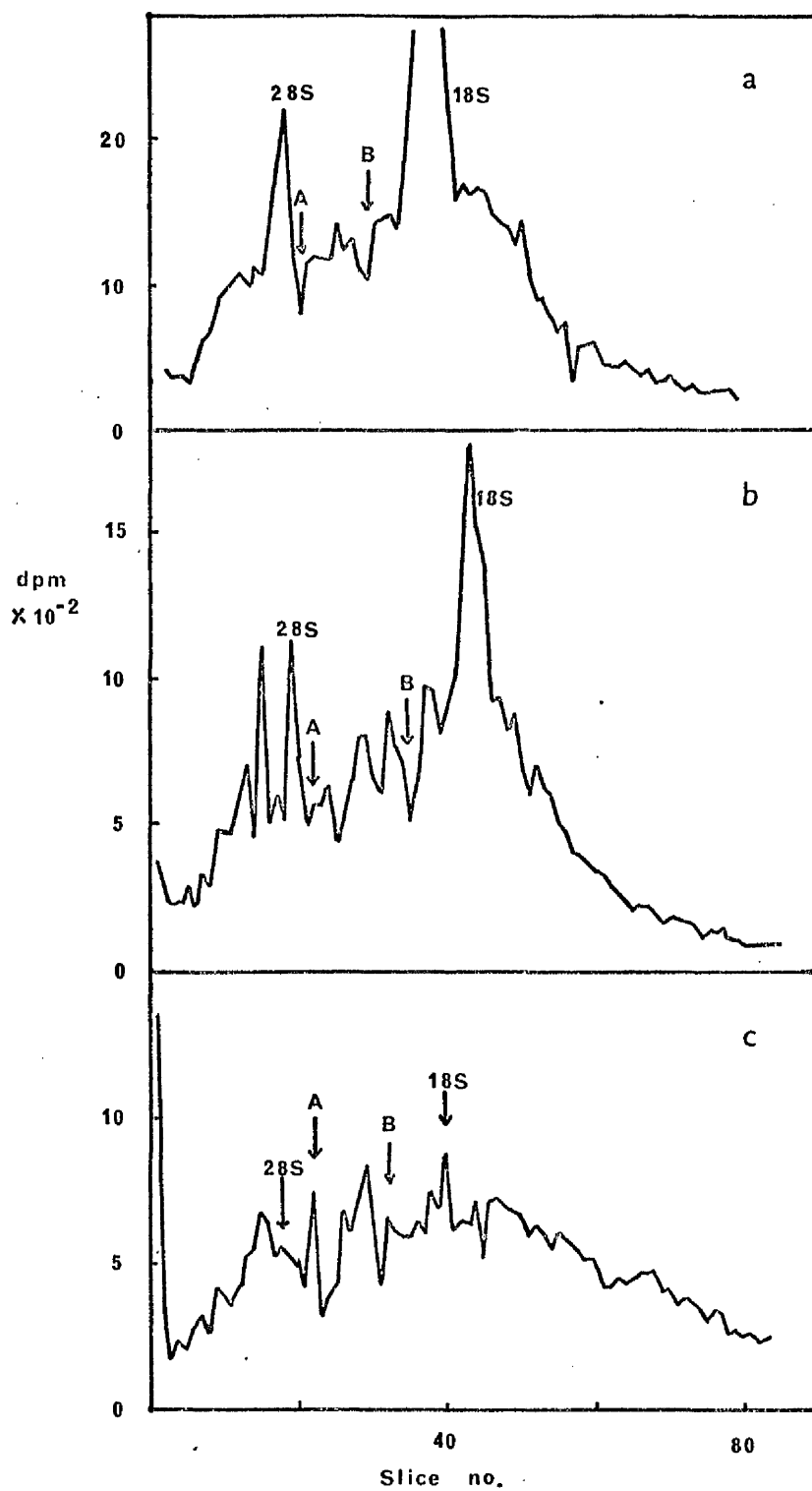
Polysomal RNA Synthesis at Three Times

After Infection

Three bottles of  $10^8$  HeLa cells were infected with PrV (20 pfu/cell) and incubated in the presence of 1.25 mCi [5,6- $^3\text{H}$ ] Uridine for the times stated below. After harvest polysomes were prepared from cytoplasmic extracts and polyadenylated RNA was isolated from whole polysomal RNA by poly-(U) sepharose chromatography. The poly-(A) containing RNA was analysed by PAGE.

Times of labelling:

- a)  $\frac{1}{2}$  -  $1\frac{1}{2}$  h post-infection
- b)  $1\frac{1}{2}$  - 3 h       "       "
- c)  $3\frac{1}{2}$  - 5 h       "       "



interpreted to give any information on the temporal control of virus RNA synthesis as it has been noted by Jean et al. (1974) that this inhibitor causes irreversible inhibition of the production of pseudorabies virus.

The cycloheximide studies may not be interpreted as the result of restricted transcription of viral RNA. When RNA is extracted from unfractionated HEp-2 cells infected with HSV in the presence of cycloheximide, viral RNA synthesis does not appear to be restricted (Frenkel et al., 1972). However in the same system the appearance of viral RNA in the cytoplasm is restricted (Kozak & Roizman, 1974). Control of the synthesis of viral polysomal RNA therefore may not occur at the level of transcription but may be exercised during post-transcriptional processing. Therefore the study of the appearance of RNA in the cytoplasm may have alternative interpretations than demonstrating transcriptional control.

## Chapter 7

### Conclusions and General Discussion

Previous studies of mRNA synthesis in pseudorabies virus infected cells have utilized molecular hybridisation methods (see Jean et al., 1974; Ben-Porat et al., 1974). Hybridisation was not used in this project, so the results presented here must partly be interpreted in the light of the conclusions drawn from other hybridisation experiments. The virus strain used in this project was grown from the same original stock as that used by Rakusanova et al. (1971) and Ben-Porat et al. (1971). This fact along with similar observed changes in polysome profiles during infection in this project and in the experiments of Ben-Porat et al. (1971) suggests that the course of infection is likely to be substantially the same in both cases. Therefore it was thought that these other studies with PrV infected cells could be used to help design experiments in this project and also assist in interpreting the results obtained here.

### Polysome Studies

Ben-Porat et al. (1971) studied polysomes in infected cells and showed that three effects were taking place:

- (i) polysomes were larger in infected cells
- (ii) cellular polysomes disaggregated during infection
- (iii) there was a switch from cellular to viral protein synthesis indicated by hybridisation data and also by examination of the amino acid composition of nascent polypeptides (see chapter 2).



Polysomes were also found to increase in size in this project and to disaggregate during infection.

Actinomycin D was found to increase the extent of disaggregation of polysomes in infected cells. Willems and Penman (1966) found that actinomycin D also increased the disaggregation of polysomes normally found during poliovirus infection of HeLa cells. This effect was later attributed to the inhibition of the synthesis of a labile cellular RNA species with a translational control function (Leibowitz & Penman, 1971). The inhibition of the synthesis of this cellular RNA species could be one mechanism whereby pseudorabies decreases the affinity of ribosomes for cellular mRNA.

Cordycepin was also found to increase polysome disaggregation in infected cells, however, this effect only occurred if virus RNA synthesis had first been allowed to occur. Cordycepin might be acting in two ways to bring about this effect:

- (i) it may be inhibiting synthesis of the translational control RNA species as discussed earlier in this section
- (ii) it may be preventing the synthesis of viral mRNA, so that only the small amount of viral messenger synthesised before the addition of cordycepin is still able to associate with ribosomes after cellular polysomes have been disaggregated.

The specificity of the inhibition of RNA synthesis by cordycepin will be discussed later in this chapter. However at this point it appears likely that cordycepin is acting in both the ways outlined above as it causes a similar disaggregation of polysomes in

uninfected cells to that caused by actinomycin D (see section 2.5) and it inhibited both rRNA and polyadenylated RNA synthesis (see section 4.3). Translational control RNA species have been reported more recently by Bester et al. (1975) and these molecules could provide a means whereby the protein synthesis machinery could distinguish between cellular and viral mRNA. If the cellular RNA control molecules bind to specific sequences which are present on cellular RNA but not viral RNA and the virus has similar virus specific control molecules then it is possible to see how the cellular mRNA might be lost from polysomes, if the RNA species required for initiation of protein synthesis is no longer synthesised in infected cells.

#### Polyadenylated RNA

The similarities between the virus strains and polysome changes during infection found in this work and in Kaplan's laboratory (Ben-Porat et al., 1971), to which I have already referred, suggest that their conclusions should also be valid for the system used here. Their results have been used to help design several experiments presented in this thesis. Ben-Porat et al. (1971) reported that cellular mRNA was not present in isolated polysomes although still found in the whole cytoplasm, it was therefore decided to isolate mRNA from polysomes and not from the whole cytoplasm. Rakusanova et al. (1972) reported that at least some pseudorabies virus mRNA was polyadenylated. This may be found in many herpesviruses and also holds for herpes simplex (Bachenheimer & Roizman, 1972; Silverstein et al., 1973). It was therefore decided to purify polyadenylated RNA from the polysomal RNA by some means. The intention was to characterise this polyadenylated RNA by PAGE and by in vitro protein synthesis.

The first problem was to find a suitable method for isolating polyadenylated RNA: the first method tried was the differential phenol extraction suggested by Brawerman et al. (1972). This method seemed to result in the appearance of a heterogeneous group of low molecular weight RNA in the polyadenylated fraction, which might have been due to the nucleolytic degradation reported by Perry et al. (1972) to be a drawback to this extraction process. Oligo-(dT) cellulose chromatography was then tried but was found to have two drawbacks under the conditions used:

- (i) rRNA bound to the oligo-(dT) cellulose
- (ii) the putative polyadenylated species were weakly bound.

The net result was that supposed mRNA was only present in low yields and then was contaminated with rRNA to a considerable extent. Poly-(U) sepharose was finally found to bind certain minor RNA species efficiently but to have little affinity for rRNA, so the supposed poly-(A) containing RNA was substantially purified by this method.

Polyacrylamide gel electrophoresis (Loening, 1969) was used to analyse the RNA which had been fractionated by poly-(U) sepharose chromatography. rRNA species were still detected in both unbound and bound RNA fractions on polysepharose. This was useful, for the rRNA species could be used for internal standards for PAGE, so that gels were more easily compared than they might have been if no rRNA had been present along with the polyadenylated RNA species. Although the analysis of RNA by PAGE cannot be used to distinguish between cellular and viral RNA, more detailed information on the sizes of mRNA species can be obtained by this technique than by molecular hybridisation methods.

The information obtained from hybridisation experiments with mixed populations of RNA is usually difficult to interpret so as to throw any light on the behaviour of a single messenger, although the appearance and disappearance of classes of RNA can often be deduced. It would be an important contribution to studies of control of herpes-virus mRNA synthesis if the conclusions drawn from hybridisation data could be substantiated by a method which monitored individual species of RNA. This was the rationale behind the decision to characterise mRNA in pseudorabies infected cells by PAGE. Two criticisms can be raised to this approach:

- (i) how is polyadenylated RNA identified as mRNA?
- (ii) how are species of RNA identified as being of viral or cellular origin?

To reply to these criticisms several experiments were performed. Polyadenylated RNA was prepared from whole polysomes but the RNA was also shown to be associated with polysomes and not with co-sedimenting ribonucleoprotein particles by dissociating polysomes with EDTA and extracting the RNA from structures that sedimented much more slowly than polysomes. In this manner the ribonucleoprotein particles were separated from the EDTA sensitive polysomal structures. The species of RNA from infected cells which normally migrated between 18S and 28S rRNA species were found to be associated with polysomes by this method (see section 4.2).

Polyadenylated RNA was isolated by poly-(U) sepharose chromatography and was found to stimulate incorporation of labelled amino acids into proteins in a cell-free wheat germ preparation. This

further supported the contention that the polyadenylated RNA isolated from polysomes contained mRNA species.

Identification of RNA species as being of viral origin is difficult without resorting to molecular hybridisation techniques. The use of conventional hybridisation methods involves the isolation of RNA much more highly labelled than the RNA used in this work. Alternatively larger amounts of individual species could be hybridised to highly labelled viral DNA but increasing the scale of the experiments also brings problems. In this work RNA was assigned a viral origin if it was not found in uninfected cells but appeared during the course of infection; this criterion was used by Honess and Roizman (1973) to class proteins as virus induced or of cellular origin. Perhaps in the future individual RNA species could be isolated by preparative PAGE and shown to code for viral proteins in a cell free system. However, it may prove difficult to isolate large enough quantities of mRNA to overcome the inevitable losses in the purification process.

It was thought to be a possibility that the minor RNA species, detected on polyacrylamide gels, might be derived in some way from rRNA. Two types of experiments were performed to find if this was the case or not. Cordycepin (3' deoxyadenosine) was reported by Penman et al. (1970) to specifically inhibit post-transcriptional adenylation of HnRNA while leaving HnRNA synthesis relatively unaffected. The result of this was that transport of mRNA into the cytoplasm is severely restricted (Adesnik et al., 1972). More recently however it has been demonstrated in vitro that the polyadenylation enzyme is inhibited to a lesser degree by cordycepin than some other enzymes involved in RNA

synthesis (Maale et al., 1975). In the cordycepin inhibition studies, presented in this thesis, rRNA was shown to be very sensitive to the inhibitor so it was not possible to draw any conclusions from these experiments as to the origin of the minor species of RNA. It had been hoped that rRNA would be resistant to the action of cordycepin, while polyadenylation would be prevented so that polyadenylated species would not appear although rRNA synthesis would still carry on. This hope was not realised and both types of RNA were strongly inhibited (see section 4.3).

It was then decided to find if rRNA synthesis could be selectively inhibited by actinomycin D. It was found that rRNA was only synthesised at a very much reduced rate in presence of actinomycin D while the minor species were inhibited to a much lesser extent (see section 4.4). This is good evidence that the minor RNA species are not derived in any way from rRNA.

Having decided that PAGE analysis of RNA, as carried out in this project, was a valid method of studying mRNA it seemed to be sensible to find if individual mRNAs were restricted to polysomes of a particular size. This was attempted but the mRNA profiles proved to be very heterogeneous and no individual peaks could be identified going from one gel to another. However the size distribution of mRNAs extracted from different polysome fractions proved to be very similar and this seemed an interesting observation. One explanation for this phenomenon is that each individual mRNA is translated with a particular frequency of initiation. If a mRNA gives rise to a very high initiation rate for polypeptide synthesis then it would be predicted

from this hypothesis that this mRNA would be associated with a larger number of ribosomes than a mRNA giving a low initiation rate. Nuss et al. (1975) have suggested that initiation of polypeptide synthesis is usually the rate limiting process in protein synthesis in HeLa cells and that some viruses such as poliovirus have mRNAs which give rise to a higher frequency of initiation than most cellular mRNAs. Several observations in this work suggest that the same mechanism is found in PrV infected HeLa cells. Polysomes in infected cells are larger than those in mock-infected cells (see section 2.2) but the mRNA size distribution is very similar in both situations (see section 5.2). This indicates that a larger number of ribosomes are found on mRNA of similar size in infected cells than in mock-infected cells. Cycloheximide, an inhibitor of eukaryotic polypeptide elongation, causes elongation and not initiation to be the rate limiting process in protein synthesis (Fukuhara, 1965; Clarke-Walker & Linnane, 1966). In this project it was found that when cycloheximide was present in low concentrations only small mRNA species were associated with small polysomes (see section 5.3) the larger mRNAs becoming uniquely associated with large polysomes. This is thought to be due to a build up of ribosomes on mRNA so that the length of the mRNA and not the frequency of initiation determined the number of ribosomes found associated with a messenger RNA species.

The size of nascent polypeptides associated with polysomes was investigated and a higher proportion of small molecular weight polypeptides was found in infected cell heavy polysomes than in the corresponding fraction from mock-infected cells (see section 5.4). This is consistent with the theory that the increase in polysome size,

observed in infected cells, is due to an increased frequency of initiation rather than a higher proportion of large molecular weight mRNAs in infected cells.

#### Control of mRNA Synthesis

Rakusanova et al. (1971) reported that when PrV infected cells are treated with cycloheximide, so that viral protein synthesis was abolished, a restricted synthesis of viral polysomal RNA occurs. Kozak and Roizman (1974) found that this phenomenon also occurs in herpes simplex infected cells. These observations have formed the basis for a theory of control of viral mRNA synthesis. Kaplan (1973) suggests that there are three types of pseudorabies virus mRNA. The first type he terms immediate-early RNA and the synthesis of this class of RNA is postulated to occur in the absence of any prior viral protein synthesis. Thus it would be expected that this class of mRNA would be synthesised in cycloheximide treated cells. The two other types of viral RNA are termed early- and late-RNA and they are defined by a requirement for prior viral protein synthesis before they can be synthesised. The late-RNA class would only be synthesised after viral DNA synthesis had begun.

Honess and Roizman (1974, 1975), working with herpes simplex virus, have followed the synthesis of virus-induced proteins following the removal of cycloheximide from cells infected in the presence of the inhibitor. They have found that proteins can be divided into three classes,  $\alpha$ ,  $\beta$  and  $\gamma$ .  $\alpha$  is first to appear and is replaced by  $\beta$  synthesis which is finally in turn replaced by  $\gamma$  synthesis. The control of the synthesis of these proteins is thought to depend on the appearance of



the proteins themselves. The  $\alpha$  proteins are thought to switch-on the synthesis of  $\beta$  mRNA; the  $\beta$  proteins are believed to have a dual role; they switch off the synthesis of  $\alpha$  proteins either at the level of mRNA synthesis or by affecting the translation of  $\alpha$  mRNA. The  $\beta$  proteins are also thought to initiate the synthesis of  $\gamma$  mRNA.  $\gamma$  proteins in turn are believed to switch off the synthesis of  $\beta$  proteins.

The control of pseudorabies virus mRNA synthesis has been investigated by molecular hybridisation (Rakusanova et al., 1971) so the results do not give any information on single species of mRNA. The herpes simplex situation had, in addition to molecular hybridisation, also been studied by monitoring the synthesis of individual proteins analysed by PAGE (Honess & Roizman, 1973, 1974, 1975; Kozak and Roizman, 1974). However none of the studies, reported at this time, have examined the synthesis of individual RNA species and the effect of cycloheximide on them.

In this project polyadenylated RNA was extracted from cells infected in the presence and absence of cycloheximide and this RNA was analysed by PAGE. Rakusanova et al. (1971) found that in cycloheximide treated PrV infected cells virus RNA sequences, present in the cytoplasm, only became associated with polysomes after the inhibitor had been removed, allowing protein synthesis to restart. This process was accompanied by a loss of cellular mRNA sequences from the polysomes. This was the basis of the methodology used in the cycloheximide studies in this project.

RNA was labelled in the presence of cycloheximide but before the cells were harvested the inhibitor was removed so that the viral mRNA

could subsequently be extracted from polysomes. When the polysomal RNA was analysed by PAGE, two species migrating between 18S and 28S rRNA were found to be absent from cycloheximide treated cells although they were present in significant amounts in untreated cells at the same time after infection (see section 6.2). The same species of RNA were found to be synthesised only after 3-4 hours post infection in the absence of cycloheximide (see section 6.3). They therefore represent likely candidates for early or late RNA and this finding adds support to the theory of Kaplan (1973) for the control of viral mRNA synthesis.

A further implication of the lack of polyadenylated RNA in the post-polysome supernatant in cycloheximide treated infected cells is that the species of RNA normally found in this fraction have become associated with polysomes. These species might be mRNAs which have a very low efficiency of initiation of polypeptide synthesis. When a small amount of cycloheximide is present movement of ribosomes along mRNA is so slowed down that there is a build up of ribosomes even on mRNA which give a low frequency of polypeptide chain initiation and so these species become associated with the polysome fraction.

The results presented in this thesis reinforce the complex picture of control of herpesvirus replication that is gradually beginning to emerge. It is slowly becoming evident that regulation of viral protein synthesis is likely to be very subtle and intricate. Different mRNAs are probably not only translated with different efficiencies but the efficiency of translation of a single mRNA is likely to be modified at various stages of infection. It is obvious that this study is still at a very preliminary stage and that many

refinements in techniques will have to be introduced before this type of approach can really reveal some of the more complex aspects of control. However, this project represents an attempt to begin to characterise single pseudorabies mRNA species. Some future work that could be built on the techniques acquired during this work are discussed in the next section.

### Future Work

Nuss et al. (1975) have suggested that some viral mRNAs give a very high frequency of initiation compared to most cellular mRNAs. These workers have found that by incubating cells in higher than normal concentrations of sodium chloride the initiation of protein synthesis is specifically inhibited. If pseudorabies virus does synthesise mRNAs which promote a high frequency of initiation of polypeptide synthesis then, according to the theory of Nuss et al. (1975), protein synthesis in infected cells should be much less inhibited than in mock-infected cells. This work should be able to be carried out in a fairly short time.

The polypeptide products of the cell free wheat germ translation system with added RNA from infected cells could be characterised by serological methods. This has been attempted but so far antibodies for single purified viral proteins have not been successfully prepared in this laboratory.

In a longer time scale the following experiments might be carried out:

- (i) Individual mRNA species, purified by preparative PAGE, could be hybridised to viral DNA and the hybrids examined by electron microscopy. It might prove to be feasible to map the gene positions for particular mRNAs by using restriction endonucleases to provide reproducible DNA fragments and using these fragments for the molecular hybridisation studies. It would be interesting, for example, to find if mRNAs, which are controlled together, are also present in the same region of the genome.

- (ii) The mechanism of virus induced disaggregation of cellular polysomes might also be amenable to further investigation. If this phenomenon is due to the virus inhibiting the synthesis of a labile cellular RNA (see Leibowitz & Perman, 1971), this species should be able to be characterised and the kinetics of its disappearance compared to the disaggregation of polysomes.

## Materials

### 1. Virus

Pig herpesvirus 1 (pseudorabies virus, PrV) was originally derived from a stock preparation (Kaplan & Vatter, 1959) and has subsequently been plaque-purified three times. The virus stock is prepared by growth in baby hamster kidney cells (BHK cells).

### 2. Tissue Culture Cells

A continuous line of baby hamster kidney fibroblasts was used for growing virus for all stock preparations. This cell line was isolated by Macpherson and Stoker (1962) and was designated BHK/21 (Cl3), it has since been adapted to grow in the absence of tryptose phosphate (Cl3A).

HeLa cells - monolayer adapted human epithelium cells, derived from a cervical carcinoma (Gey et al., 1952) were used throughout in the study of the infective process of PrV.

### 3. Radiochemicals

All isotopically labelled compounds were obtained from The Radiochemical Centre, Amersham, Bucks.

### 4. Chemicals for Liquid Scintillation Counting

The chemicals for liquid scintillation counting were obtained as follows:

2,5 diphenyloxazole (PPO)	}	Koch Light Laboratories Ltd.,
Toluene		Colnbrook, Bucks.

p-Bis(o-methylstyryl)benzene	Eastman Biochemicals,
(Bis MSB)	Kodak Co.
Triton X100	Rohm & Haas (U.K.) Ltd.,
	Croydon, CR9 3NB.
Hydrogen peroxide (100 vol)	BDH, Poole.

#### 5. Polyacrylamide gel materials

Acrylamide, NN'methylenebisacrylamide and TEMED (NNN'N' tetramethylethylenediamine) were obtained from Koch-Light Laboratories Ltd. Ammonium persulphate was obtained from BDH.

#### 6. Column Chromatography Materials

Poly (U) sepharose 4B was obtained from Pharmacia, Upsala, Sweden; formamide from B.D.H. and N-Lauroyl-Sarcosine from Sigma Chemical Company, London.

Oligo d-T cellulose from Collaborative Research Inc., Waltham, Mass.

#### 7. Other Materials

All other chemicals were 'Analar' or its equivalent and were purchased mainly from B.D.H. or Sigma. In particular:

Cordycepin (3' deoxyadenosine)	Sigma
Cycloheximide	Sigma
Deoxyribonuclease 1 from	
Bovine Pancreas, Electrophoretically	
Purified, free of ribonuclease	Sigma
Actinomycin D	Calbiochem, La Jolla,
	California.

8. Media and Solutions

## (i) Eagles Minimal Essential Medium (MEM) (Modified)

A modification of Eagle's medium (Busby, House and Macdonald, 1964) was used; this contained:

<u>Amino-Acids</u>	<u>mg/l</u>	<u>Vitamins</u>	<u>mg/l</u>
L-arginine	126.4	D-calcium pantothenate	2.0
L-cystine	24.0	Choline chloride	2.0
L-glutamine	292.0	Folic acid	2.0
L-histidine HCl	41.9	Inositol	4.0
L-isoleucine	52.5	Nicotinamide	2.0
L-leucine	52.5	Pyridoxal-HCl	2.0
L-lysine HCl	73.1	Riboflavin	0.2
L-methionine	14.9	Thiamine HCl	2.0
L-phenylalanine	33.0		
L-threonine	47.6		
L-tryptophan	10.2		
L-tyrosine	36.2		
L-valine	46.8		

<u>Inorganic Salts and Other Components</u>	<u>mg/l</u>
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	393
KCl	400
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	200
NaCl	6800
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	140
$\text{NaHCO}_3$	2200



Inorganic Salts and Other Components (cont.) mg/l

Phenol Red	17.0
D-glucose	4500
Penicillin	$10^5$ units
Streptomycin	$10^5$ $\mu$ g/ml

(ii) EC 10

90% modified Eagles MEM + 10% calf serum (v/v), from Flow Laboratories.

(iii) Trypsin/Citrate

0.25% (w/v) trypsin, 10.5 mM NaCl, 1.0 mM sodium citrate and 0.002% phenol red; adjusted to pH 7.8 with NaOH.

(iv) Versene

0.6 mM diaminoethanetetraacetic acid, disodium salt (EDTA), 0.17 M NaCl, 3.4 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 2 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4.

(v) Trypsin/Versene

Trypsin/citrate 1 part, Versene 4 parts (v/v).

(vi) LETS Buffer

0.1 M LiCl, 0.01 M EDTA, 0.01 M Tris-HCl, 0.2% SDS, pH 7.4.

15% and 30% Sucrose solutions (w/v) in LETS were prepared; diethyl pyrocarbonate was added to all solutions ( $\leq 0.01\%$ ), and allowed to stand overnight at 20°C before autoclaving at 5 lbs/in<sup>2</sup> for 50 min.

(vii) Reticulocyte Standard Buffer (RSB)

0.01 M NaCl, 0.003 M  $\text{MgCl}_2$ , 0.01 M Tris/HCl, pH 7.4.

15% and 30% Sucrose solutions (w/v) in RSB were prepared; diethyl pyrocarbonate was added to all solutions ( $\leq 0.01\%$ ) and allowed to stand overnight at 20°C before autoclaving at 5 lbs/in<sup>2</sup> for 50 min.

(viii) TESSEN

0.01 M TES (N-tris [Hydroxymethyl] methyl 2 aminoethane sulphonic acid), 0.01 M EDTA, 0.001 M NaCl, pH 7.4.

15% and 30% Sucrose solutions (w/v) were prepared, treated with diethyl pyrocarbonate and autoclaved as with RSB solutions.

(ix) Solutions for Electrophoresis of RNA(a) Electrode Buffer (Loening, 1969)

36 mM Tris, 30 mM  $\text{NaH}_2\text{PO}_4$ , 1 mM  $\text{Na}_2\text{EDTA}$ , 0.2% (w/v) SDS, pH to 7.7 with glacial acetic acid.

(b) Gel Solution

36 mM Tris, 30 mM  $\text{NaH}_2\text{PO}_4$ , (pH 7.7), 1 mM  $\text{Na}_2\text{EDTA}$ , 0.2% (w/v) SDS, 2.6% (w/v) acrylamide, 0.13% (w/v) bis-acrylamide, 0.18% (v/v) TEMED, 0.21% (w/v) ammonium persulphate.

(x) Solutions for Electrophoresis of Proteins(a) Electrode Buffer

0.025 M Tris-HCl, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.5.

(b) Main Gel Solution

0.375 M Tris-HCl, 0.1% (w/v) SDS, 0.03% (v/v) TEMED, 0.035% (w/v) ammonium persulphate, 7% (w/v) acrylamide, 0.18% (w/v) bis-acrylamide.

(c) Stacker Gel Solution

0.125 M Tris-HCl, 0.1% (w/v) SDS, 0.03% (v/v) TEMED, 0.07% (w/v) ammonium persulphate, 3% (w/v) acrylamide, 0.08% bis-acrylamide.

(xi) Eluting Buffer

10 mM  $\text{K}_2\text{HPO}_4$ , 10 mM  $\text{Na}_2\text{EDTA}$ , 0.2% (w/v) SDS in 90% formamide, pH 7.5.

The formamide was deionised by stirring with Bio-Rad Mixed-Bed Ion Exchange Resin AG 501-X8, 20 - 50 mesh.

(xii) Concentrated Salt Buffer (CSB)

0.7 M NaCl, 50 mM Tris-HCl, 10 mM Na<sub>2</sub> EDTA in 25% formamide,  
pH 7.5.

The formamide was deionised, as above.

(xiii) Starting Buffer

0.1 M Tris-HCl, 0.5 M KCl, pH 7.5.

(xiv) Elution Buffer

0.01 M Tris-HCl, pH 7.5.

(xv) pH 7.6 RNA Extraction Buffer

150 mM Tris-HCl, 50 mM KCl, 1 mM MgCl<sub>2</sub>, 0.5% (w/v) SDS,  
pH 7.5.

(xvi) pH 9.0 RNA Extraction Buffer

0.1 M Tris-HCl, 0.5% (w/v) SDS.

(xvii) Extract Buffer

20 mM Hepes, 100 mM KCl, 1 mM magnesium acetate, 2 mM CaCl<sub>2</sub>,  
6 mM mercaptoethanol; adjust the pH with KOH to 7.6 and autoclave,  
then add the mercaptoethanol.

(xviii) Column Buffer

20 mM Hepes, 120 mM KCl, 5 mM magnesium acetate, 6 mM  
mercaptoethanol; adjust the pH to 7.6 with KOH and autoclave before  
adding the mercaptoethanol.

(xix) Energy Mix (20X final concentration in assay mix)

0.06 g ATP, 0.001 g GTP, neutralise these then add 0.2 g  
creatin phosphate and adjust to pH 7.6 with KOH. Dilute to 5 ml and  
freeze in small aliquots.

(xx) Amino acid mixture (400  $\mu$ M = 20X final assay concentration)

The amino acids were glutamine, asparagine, glutamic acid, methionine, glycine, cysteine, aspartic acid, histidine, arginine, lysine, tyrosine, tryptophan, serine, valine, threonine, alanine, isoleucine, proline and phenylalanine. Heat to dissolve, adjust pH to 7.6 with KOH, filter through a 0.45 micron HA millipore filter and freeze in small aliquots.

(xxi) Salt Mixture (50X the final assay concentration)

2 ml of 1 M Hepes pH 7.6 + 0.03 g KCl + 8 mg spermidine.  
Freeze in small aliquots.

(xxii) Dithiothreitol

0.12 M DTT, bubble  $N_2$  through for 10 min and freeze in small aliquots.

(xxiii) Creatin kinase

10 mg/ml in 80% glycerol.

(100  $\mu$ l of 80% glycerol was added to 1 mg creatin kinase).

(xxiv) [ $^3$ H] leucine; 1 mCi/ml; specific activity 54 Ci/mmol.(xxv) [5,6- $^3$ H] Uridine; 1 mCi/ml; specific activity 43 Ci/mmol.(xxvi) ATP mix (made up fresh each time just before use).

Energy mix 25  $\mu$ l, salt mix 10  $\mu$ l, amino-acids 25  $\mu$ l, DTT 10  $\mu$ l, creatin kinase 5  $\mu$ l,  $^3$ H leucine 10 - 20  $\mu$ l,  $H_2O$  add to 100  $\mu$ l total volume.

(xxvii) Assay Mix

ATP mix 10  $\mu$ l, wheat germ 20  $\mu$ l, RNA or  $H_2O$  20  $\mu$ l.

(xxviii) Sodium acetate

- a) 2 M sodium acetate
- b) 0.01 M sodium acetate

Both solutions were made approximately 0.01% with diethyl pyrocarbonate, allowed to stand overnight and then autoclaved at 15 lb/in<sup>2</sup> for 20 minutes.

(xxix) Triton-Toluene Scintillant

650 ml Toluene, 350 ml Triton X-100, 5 g PPO, 0.5 g Bis MSB.

(xxx) Toluene-PPO Scintillant

0.5% (w/v) PPO in Toluene.

## Methods

### I Cell Culture Techniques

#### (i) Propagation of cells

HeLa and BHK/21 (C13A) were routinely cultured as monolayers in 80 oz Winchester bottles (burlers) according to the method of House and Wildy (1965). The burlers were gassed to give an atmosphere of 5% CO<sub>2</sub>/95% air. 0.02% phenol red was used to indicate acidity.

For serial passaging, the cells were removed from the glass by treatment with trypsin/versene, suspended in EC10, counted and dispensed into sterile burlers containing 180 ml of EC10, in aliquots of 18 - 22 x 10<sup>6</sup> cells. Cells were not used beyond a passage number of 15 (Shedden & Wildy, 1966). These cultures were maintained by the staff of the Wellcome Cell Culture Unit of this department.

#### (ii) Contamination Checks

All sterile media, and passaged cells, were checked regularly for bacterial, fungal or PPLO infection as follows:

Bacterial Contamination: aliquots were placed on blood agar plates and brain-heart infusion broth at 37°C. Results were considered to be negative if no growth was seen within 7 days.

Fungal Contamination: a small volume of the sample to be tested was added to Sabouraud's medium and incubated at 32°C. No growth in 7 days was assumed to indicate the absence of fungal contamination.

PPL0 infection: agar plates were seeded with passaged cells by piercing the agar surface with a charged pasteur pipette. The plates were grown in an atmosphere of 5% CO<sub>2</sub> in N<sub>2</sub> at 37°C.

Infected cultures resulted in the occurrence of the characteristic 'fried-egg' appearance of PPL0 colonies on examination of the plates under the microscope. These cultures were discarded.

(iii) Propagation of Virus

Monolayer cultures of BHK/21 (Cl3A) cells (about 10<sup>8</sup> cells per burler) maintained in EC10 were infected at a multiplicity of 1 plaque-forming unit (pfu) per 300 cells in 20 ml medium. Virus was allowed to adsorb for 1 hour, then the inoculum was removed and replaced with 100 ml EC10. The cultures were rolled at 37°C for 36 hours, then harvested aseptically by shaking the bottle to dislodge cells into the medium, transferring to centrifuge bottles and spinning at 600 g for 10 minutes. This pellets the cells, the supernatant was then spun at 15,000 g for 2 hours to pellet the virus. The pelleted supernatant virus was resuspended in EC10 (1 ml for every burler used), and gently sonicated to make the suspension uniform. Aliquots were stored at -70°C and thawed only once before use.

Cell associated virus, prepared by resuspending and sonicating the cell pellets, gives a low titre and was used only for production of virus stocks.

Plaque-assays were carried out on monolayer cultures of BHK/21 cells and normally gave titres of 10<sup>9</sup> - 10<sup>11</sup> pfu/ml.

(iv) Plaque assay for PrV

Confluent monolayers of BHK/21 cells in 50 mm Petri dishes, which had been seeded at  $4 \times 10^6$  cells/dish 18 hours previously, were infected with serial dilutions of PrV in 0.2 ml EC10. After 1 hour adsorption at  $37^\circ\text{C}$ , the excess was poured off, 4 ml EC10 was added and incubation continued. After a further hour, 50  $\mu\text{g/ml}$  heparin was added to the medium to prevent vertical transmission of the virus. 28 hours after infection incubation was arrested, the cell sheet was washed with PBS and fixed with 3 ml formol-saline per dish (30 min at room temperature). Plates were then stained with 0.5 ml Giemsa stain/dish for 1 hour at room temperature, excess stain was washed off very gently with  $\text{H}_2\text{O}$  and plaques were counted under a low power microscope.

(v) Growth of HeLa Cells for Experimental Use

Cells were normally seeded at  $20 \times 10^6$  per burler and incubated at  $37^\circ\text{C}$  for 2 days in 5%  $\text{CO}_2$  until the cell-density had reached approximately  $60 - 80 \times 10^6$ . HeLa cells are still growing exponentially at this cell density. Cells grown in Roux bottles were seeded at  $5 \times 10^6$  per bottle to give an equivalent cell density to that obtained with cells grown in burlers.

(vi) Infection of Cells

Cells were normally infected at 20 pfu/cell in 20 ml slightly acid medium as adsorption is more efficient at lower pH's. After 1 hour, the excess was decanted, and 25 ml of fresh medium added. If amino acids were being used to label with the medium was deficient in the



corresponding amino acids. The radio-active isotope was added at the appropriate time and, at the end of the labelling period, the cells were harvested.

(vii) Harvesting of Cells

a. Mechanical: the growth medium was poured off and the bottle rotated in an ice bath. 10 ml ice-cold EC10 was then pipetted into the burler and the cells removed from the glass by means of a rubber scraper, and pelleted by centrifugation at 800 g for 5 mins at 0°C. The pellet was resuspended in 2.5 ml RSB and the cells allowed to swell for 5 mins before being disrupted by gentle homogenisation (3 strokes in a loose fitting teflon/glass homogeniser tube). The nuclei and large cell debris were pelleted at 2,200 g for 5 mins. The cytoplasmic extract was used immediately.

b. Trypsin/Versene: where cells were to be reseeded they were removed enzymically from the glass. The cell sheet was washed twice with trypsin/versene, the second lot of trypsin/versene was poured off as the monolayer became opaque. When cells slid off the glass, 10 ml warm EC10 was added and the cells resuspended by gentle pipetting.

II Biochemical Techniques

(i) Isolation of Polysomes

Cytoplasmic extracts of cells were prepared (as detailed in I(vii)a) and layered onto 35 ml 15 - 30% sucrose (w/v) gradient in RSB. This was then centrifuged in a SW27 rotor at 27,000 rpm for 110 min at 0°C. A needle was carefully placed onto the bottom of the tube, from the top,

and the gradient was pumped through a Gilford 2000 recording spectrophotometer. The absorbance was continuously monitored at 260 nm and the gradient was divided into fractions. Fractions containing polysomes were pelleted by centrifuging in a SW27 rotor at 20,000 rpm for 12 h at 0°C.

(ii) Extraction of RNA

Polysomes were suspended in LETS buffer (to a maximum concentration resulting in an absorbance at 260 nm of between 3 and 4) and an equal volume of a 1:1 mixture of phenol and chloroform (w/w) was added. The organic and aqueous phases were well mixed by repeated pipetting at 25°C for 15 min. The phases were separated by centrifugation at 2000 rpm for 10 min at 8°C in an MSE Major swing out centrifuge. The aqueous phase (on top of the organic phase) was carefully removed (and retained), and the extraction procedure repeated until no more protein could be seen at the interface. The RNA was precipitated by addition of  $2\frac{1}{2}$  - 3 volumes of ethanol and standing overnight at -20°C.

(iii) Isolation of Poly-(A) containing RNA

a) Poly-(U) Sepharose Chromatography

Poly-(U) sepharose was suspended in 1 M NaCl for 5 min, washed twice with 0.1 M NaCl (100 ml/g of dry powder) and poured into a column. The column was then washed with EB (100 ml/g of poly-(U) sepharose), and finally was equilibrated with CSB.

RNA precipitates were first dissolved in the minimum volume of LETS; this was diluted with 5 volumes of CSB and the sample applied to the column. The unbound RNA was washed off the column with 2 bed

volumes of CSB and this fraction was retained. The column was further washed with 10 bed volumes of CSB and this wash was discarded. The poly-(A) containing RNA was eluted from the column with 2 bed volumes of EB. RNA in both the unbound and bound fractions was precipitated by adding  $2\frac{1}{2}$  - 3 volumes ethanol and standing overnight at  $-20^{\circ}\text{C}$ .

(iii) b) Oligo-(dT) Cellulose Chromatography

Oligo-(dT) cellulose was suspended in Starting Buffer (see Materials 8.xiii) and poured into columns (approx. 0.1 g dry wt. of powder/column). RNA was dissolved in the same buffer and applied to the column, the unbound material was removed by further washing with the starting buffer. Bound-RNA was eluted by washing with Elution Buffer (8.xiv).

(iii) c) Differential Phenol Extraction

Polysomes were suspended in 4 ml of pH 7.6 RNA Extraction Buffer at  $0 - 4^{\circ}\text{C}$  and an equal volume of water-saturated phenol was added. The mixture was shaken for 5 min, centrifuged at 12,000 g for 10 min, and the aqueous phase removed. 4 ml of 0.1 M Tris (pH 7.6) was added to the non-aqueous residue, including the interface and the extraction procedure was repeated. These two aqueous samples were pooled. The non-aqueous residue was re-extracted successively with pH 9.0 RNA Extraction Buffer and 0.1 M Tris (pH 9.0) and the two alkaline extracts were combined.

(iv) Polyacrylamide Gel Electrophoresis of RNA

RNA was dissolved in 50  $\mu\text{l}$  Electrode Buffer (see 8.ix.a) and bromophenol blue and glycerol were added to final concentrations of

approximately 0.005% and 20% respectively. The samples, so prepared were applied to the top of 100 mm long, 6 mm diameter disc gels which had been polymerised on top of a disc of glass-fibre paper (Whatman GFC) which was held in place by a constriction at the bottom of the tube. Electrophoresis was carried out at a constant current of 5 ma/tube, until the bromophenol blue had travelled to within 15 mm of the bottom of the tube. The gels were removed from the tubes by inverting and gently pumping air into the constricted end, using a syringe. The gels were scanned for absorbance at 260 nm in a Gilford 2000 Gel Scanner Recording Spectrophotometer. For radioactivity determinations the gels were frozen on Drikold and cut longitudinally into 1 mm slices using a Mickle Gel Slicer.

(v) Polyacrylamide Gel Electrophoresis of Proteins

The gels were comprised of 20 mm of stacker gel on top of 80 mm of main gel in 6 mm diameter perspex tubes. Immediately prior to electrophoresis the proteins were denatured and dissolved by the addition of concentrated reagents to yield final concentrations of 0.05 M Tris-HCl (pH 7.0), 2% (w/v) SDS, 5% (v/v)  $\beta$ -mercaptoethanol, and 0.005% bromophenol blue, followed by boiling for 2 min. Samples were between 50 and 200  $\mu$ l and were subjected to electrophoresis at a constant current of 3 to 4 ma per gel cylinder. Electrophoresis was monitored by following the migration of the bromophenol blue. The gels were sliced in the same way as for the gels used to separate RNA.

(vi) Sucrose Gradient Analysis of RNA

12 ml (SW40) and 4.2 ml (SW65) 15% to 30% sucrose gradients in

LETS buffer were used to separate RNA species. RNA was dissolved in LETS and carefully layered onto the preformed gradients which were subsequently centrifuged at 20°C. Conditions of centrifugation were varied so that different sizes of RNA could be examined, for example, in the SW40 after centrifugation for 10½ h at 40,000 rpm 28S RNA was near the bottom of the gradient. 12 ml gradients were harvested in the same manner as was used for polysome gradients.

(vii) Sucrose Gradient Analysis of Ribonucleoprotein Particles  
Resulting from EDTA treatment of Polysomes

The methodology was identical to that employed for RNA except that TESEN buffer was used instead of LETS. The SW40 was also used for this technique, centrifugation at 30,000 rpm/24 h resulted in large ribosomal subunits being near the foot of the gradient.

(viii) Wheat Germ Cell-free Protein Synthesis  
 a) Preparation of Wheat Germ Extract

6 g wheat germ + 6 g of acid washed (2 N HCl) and baked sand + 28 ml of cold extract buffer (8.xvii) were ground with a mortar and pestle (on ice) for 8 min until the sand was a fine powder. This was centrifuged at 12,000 g for 10 min and the supernatant fraction was removed (avoiding the layer of fat). To the supernatant fraction was added 180 µl of 0.1 M ATP (pH 7.6), 90 µl 0.0436 M GTP, 450 µl of 0.3 M creatin phosphate (pH 7.6), 250 µl of 0.12 M dithiothreitol, and 70 µl of creatin kinase (10 mg/ml in 80% glycerol). After 10 - 15 min at 30°C this preincubated mixture was placed onto a Sephadex G-25 column (52 x 2 cm) equilibrated with column buffer (8.xviii). The column had previously

been baked at 200°C, and the G-25 (wet) and all tubing was autoclaved. The most turbid fractions were collected and pooled and then frozen in small aliquots at -80°C.

b) Incubation

The assay mix, containing added RNA or water was incubated at 30°C for 30 min. 10 µl aliquots were removed for determination of radioactivity incorporated into protein. The aliquots are put on paper discs and these are placed in cold 10% TCA,  $10^{-5}$  M leucine, then washed for 10 min in 5% TCA (cold) then in 5% TCA at 90°C. The filters were dried in ethanol:diethyl ether (1:1 (v/v) ) and then in ether. The filters were then dried in air and then under a heat lamp for 5 min prior to counting in toluene-PP0 (Efficiency 5 - 6%).

(ix) Determination of Radioactivity

a) Polyacrylamide Gels

Gel slices were placed in 5 ml plastic insert vials (Sterlin) and dried overnight at 60°C, 0.2 ml hydrogen peroxide was then added and the gels were incubated at 37°C for a further 8 - 12 h. 2 ml of triton-toluene scintillant was then added to each vial. The radioactivity was determined in a Phillips Scintillation Spectrometer. Efficiency of counting for [ $^3\text{H}$ ] was approximately 30%.

b) Sucrose Gradients

Aliquots were added to 10 volumes of triton-toluene scintillant and counted as above. Efficiency of counting for [ $^3\text{H}$ ] was approximately 10%.

c) Assays

Dried Whatman No. 1 discs containing the [ $^3\text{H}$ ] labelled sample were placed in Toluene-PPO scintillant and counted with an efficiency of between 5 and 6%.

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