Characterisation of *In Vitro* Translation Conditions for Messenger RNA Prepared from Pseudorabies Virus Infected Cells

A thesis submitted to the University of Glasgow in candidature for the degree of Doctor of Philosophy in the Faculty of Science

by

Bernadette M. McGrath, B.Sc.

Department of Biochemistry,

University of Glasgow

November, 1978.

ACKNOWLEDGEMENTS

I would like to thank Professor R.M.S. Smellie and Professor A.R. Williamson for making the facilities of the Biochemistry Department available for this research. I am very grateful to the Association of Commonwealth Universities for a Commonwealth Scholarship.

It is my pleasure to thank my supervisor Dr. W.S. Stevely for his guidance, encouragement and much useful discussion throughout the course of the experimental work and during the preparation of this thesis.

I would like also to thank the following: Mrs. H.H. Singer for advice and discussion on the wheat germ *in vitro* translation system; Dr. S.A. Laidlaw for the use of his messenger dependent reticulocyte lysate; Dr. D.P. Leader for advice on the Krebs II ascites *in vitro* translation system and Mr. M. McGarvey for providing ascites cells; Mrs. E. Blakely, for preparation of antisera; Mr. D. Mease for maintaining virus stocks; the staff of the Wellcome Cell Culture Unit for providing cell stocks and finally the staff, students of labs. A16, A3, A7 and C30 for much help and encouragement.

I am also very grateful to Miss N. Golder for typing this manuscript.

ABBREVIATIONS

These are as laid down in the Biochemical Journal Instructions to Authors (revised 1978) with the following additions:-

PrV	pseudorabies virus
HSV	herpes simplex virus
EBV	Epstein Barr virus
MDV	Marek's disease virus
EHV	Equine herpes virus
VSV	Vesicular stomatitis virus
EMC virus	Encephalomyocarditis virus
pfu	plaque forming unit
moi	multiplicity of infection
PPLO	pleuropneumonia like organism
IgG	immunoglobulin G
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene-glycol-bis (2-aminoethylether)-N,N'tetraacetic acid
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
DMSO	dimethyl sulphoxide
PPO	2,5 diphenyloxazole
DATD	N,N'diallyltartardiamide
TEMED	N,N,N'N' tetramethylethylenediamine
HEPES	N'-2-ethanesulphonic acid
DTT	dithiothreitol
PEG	polyethylene glycol
ICNV	International Committee for the Nomenclature of Viruses
ICTV	International Committee for the Toxonomy of Viruses

ds	double stranded
MDL	messenger dependant lysate
GAR	goat-antirabbit serum
HART	hybrid arrested translation
ara C	cytosine arabinoside

SUMMARY

Poly-(A) containing RNA was isolated by affinity chromatography from HeLa cells 5h after infection with Pig Herpesvirus I (Pseudorabies virus, PrV) or mock-infection. 54% of the RNA from infected cells was shown by molecular hybridisation to be complementary to virus specific sequences. Both species of poly-(A) containing RNA stimulated incorporation of radioactive amino acids into TCA precipitable material in a wheat germ cell free translation system. It was concluded, therefore, that these RNAs contained significant amounts of mRNA.

The in vitro translation products were examined by polyacrylamide gel electrophoresis and fluorography. Preliminary classification of the in vitro products of infected cell mRNA as viral or cellular coded was carried out by comparison of their electrophoretic mobilities with those of the in vitro products of mock-infected cell mRNA. The mobilities of the in vitro products were also compared with those of the polypeptides present in infected and mock-infected cell lysates and, with a few exceptions, in vivo labelled polypeptides which comigrated with those synthesised in vitro could be identified. Further evidence for the viral origin of eight infected cell mRNA products was obtained by immune-precipitation of in vitro products with antisera to the major capsid protein of PrV and by examining the products synthesised in vitro when infected cell mRNA had been hybridised to PrV DNA prior to addition to the translation system.

The mRNAs were also translated in a mRNA dependent reticulocyte lysate and a similar spectrum of products was obtained. This cell free system, however, was more efficient in the synthesis of high molecular weight polypeptides. Translation of the mRNAs in a Krebs II ascites cell free system was also investigated.

The/

The *in vitro* translation systems were used to examine the applicability of the model for virus-induced shut-off of host cell protein synthesis proposed by Carrasco (1977). This hypothesis attributes the virus-induced shut-off to an increase in monovalent cation concentration in infected cells. In the wheat germ system the optimum conditions for translation of infected and mock-infected cell mRNA were similar whereas reticulocyte mRNA and encephalomyocarditis virus RNA showed slightly different requirements. Investigation of the optimum $\{K^+\}$ for translation in the Krebs II ascites system also suggested that translation conditions for infected and mock-infected cell mRNA were similar. Hence it was concluded that changes in the intracellular monovalent cation concentration are unlikely to have a role in the PrV-induced shut-off of host cell protein synthesis.

Possible differences in the initiation rates for viral and cellular mRNA were also investigated *in vivo*. Protein synthesis in cells grown in hypertonic medium was compared with synthesis under isotonic conditions both early (2h) and late (6h) after PrV-infection or mock-infection. After infection with a number of other viruses protein synthesis has been found to be less susceptible to a hypertonic initiation block than synthesis in uninfected cells and the resistant polypeptides have been shown to be virus coded (Nuss *et al.*, 1975). PrV infection did not result in any resistance to this initiation block and it was concluded that PrV mRNA is not more readily translated than HeLa cell mRNA under limiting conditions. Hence it did not seem likely that the PrV-induced inhibition of cellular protein synthesis could be due to an overall inhibition of protein synthesis because under such conditions PrV mRNA translation would be inhibited also.

The increase in average polysome size in PrV-infected cells which has/

has been reported by Ben-Porat et al., (1971) and which has also been observed in this laboratory could be due to an increase in the average length of the translated region of infected cell mRNAs. This was investigated by allowing completion in vitro of the nascent polypeptide chains on different size classes of polysomes isolated from cells late after infection or mock-infection. The results showed that such a model could not account for the increased loading phenomenon.

Thus no differences between PrV mRNA and HeLa cell mRNA which might account for the changes in protein synthesis which occur in PrV-infected HeLa cells could be detected.

CONTENTS

CHAPTER 1

Introduction

1.1. General Introduction.

Most mammalian cells possess the genetic information to direct synthesis of more proteins than are ever synthesised by any one normal cell. This implies a control of protein synthesis which by the nature of the eucaryotic cell may act at several sites. Control of transcription is the basis of regulation but because of the physical separation of the transcriptional and translational processes and the longer half-life of mRNA molecules in eucaryotic cells other controls are possible. Primary transcripts differ from functional mRNA molecules and therefore processing as well as translocation from the nucleus to the cytoplasm is necessary. Both these processes are likely to be regulated. In the cytoplasm translation of the mRNA may be regulated in a number of ways: mRNA may be sequestered in non-translatable complexes; it may fail to initiate or once initiated elongation may be inhibited. In a cell synthesising a large number of mRNA molecules and proteins these controls are difficult to study.

Virus-infected cells in which the synthesis of host proteins is inhibited and a more limited number of proteins are synthesised provide an important experimental system. The mechanism of inhibition of host protein synthesis is unknown and cannot be explained by disappearance of host mRNA. The protein synthesising apparatus discriminates between host and viral mRNA. The availability of cell free protein synthesising systems has made it possible to study this discrimination and other changes in translation in infected cells in isolation from transcriptional and other controls.

Elucidation of the regulatory mechanisms in virus-infected cells should/

should provide an insight into the controls operating in uninfected cells.

1.2. Aim of this project.

The aim of this project was to characterise and compare the *in vitro* translation of Pig Herpesvirus 1 (pseudorabies virus, PrV) mRNA and HeLa cell mRNA with a view to gaining some understanding of the translational control mechanisms in infected cells. Where applicable, these studies were extended by *in vivo* experiments.

1.3. The Nature of Viruses.

Viruses are infectious potentially pathogenic entities which are totally dependent on living cells for propagation. They possess no system for energy production or protein synthesis and utilise cellular machinery to direct synthesis of specialised particles which transfer the genome to other cells (Lwoff, 1957; Luria and Darnell, 1968).

The mature extracellular virus particle is termed the virion. The viral genome, which can be either DNA or RNA, is enclosed in a protein coat (the capsid). In complex virions the capsid may in turn be enclosed in a lipoprotein membrane called the envelope (Caspar *et al.*, 1962).

1.4. The Herpesviruses.

1.4.1. Classification.

Attempts to define a taxonomic system for viruses have been fraught with controversy generated by the difficulties inherent in integrating the ideasof scientists working on similar viruses isolated from hosts from disparate taxonomic groups. The first approach of the International Committee/

Committee of Nomenclature of Viruses (ICNV) was to set up four specialist subcommittees to investigate the taxonomy of vertebrate, plant and invertebrate viruses and bacteriophage (Wildy, 1971). Further classification is based on the following criteria:

(i) Intrinsic properties of the virus including type of nucleic acid, symmetry of nucleocapsid, presence or absence of envelope and number of capsomeres (Tournier and Lwoff, 1966).

(ii) Clinical features of the disease.

The division of viruses according to host species has now been shown to be inappropriate because viruses with similar intrinsic properties have been found in more than one phylum (Fenner, 1976).

The herpesvirus group was recognised as a genus in 1970 (Wildy, 1971). In 1975 the International Committee for Taxonomy of Viruses (ICTV, formerly the ICNV) raised the status of the genus to that of a family (Herpetoviridae) and defined it as viruses with the following characteristics:

"Virion consists of a capsid 120-150mm in diameter surrounded by a lipid-containing evelope. Buoyant density (CsCl) of virion 1.27 - 1.29g/cm^3. Capsid icosadeltahedral with 162 partially hollow capsomeres. Buoyant density (CsCl) of capsid 1.305g/cm^3. Capsid surrounds a core which consists of DNA wrapped around a protein spool. About 33 protein species in virion with molecular weights up to 290,000.

Genome consists of linear DNA with both terminal reiterations and internal repetition of terminal sequences, G+C content 33-74%, molecular weight 92 - 102 x 10^6.

Viral multiplication begins in the nucleus and is completed by the addition/

addition of a glycoprotein-membrane as the virus passes through the inner lamella of the nuclear membrane into the endoplasmic reticulum. Margination of chromatin and intranuclear inclusion bodies are characteristic." (Fenner, 1976).

Representatives of the Herpetoviridae are shown in Table 1.4.1.

The ICTV approved the definition of one genus to be known as Herpesvirus. At present the genus includes four species; human herpesviruses 1 & 2 and cercopithecid herpesviruses 1 & 3. The extent of this genus was determined by comparative serology. It has been suggested that serological cross-neutralisation may prove to be a useful criterion for systematic taxonomy of other viruses (Honess and Watson, 1977).

In 1971 the ICNV appointed a Herpesvirus Study Group to make recommendations on the nomenclature of the viruses. Thus, the following provisional system of labelling was proposed.

(i) The label for each herpesvirus would be an anglicised 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.

(Herpesvirus Study Group, 1973).

These proposals, however, have not been consistently applied and are not widely adopted (Honess and Watson, 1977). Hence, the trivial names shown in Table 1.4.1. are frequently used.

TABLE 1.4.1. (Adapted from Herpesvirus Study Group, 1973).

A list of some of the herpesvirus

Recommended Name	Trivial Name (with common abbreviation)
Human herpesvirus 1	Herpes simplex virus type 1 (HSV-1)
2	Herpes simplex virus type 2 (HSV-2)
3	Varicella-zoster virus
4	Epstein-Barr virus (EBV)
5	Cytomegalovirus (CMV)
Cercopithecid herpesvirus 1	B virus
2	SA6
3	SA8
Equid herpesvirus 1	Equine abortion virus (EAV)
Pig herpesvirus 1	Pseudorabies virus (PrV)
2	Pig cytomegalovirus
Phasianid herpesvirus 1	Infectious laryngotracheitis virus
2	Marek's disease virus (MDV)
Cebid herpesvirus 2	Herpesvirus saimairi (HVS)

1.4.2. The Herpesvirion.

Roizman & Furlong (1974) described four major architectural components in the virion. The innermost, the core, is surrounded by three concentric structures, the capsid, the tegument and the envelope. The virion contains at least 33 polypeptides, DNA, lipid and polyamine.

1.4.2.1. The Core.

In thin section of virions the core appears as an electron dense ring of 25 - 35nm in diameter surrounding an electron translucent centre or as an electron dense bar (Furlong et al., 1972). Two lines of evidence suggest that the core contains the DNA. Firstly, the structure is sensitive to DNAase but not to RNAase or proteolytic enzymes (Epstein, 1962; Zambernard & Vatter, 1966; Chopra et al., 1970). Secondly, use of a technique which selectively removes uranyl ions bound to DNA in stained preparations showed that the electron dense area contains DNA and led to the conclusion that the DNA was in the form of a toroid surrounding a protein plug (Furlong et al., 1972). This structure has been confirmed in virions of several herpesviruses (Nazerian, 1974; Perdue et al., 1976).

In addition to DNA and protein, it appears likely that the spermine found in capsid preparations is located in the core (Gibson & Roizman, 1971; Roizman, 1978).

1.4.2.2. The Capsid.

The capsid has been extensively studied by electron microscopy. In thin section it appears as a moderately electron dense structure separated from the core by an electron translucent shell. The outer diameter/

diameter of the capsid has been reported to range from 85 - 110nm (Roizman and Furlong, 1974). It is not clear whether this variability is artefactual or reflects inherent differences among the herpesviruses.

The morphological subunits of the capsid, the capsomeres, are arranged to show 2- 3- and 5-fold symmetry. Consideration of the number of capsomeres along the side of the triangular faces and the axis of symmetry leads to the conclusion that there are 162 capsomeres arranged in the form of an icosadeltahedron. Capsomeres are hexagonal in shape and appear to be 12.5nm long. The end projecting outside the capsid has a diameter of 8.0 - 9.0nm and a hole 4nm in diameter runs through the axis of the capsomere (Wildy et al., 1960). In the intact capsid the hole appears to be blocked at the proximal end (Roizman & Furlong, 1974).

Evidence suggests that the electron translucent region between the core and the capsid, the pericore, is not empty. The core remains in the centre of the capsid even after prolonged centrifugation and pelleting (Gibson & Roizman, 1972). In capsids treated with NP-40 for 30 minutes the core loses its shape and can be seen lying next to the capsid which does not appear to be morphologically altered (Abodeely et al., 1970). The nature of the material in the pericore is unknown and it is possible that it is an extension of the protein plug on which the DNA is wound. Smid et al., (1977) have described a structure in freeze fracture preparations which might be a pericore.

1.4.2.3. The Tegument.

Roizman & Furlong (1974) define the structure located between the capsid and the envelope as the tegument. Evidence for such a structure has been recognised by numerous workers (for references see Roizman

&/

& Furlong, 1974). Comparison of the polypeptides present in virus treated with detergent to remove the envelope with purified capsid proteins showed the existence of polypeptides external to the capsid (Gibson & Roizman, 1972; 1974). These polypeptides have been assigned to the tegument (Roizman & Furlong, 1974).

1.4.2.4. The Envelope.

The outermost structure of the virus is the envelope which consists of a trilaminar membrane with spikes projecting from its outer surface (Fong et al., 1973; Wildy et al., 1960). It is composed of glycoproteins, glycolipids and lipids (Morgan et al., 1954), and evidence suggests that it is derived from host cell membrane (Epstein & Holt, 1963; Watson & Wildy, 1963). However, the virus envelope appears to be more fragile than the cellular membrane.

1.4.3. Pseudorabies Virus.

Pseudorabies Virus is one of the most widely studies herpesviruses and has typical physical characteristics of the family. The ICTV recognised it as a possible member of the genus Herpesvirus (Fenner, 1976); however serological studies suggest that it is not closely related to other members of the genus (Killington et al., 1977; Honess and Watson, 1977).

PrV DNA has a molecular weight of 90 x 10^6 - 95 x 10^6 (Stevely, 1977) and a G+C content of 72mol% (Rubenstein & Kaplan, 1975). Twenty polypeptides have so far been described in purified virions (Stevely, 1975).

McKercher (1973) has reviewed the manifestations of PrV infection in/

in vivo. Pseudorabies was among the earliest of the viral diseases recognised (Aujeszky,1902). It occurs most frequently in swine, cattle, sheep, dogs and cats and to a limited extent in rats and mice (Gustafson, 1970).

The virus causes two types of infection. In the pig, which is the natural host, the infection is latent in the adult but severe in the newborn. Mortality ranges from 100% in the newborn to virtually nil in the adult (Gustafson, 1970). In cattle and most other susceptible species, it is manifested as a rapidly fatal systemic disease characterised by signs referable to the central nervous system. The name "pseudorabies" comes from the clinical similarity to rabies.

Naturally occurring PrV infection is acquired by droplet infection or ingestion (Gustafson, 1970). Virus multiplication in the respiratory tract of swine facilitates its spread within the body since leukcocytes attracted to the infected area pick up virus and carry it to various body organs, in paricular the placenta, from whence it invades the foetus and causes abortion. Transmission of the virus along nerve tracts and hence to the medulla has been shown to occur in rabbits (Hurst, 1934).

PrV has a wide cell culture host range, replicating in rabbit, dog, and monkey kidney, in HeLa cells, mouse fibroblasts, and in chicken embryos (Kaplan, 1969). In cell monolayers the virus produces either syncytium formation or granulation and clumping of cells with eventual lysis.

1.5. Replication of Herpesviruses in Permissive Cells.

Herpesviruses can cause three types of infection. The most widely/

widely studied is productive infection where virus multiplication takes place in permissive cells. Herpesviruses may also infect cells latently giving rise to no gross effect or oncogenically causing cellular transformation. This section will be concerned mainly with productive infection.

Virus multiplication can be divided into several phases. The first event is attachment of the viral particle to the cell. This is followed by penetration. After penetration the virus is rapidly uncoated and for several hours no infectious virus can be isolated from the cell (eclipse phase). During the eclipse, viral macromolecular synthesis takes place. Finally, infectious viral particles are assembled and released from the cell (Watson, 1973b).

The duration of the reproductive cycle varies from virus to virus and is dependent on the host cell, the multiplicity of infection (moi), the temperature of incubation and the nutritional properties of the medium (Roizman, 1978). At 37°C and 50 pfu/cell, the cycle of HSV-1 in HEp-2 cells lasts 17 hours (Roizman & Furlong (1974). The cycle of PrV in BHK 21 cells infected at 40 pfu/cell is somewhat shorter being about 12 hours (Tyler et al., 1973).

1.5.1. Attachment.

Little is known of the receptors on the cell surface to which herpesviruses adsorb. Adsorption is volume and cation dependent (Roizman, 1978), and varies for different viruses (Darlington & Granoff, 1973). Attachment occurs at 4°C (Gostling and Bedson, 1956) but penetration requires warming (Holmes & Watson, 1963). Enveloped particles are preferentially adsorbed.

1.5.2. Penetration and Uncoding.

Most of the information on the mode of penetration comes from electron microscopy and such studies must be cautiously interpreted (Watson, 1973b; Roizman & Furlong, 1974). Two mechanisms are currently proposed. One suggests that the virus enters by pinocytosis and the second that entry is brought about by fusion of the viral envelope with the plasma membrane (see Watson, 1973b). Both proposals provide for release of the capsid from the envelope into the cytoplasm. The mechanism of release of DNA from the capsid is not known. Soon after infection, however, viral DNA can be detected in the nucleus (Hochberg & Becker, 1968) and synthesis of virus specific macromolecules commences.

1.5.3. Viral RNA Synthesis.

The first synthetic event in replication is synthesis of viral RNA. Viral RNA is defined as RNA complementary to viral DNA. Viral mRNA refers to viral RNA sequences isolated from polysomes.

1.5.3.1. Site of Transcription and Enzymes Transcribing Viral DNA.

Viral RNA is transcribed from viral DNA in the nucleus (WAgner and Roizman, 1969a,b; Roizman et al., 1970). An α-amanitin-sensitive RNA polymerase similar to RNA polymerase II is used to transcribe viral DNA (Alwine et al., 1974; Kemp et al., 1976; Ben Zeev et al, 1976; Ben-Zeev and Becker, 1977; Costanzo et al., 1977). There is no evidence for synthesis of a viral induced polymerase activity (Lowe, 1978), but some studies suggest that the activity is altered (Saxton and Stevens, 1972; Kemp et al., 1976; Preston and Newton, 1976) and viral-induced modification of the host enzyme/

enzyme has not been ruled out.

1.5.3.2. Physical Characteristics of Viral RNA.

The primary transcripts are larger than cytoplasmic viral RNA and processing similar to that of cellular mRNA occurs. Viral RNA is cleaved to yield message sized transcripts (Roizman et al., 1970; Wagner & Roizman, 1969a,b). Polyadenylic acid sequences (poly-(A)) are covalently linked to cytoplasmic viral RNA (Rakusanova et al., 1972; Backenheimer & Roizman, 1972). The poly-(A) is added post-transcriptionally in the nucleus (Bachenheimer & Roizman, 1972) and cytoplasmic poly-(A) containing RNA represents most if not all viral RNA sequences present in the cytoplasm (Silverstein et al., 1976). Viral RNA lacking poly-(A) tracts has been found on polysomes but appears to share the same sequences as poly-(A) containing RNA. However, a significant fraction of the polysome associated viral RNA lacking poly-(A) is larger than the corresponding viral poly-(A) containing RNA (Stringer et al., 1977).

Viral mRNA contains a 5' terminal methylated oligonucleotide of the form commonly found in eucaryotic mRNAs (Bartkoski & Roizman, 1976, 1978; Moss et al., 1977). Early viral transcripts contain internal methylated nucleotides of the form 6methyl adenosine but internal methylation of late viral transcripts is inhibited (Bartkoski & Roizman, 1976, 1978).

1.5.3.3. Characterisation of the Viral Transcripts Present at Different Stages of Infection.

The programme of transcription of HSV-1 DNA has been examined by two groups.

Roizman and coworkers have found that in HSV-1-infected HEp-2 cells/

cells viral transcripts complementary to at least 40% of the DNA are present at all stages of infection. The transcripts present early in infection or in cells in which viral protein synthesis is inhibited by cycloheximide do not differ from those found late in infection. The concentration of transcripts is always lower in the cytoplasm than in the nucleus and the nucleus contains transcripts not present in the cytoplasm. RNA complementary to 50% of the DNA is synthesised in cells treated with cycloheximide but the cytoplasmic transcripts are complementary to only 10% of the DNA. On release of the cycloheximide block, new RNA synthesis is required before more transcripts appear in the cytoplasm thus suggesting that non-functional transcripts are synthesised. This has led to the conclusion that translocation of transcripts is regulated in such a way that only functional transcripts are transported (Frenkeland Roizman, 1972; Kozak and Roizman, 1974; Roizman et al., 1974).

Nuclear viral RNA contains complementary sequences transcribed from at least 30% of the genome (Jaquemont and Roizman, 1975). There is some evidence that this fraction of self-annealing RNA accounts for the fraction of nuclear RNA sequences that is not translocated to the cytoplasm (Kozak and Roizman, 1975).

In contrast, Wagner's group, using a similar technique, have described restricted transcription of HSV-1 in the presence of inhibitors of DNA and protein synthesis and suggest that protein synthesis is required for transcription of some regions of the DNA (Swanstrom et al., 1975). In agreement with these results Clements et al., (1977) have shown that RNA transcribed in HSV-1-infected cells treated with cycloheximide hybridised to restricted portions of the genome. Similarly, Rakusanova et al., (1972) define an immediate-early/

early class of viral RNA which is synthesised in the PrV infected rabbit kidney (RK) cells in the presence of cycloheximide. Jean et al., (1974) claim to have shown that immediate-early RNA is complementary to only 25% of the DNA by techniques with comparable sensitivity to those used by Frenkel et al., (1973).

Roizman's group, however, found that transcription in HSV-2-infected cells in the absence of cycloheximide is restricted to 21% of genome early in infection (Frenkel et al., 1973). Hence, it is possible that different transcriptional controls operate for different viruses. However the differences described for HSV-1 are at present unresolved.

1.5.4. Viral Protein Synthesis.

1.5.4.1. Characteristics of Infected Cell Protein Synthesis.

Following synthesis and processing of viral RNA, viral protein synthesis commences in the cytoplasm. The overall pattern of protein synthesis depends on the host-virus system and the moi. In some cases there is an initial decline in the uptake of amino acids and the number of polysomes followed by a recovery as viral protein synthesis increases (Roizman et al., 1965; Sydiskis and Roizman, 1966) while in other cells there is a smooth switch from host to viral protein synthesis with no measurable decline (Hamada and Kaplan, 1965; Kaplan, 1973). Late in infection the rate of protein synthesis decreases and does not recover.

The switch to viral protein synthesis is reflected in the polysome size. Infection of cells with HSV and PrV results in an increased proportion of rapidly sedimenting polysomes and a decrease in the polysome to monosome ratio. This disaggregation of polysome increases as infection proceeds (Sydiskis and Roizman, 1966, 1967; Ben Porat

et al., 1971). The increase in polysome size correlates with the shift to viral protein synthesis. Kaplan et al., (1970) showed that proteins synthesised by PrV-infected RK cells have a lower lysine to leucine ratio than the proteins synthesised by uninfected cells. It was then demonstrated that nascent polypeptides on large polysomes had the characteristic viral amino acid content. Hybridisation of polysomal RNA to viral and cellular DNA showed the predominance of viral sequences in large polysomes (Ben-Porat et al., 1971).

1.5.4.2. Criteria for Classification of Infected Cell Polypeptides as Viral.

Difficulties in classification of herpesvirus infected cell polypeptides have arisen because of the large coding potential and complexity of the herpesviruses and the incomplete shut-off of host protein synthesis. Hence, several criteria have been used.

Honess and Roizman (1973) classified infected cell proteins as virus-specific if they met one or more of the following criteria:

(i) stimulation in the rate of synthesis post-infection.

(ii) variations in properties of the protein as a function of the virus strain infecting the cell.

(iii) immune precipitation of infected cell proteins by antisera reactive solely with virus antigens.

On this basis 53 polypeptides were identified in HSV-1-infected cells (Honess & Roizman, 1973; Heine et al., 1974).

Marsden et al., (1976) further extended Honess & Roizman's second/

second criterion by examining the polypeptides synthesised in cells infected with ts mutants of HSV-1 at restrictive temperatures. They identified 52 viral polypeptides including six low molecular weight species not identified by Honess & Roizman. Classification of HSV-2 polypeptides has proven to be easier because host protein synthesis is rapidly shut-off. 50 polypeptides have been identified as viral by the above criteria (Powell & Courtney, 1974; Strnad & Aurelia, 1974). Similar analyses have not been carried out for other herpesviruses but the virions have similar numbers of polypeptides (Killington et al., 1977) and there is no reason to suspect that they may be less complex.

1.5.4.3. Regulation of Synthesis of Viral Proteins.

Controlled synthesis of virus specific proteins in HSV-1-infected HEp-2 cells has been described. Viral proteins form at least three groups whose synthesis is coordinately regulated and sequentially ordered. Analysis of the rate of synthesis of viral polypeptides at different times after infection showed the presence of groups differing in kinetics of synthesis (Honess & Roizman, 1973). Further studies with inhibitors of protein synthesis allowed definition of three groups which have been designated α, β and γ. The α group is synthesised early in infection and maximally 3-4 hours post-infection. β synthesis is maximum at 5-7 hours and γ proteins are synthesised at increasing rates until 12 hours post-infection (Honess and Roizman, 1974, 1975a). Similar sequential ordering has been described in HSV-2-infected cells (Powell & Courtney, 1974; Powell et al., 1975).

α polypeptide synthesis requires no prior viral protein synthesis and the polypeptides are synthesised immediately on removal of a cycloheximide block imposed at the time of infection. Three α polypeptides, two/

two non-structural and one virion associated, have been identified in HSV-1 and 2-infected cells. They are all translocated to the nucleus immediately after synthesis, and are post-translationally modified (probably in the nucleus) and phosphorylated (Fenwick & Roizman, 1977; Pereira et al., 1977).

Synthesis of β polypeptides is dependent on α polypeptide synthesis and functional β polypeptides are required to shut-off α polypeptide synthesis. This shut-off is, at least in part, a translational event. β polypeptides have now been shown to form a heterogeneous class. A subset of the group is made in HSV-1 and 2-infected cells treated with amino acid analogues from the time of infection and thus synthesising altered α polypeptides. The β class contains both structural and non-structural proteins. Some of the β polypeptides have been shown to be post-translationally modified and translocated to the nucleus (Honess & Roizman, 1974, 1975a; Pereira et al., 1977).

The relationship between γ and β polypeptides is similar to that between the β and α classes. γ polypeptides are mainly the major structural proteins (Honess and Roizman, 1974, 1975a). γ polypeptide synthesis coincides with viral DNA synthesis and has been found to be decreased in its absence (Honess & Roizman, 1974; Roizman et al., 1974; Powell et al., 1974). It has been suggested that transcription of progeny DNA is necessary for optimal γ polypeptide synthesis (Roizman 1978). Powell et al., (1974) have suggested the existence of another class of proteins designated γ_2 which are totally dependent on DNA synthesis. Further, Ward and Stevens (1975) failed to detect synthesis of any γ proteins when DNA synthesis was inhibited with araC. This discrepancy is unresolved but it is possible that the γ proteins are made but/

not detected by the methods employed (Roizman, 1978).

Such a cascade model is supported by the studies of Marsden et al., (1976) on protein synthesis in cells infected with mutants of HSV-1 at non-permissive temperatures. However, their results suggest that this scheme is an over simplification and a more intricate series of control steps may operate.

At present the extent to which this scheme applies to other herpesviruses is not known. The immediate-early proteins synthesised in PrV-infected RK cells after release of a cycloheximide block correspond to the α class. These proteins appear to be more numerous than the α proteins of HSV-1 and -2 and no structural proteins have been detected (Ben-Porat et al., 1975). However, the different transcriptional programs described for various herpesviruses (see 1.5.3.3.) and the irreversible effects of cycloheximide on PrV and HSV-2 replication (Jean et al., 1974; Frenkel et al., 1973; Powell and Courtney, 1974) suggest that the scheme must be extended only cautiously.

1.5.4.4. Post-translation Translocation and Modification of Viral Proteins.

Newly synthesised proteins migrate from the cytoplasm to the nucleus of infected cells (Fujiwara and Kaplan, 1967; Olshevsky et al., 1967). Specifically, virus structural proteins enter the nucleus (Spear and Roizman, 1968; Ben-Porat et al., 1969) where nucleocapsids are assembled but non-structural proteins are also translocated (Spear and Roizman, 1968; Pereira et al., 1977; Fenwick and Roizman, 1977). Polypeptides probably enter the nucleus through the nuclear pores and nuclear accumulation is likely to be determined by the affinity of polypeptides for constituents of the host cell nucleus (Ben-Porat and Kaplan, 1973; Fenwick et al., 1978).

Polypeptides/

Polypeptides can be modified in three ways (Roizman, 1978). Rapid post-translational cleavages such as those seen in picornavirus-infected cells do not occur (Honess and Roizman, 1973). There is, however, evidence for slow modification of an HSV polypeptide during capsid formation (Gibson and Roizman, 1974). Phosphorylation of structural and non-structural polypeptides of HSV (Gibson and Roizman, 1974; Pereira et al., 1977) and of structural proteins of PrV (Stevely, 1975) has been reported. Erickson (1976) has detected PrV-specified sulphated polypeptides.

Glycosylated polypeptides have been found in all purified herpesvirions analysed to date (Roizman, 1978). However, most of the information on their synthesis comes from HSV. Polysaccharide moieties are added to completed precursor polypeptides by stepwise addition of heterosaccharide chains late in infection (Honess and Roizman, 1975b). Spear and Roizman (1970) showed that HSV-specified glycoproteins partition with membranes. This suggested that the polypeptides are glycosylated in situ. Four antigenically distinct viral-specified polypeptides give rise to all the major glycosylated species detected in HSV-1-infected cells (Spear, 1975; 1976). PrV-infected RK cells produce a non-structural sulphated glycoprotein most of which is excreted from the cell (Kaplan and Ben-Porat, 1976).

1.5.5. Viral DNA Synthesis.

Protein synthesis after infection is necessary to initiate the synthesis of viral DNA. However, the dependence decreases as the infective process proceeds and once DNA synthesis is established inhibition of protein synthesis diminishes, but does not abolish, it. The pool size of all the dNTPs in infected cells increases and the overall/

overall rate of DNA synthesis is higher than in uninfected cells (Cheng et al., 1975). Increased uptake of thymidine is dependent on viral transcription and translation (Bittlingmaier et al., 1975), hence, the dependence on protein synthesis may be related to the pool size.

1.5.5.1. Site of Replication and Enzymes replicating viral DNA.

Histochemical and biochemical evidence indicates that viral DNA synthesis takes place in the nucleus, (Roizman and Furlong, 1974).

New DNA polymerase activities have been reported in cells infected with HSV, PrV, MDV, cytomegalovirus and equine herpesvirus (for references see Allen et al., 1977) varicella-zoster virus (Miller & Rapp, 1977) and EBV (Miller et al., 1977). The physico-chemical properties of the activities differ from those of cellular polymerases. Viral polymerases are stimulated by salt (Boezi et al., 1974) and inhibited by phosphonoacetic acid (Huang, 1975) and temperature labile activities have been described in temperature-sensitive mutants (Aron et al., 1975). There is evidence that more than one gene of HSV-2 is involved in polymerase synthesis (Hay et al., 1976; Purifoy and Benyesh-Melnick, 1975). However the full extent to which the polymerase activity is viral coded is not known and its importance to viral replication is not clear. In S phase cells EHV-1 DNA synthesis is carried out by cellular DNA polymerase before viral polymerase activity is detectable and there is evidence that the maximal levels of HSV-2 DNA polymerase occur after DNA synthesis has begun to decline (Purifoy and Benyesh-Melnick, 1975; Cohen et al., 1977).

Induction of thymidine kinase and DNAase have also been reported in herpes virus infected cells. There is strong evidence that the thymidine kinase is viral coded. The importance of these enzymes to/

to viral DNA synthesis is not known (Ben-Porat & Kaplan, 1973).

1.5.6. Assembly, Envelopment and Release of Virus Particles.

The core and capsid of herpesviruses are assembled in the nucleus. Many different nuclear particle formations have been described (see Roizman & Furlong, 1974), but the flow of events leading to assembly has not been unequivocally established. Perdue et al., (1976) have produced a model for core formation which suggests that DNA is inserted into capsids without a preformed core and organises core formation. There is some other support for this (Friedmann et al., 1975) but the alternative that DNA is incorporated into the capsid during initial assembly has not been ruled out.

The virion envelope is acquired by budding through a cytoplasmic membrane. Thin-section (see Watson, 1973b) and freeze-etch studies (Rodriguez & Dubois-Dalcq, 1978) show particles budding from the inner nuclear membrane, and it is generally accepted that this is the mode of envelopment. However, there are numerous descriptions of naked particles in the cytoplasm and other sites of envelopment have been proposed. Friedmann et al., (1975) have suggested that assembly can occur in the cytoplasm of cells which do not show nuclear changes as a result of infections.

Capsids budding through the inner nuclear membrane pick up dense material that is present on the nuclear side of the lamella. The location of this material allows its definition as tegument but it is not known whether it is the only material in the tegument. Unenveloped capsids covered with a layer of material have been seen in the nucleus, (Roizman & Furlong, 1974).

Several mechanisms of release have been proposed. There is evidence/

evidence for each and it is possible that the process depends on the virus (Watson, 1975b). One pathway provides for continuous partition from the cell cytoplasm. Thus the virion may bud into release channels which lead to the extracellular space (Schwartz & Roizman, 1969) or be transported in vacuoles which are released by reverse phagocytosis (Morgan et al., 1959). Evidence suggests that in some cells release is effected by cell lysis (Watson et al., 1964).

1.6. Alterations in Cell Structure and Function During Productive Infection.

All available evidence indicates that the consequence of productive infection with herpesviruses is cell death (Roizman, 1978). The causes of cell death are obscure but are undoubtedly related to the morphological and biochemical changes seen in infected cells. Thus, host cell macromolecular synthesis is reduced or halted, the cell membrane is altered and other structural changes occur.

1.6.1. Structural Alterations.

The infected cell nucleus exhibits gross changes in the structure of the nucleolus, chromatin and nuclear membrane. During infection, the nucleolus becomes enlarged, and is frequently displaced toward the nuclear membrane and the nucleolar material is altered. Margination of chromatin occurs early in infection and is correlated with distortion of the nucleus and chromosome breakage. Late in infection long stretches of nuclear membrane folded upon themselves are observed (see Roizman, 1978).

No unique feature characteristic of herpesvirus infection is seen in the cytoplasm (Roizman, 1978).

1.6.2. Cellular RNA Synthesis.

Infection of cells with herpesviruses leads to a gradual decrease in the rate of RNA synthesis (Ben-Porat and Kaplan, 1973). The decrease in synthesis is least for RNA greater than 28S and most pronounced for 4S RNA (Roizman, 1978). The appearance of newly synthesised 28S and 18S rRNA declines rapidly after infection. This shut-off is not entirely due to the decrease in synthesis of 45S RNA or to changes in its methylation, but rather to abortive processing (Roizman et al., 1970).

Changes in the rates of synthesis of the other cellular RNAs are less well characterised.Roizman et al., (1970) have shown that transport of non-viral RNA is different from that of viral RNA. Rakusanova et al., (1972) have evidence that cellular RNA species accumulate in infected cells and that these species differ from those present in uninfected cells. Other results show that cellular DNA is transcribed late in infection and some cellular transcripts become associated with polysomes (Stringer et al., 1977). Most of this information comes from DNA-RNA hybridisations and because cellular RNA contains many species present in different abundances and total cellular DNA contains reiterated and unique sequences the technique is limited. Hybridisation of RNA from HSV infected polyoma-transformed BHK cells to polyoma virus DNA does not suffer from these limitations and such studies have been used to show that transcription of polyoma DNA is specifically inhibited in HSV-infected cells (Pizer and Beard, 1976). Thus the overall cellular mRNA synthesis probably reflects different changes in the transcription of different sequences.

The mechanism of inhibition of host RNA synthesis is unknown. Sasaki et al., (1974) noted the presence of an inhibitor of nucleolar RNA/

RNA polymerase in HSV-infected cells but its role is unknown.

1.6.3. Cellular Protein Synthesis.

The overall inhibition of cellular protein synthesis in cells infected with herpesviruses has been established by the criteria used to identify viral polypeptides in infected cells (see 1.5.4.2.). However, the absolute degree of shut-off is not known. Cellular mRNA species have been found on polysomes late after infection (Stringer et al., 1977), and several polypeptides synthesised at this stage of infection have been identified as cellular (Honess and Roizman, 1973; Powell and Courtney, 1974).

The method of shut-off of host protein synthesis is not understood. The inhibition of host RNA synthesis will account for some decline in protein synthesis and there is evidence for some degradation of mRNA (Nishioka & Silverstein, 1977). In addition, it seems likely that some translational control operates.

1.6.4. Cellular DNA Synthesis.

In general, infection of susceptible cells with herpesviruses leads to a decrease in the rate of cellular DNA synthesis (Ben-Porat and Kaplan, 1973). Exceptions are viruses such as EBV and MDV which can cause leucocyte proliferation and consequently increase DNA synthesis (Gerber and Hoyer, 1971; Lee, 1972). However, the synthesis of cellular DNA is inhibited in EBV-infected cells in which early viral antigens are synthesised (Gergely et al., 1976 a,b; Nonoyamaand Pagano, 1972).

The time of shut-off of cellular DNA synthesis is dependent on the moi but is generally an early event. The mechanism of shut-off is/

is unknown. Ben-Porat and Kaplan (1965) have shown that inhibition is dependent on protein synthesis. Thus, two mechanisms are possible: virus induced proteins might act as inhibitors of cellular DNA replication, or the inhibition of cellular protein synthesis may lead to a decrease in synthesis of a cellular protein which is essential for cellular DNA synthesis. Ludwig and Rott (1975) have shown that inhibition of glycosylation in PrV-infected cells by 2-deoxy-glucose allows concomitant synthesis of viral and cellular DNA implying that a glycoprotein is involved. Bittlingmaier et al., (1975), however, have evidence that the early inhibition of synthesis is independent of the genome and raise the possibility that the inhibition may be due to changes in dTTP pools.

The physical changes in cellular DNA which have been noted (1.6.1.) may also be related to control of synthesis.

1.6.5. Alterations in Cell Membranes.

Cells infected with herpesviruses acquire virus-specific antigens on their surface (Roane and Roizman, 1964). In EBV and HSV infection these antigens seem to be identical to those present on mature virions (Pearson et al., 1970; Roizman and Spring, 1967). Comparison of the electrophoretic mobilities of membrane polypeptides with virion polypeptides has led to the conclusion that the viral polypeptides inserted into membranes are identical to those that become part of the envelope of mature virions (Roizman, 1978). The reason for virus-induced alterations of the plasma cell membrane is not obvious because unlike the nuclear membrane this membrane does not form part of the mature virion. It is likely, however, that these changes are related to alterations in the tendency of infected cells to interact with each other (their "social behaviour" Ejercito et al/

al., 1968), and to the membrane's role in the infective process (Manservigi et al., 1977).

The social behaviour of infected cells is dependent on the virus strain and the cell type and infected cells may clump or fuse. Manservigi et al., (1977), have shown that there are at least two genes involved in determining the social behaviour of infected cells and the existence of fusion promoting and fusion inhibiting activities has been proposed. These activities have been related to the presence or absence of two glycoproteins in the cell membrane of infected cells. There is also evidence that the pattern of glycolipid synthesis is altered in fusion-producing mutants (Ruhlig and Person, 1977). At present the mechanism of fusion and the relationship of macromolecules to the process has not been established.

1.7. Translational Control of Protein Synthesis in Eucaryotic Cells.

As discussed in the general introduction to this thesis (1.1.) control of protein synthesis may occur at a number of levels. However, since the purpose of this project was an investigation of translational control, this review will be confined to the mechanisms by which translation of mRNAs present in the cytoplasm can be regulated in eucaryotic cells. Translational control also occurs in procaryotic cells. This subject has been reviewed recently (Lodish, 1976) and will not be considered here.

The mechanisms of control available are governed by the components of the system and hence it is useful to consider the apparatus and mechanism of protein synthesis.

1.7.1./

1.7.1. The Mechanism of Translation.

A pathway for initiation of protein synthesis has been proposed recently by Staehelin and coworkers (Trachsel et al., 1977; Schreier et al., 1977). This can be summarised as follows: The first step is the formation of a ternary complex between met-$tRNA_f$ GTP and the initiation factor eIF-2. The complex then binds to the 40S ribosomal subunit and this quaternary complex is stabilised by eIF-1 and eIF-3. The subsequent binding of mRNA requires eIF-3, eIF-4A and eIF-4B and is accompanied by ATP hydrolysis. eIF-4C, like eIF-1, apparently has a role in stabilisation. The {40S·met-$tRNA_f$·GTP·mRNA·initiation factor} complex then binds the 60S subunit in the presence of eIF-5 to yield an 80S initiation complex {80S·met-$tRNA_f$·mRNA} where the met-$tRNA_f$ is in the peptidyl site of the ribosome.

The intermediates proposed in this pathway have been described by a number of workers and the scheme is very similar to that proposed by Benne and Hershey (1978). However, most of the evidence has been obtained using reconstituted systems where the rate of protein synthesis is only a fraction of that observed in vivo and so it may be an over-simplification. In particular, there is still no agreement on the total number of initiation factors. Two groups have identified a factor eIF-4D the role of which has yet to be confirmed (Kemper et al., 1976; Benne and Hershey, 1978) and very recent work on the mode of action of the haem controlled repressor (see 1.7.2.3.) has shown that the eIF-2 activity may be modified by another protein. Furthermore, the proposed pathway does not take into account possible cooperation of eIF-1 and eIF-4C in 60S subunit joining and the dissociation role of eIF-3 (Trachsel et al., 1977; Benne and Hershey, 1978).

Elongation, as far as is known at present, involves only two factors.

The subject has been reviewed by Weissbach and Ochoa (1976) and can be summarised as follows: The appropriate aminoacylated tRNA is brought to the A site on the ribosome as a ternary complex consisting of GTP, elongation factor EF-1 and aminoacylated tRNA. Binding involves hydrolysis of GTP and the GDP·EF-1 complex is released and recycled. The ribosomal peptidyl transferase then catalyses formation of the peptide bond. Translocation requires EF-2 and GTP. EF-2 and GTP form a stable complex which binds to the ribosome resulting in translocation and GTP hydrolysis. The EF-2 GDP is then released and recycled. It is not clear how recycling of the factors occurs and it is possible that other factors may be involved in a recycling process such as occurs with EF-Tu and EF-Ts in procaryotes.

The termination process allows release of the completed peptide chain from the ribosome-mRNA complex. The peptide is thought to be located on the P site and its release requires a termination codon in the A site, a release factor (RF) and GTP. Available evidence suggests that the first step is GTP-dependent binding of RF to the A site. This activates peptidyl transferase so that the terminal aminoacyl bond is hydrolysed and the completed polypeptide is released. GTP hydrolysis is required for the dissociation of RF from the A site.

1.7.2. Translational Control Mechanisms.

Translational controls can be seen as methods of regulating the availability of the components of the translational apparatus or of changing their efficiency. These components are now considered individually.

1.7.2.1. Low Molecular Weight Substances and tRNA.

Glucose starvation and amino acid starvation inhibit protein synthesis (Jackson, 1975). While such factors probably act at a number of levels, there is evidence that they affect translation directly. In both cases the energy supply may become limiting and small changes in ATP and GTP concentrations inhibit initiation and elongation (Rupniak and Quincey, 1975).

Amino acid starvation may, however, have effects beyond those on energy metabolism or on the concentration of aminoacylated tRNAs. Thus, if the concentration of a charged tRNA other than the initiator tRNA is severely decreased, elongation of peptides might be limited. It has been shown, however, that when the charging of $tRNA^{his}$ is inhibited in HeLa cells with L-histidinol both initiation and elongation are affected (Vaughan and Hansen, 1973). This led to the suggestion that deacylated tRNAs inhibit initiation directly. Such a hypothesis is supported by the observation that deacylated tRNAs inhibit the AUG dependent 80S complex formation *in vitro* (Kyner *et al.*, 1973). The details of this effect are not fully characterised. Kyner *et al.*, (1973) suggested that deacylated tRNA competed with the initiation tRNA for the P site confining the effect to the early stages of initiation. Some evidence supports this (Hayes *et al.*, 1975; Pain and Henshaw, 1975) but it has been suggested that the lesion is between 80S complex formation and elongation (Warrington *et al.*, 1977). A major difficulty is assessing the correlation between experiments using amino acid analogues and amino acid starvation studies.

It has also been suggested that the effect of tRNA might be related to the energy supply. An enzyme system which degrades GTP to guanine when uncharged tRNA is in the A site has been demonstrated *in vitro*/

vitro (Grummt and Speckbacher, 1975). Further, lowering the charging of $tRNA^{his}$ with L-histidinol depresses first the GTP then the ATP levels (Grummt and Grummt, 1976). It is of interest that in procaryotes modification of GTP to (p)ppGpp is involved in suppression of protein synthesis in amino acid starvation (Block & Haseltine, 1974).

A direct effect of tRNA on elongation has been demonstrated and is better characterised. Protein synthesis can be regulated by the availability of one or more iso-accepting tRNAs. In a tRNA-dependent cell free system oviduct mRNA and EMC virus RNA are not translated efficiently or with fidelity in the presence of rabbit reticulocyte tRNA although globin mRNA is, but efficient synthesis is achieved by addition of rabbit liver or ascites tRNA (Sharma et al., 1976a). In addition, the amount of synthesis in vitro can be modulated by specific tRNAs (Sharma et al., 1976b). In vivo, changes in the relative amounts of tRNAs and the population of iso-accepting species have been correlated with changes in protein synthesis (Viotti et al., 1978). These results may be related to the different amino acid compositions of the proteins being synthesised or to different mRNA requirements for iso-accepting tRNAs.

1.7.2.2. mRNA.

Regulation of the translation of mRNAs present in the cytoplasm can be achieved in three ways. Firstly, specific degradation of mRNA may occur. Secondly, mRNAs may differ in their ability to be recognised by the translational machinery. Thirdly, mRNAs may be made unavailable by complexing with other molecules.

The first two controls are dependent on differences in mRNA structure. At present only general features of mRNA structure are known and thus little is known about specific controls. Most

eucaryotic/

eucaryotic mRNAs have at the 3' end a poly-(A) tract (Brawerman, 1975) which probably has a role in preventing degradation (Revel and Groner, 1978). With the exception of the picornavirus and satellite tobacco necrosis virus mRNAs, all eucaryotic mRNAs so far examined carry a 7^{m} G "cap" joined to the 5' terminus by a 5'-5' pyrophosphate link. This may be followed by one or two 2'O-methylated residues (Shatkin, 1976). The cap protects mRNA from 5' exonucleolytic degradation. It is required for initiation and is recognised by eIF-2 (Kaempfer et al., 1978) and eIF-4B (Shafritz et al., 1976) the latter of which may be limiting in poliovirus infected cells (Rose et al., 1978). Other structural features such as internal methylation have not yet been correlated with control.

mRNA translation can be controlled by sequestering mRNAs in messenger ribonuclear particles (mRNPs) non-associated with ribosomes. Sea urchin embryos, for example, store histone mRNAs which are released for growth (Kedes, 1976). Active mRNA can be regained from immature duck erythrocytes by deproteinisation (Civelli et al., 1976), but Kennedy and Heywood (1976a) find that it is necessary to denature the RNA and suggest that translational control RNA (tcRNA) is involved in sequestration (see 1.7.2.7.).

1.7.2.3. Initiation Factors.

The total number of the initiation factors has not been completely defined and there is some disagreement on the way in which they regulate translation. On the one hand, cells could regulate the overall rate of chain initiation by the availability of factors which would be expected to affect all mRNAs. Alternatively, the cells could utilise message-specific components that would lead to changes in the types of mRNAs translated/

translated. These mechanisms are not, however, mutually exclusive.

The availability of cell free systems which translate heterologous mRNAs at rates very close to those observed *in vivo* suggests that the translational apparatus has the same specificity in all cells. Furthermore, the demonstration that the initiation factor which stimulates EMC virus RNA translation (Wigle and Smith, 1973) is eIF-4A (Staehelin *et al.*, 1975) and that the preferential translation of EMC virus RNA in a mixed viral/cellular mRNA population can be alleviated by eIF-4B. (Golini et al., 1976) suggests that apparent specificities may be due to different requirements of mRNAs for the same initiation factors.

Non-specific control of translation has been demonstrated in haem-deficient reticulocytes and it is becoming clear that this is due to inactivation of an initiation factor. In reticulocytes in the absence of haem protein synthesis proceeds for several minutes and then declines rapidly and disaggregation of polysomes and depletion of 40S·met $tRNA_f$ complexes occurs. A cAMP-independent protein kinase which phosphorylates eIF-2 can be isolated from reticulocytes incubated without haem and, if added back to lysates, the kinase causes inhibition of protein synthesis (Farrell *et al.*, 1977). Phosphorylated eIF-2 has normal activity in promoting formation of 40S·met $tRNA_f$ complexes in reconstituted systems where relatively large amounts of purified initiation factors are used. However, Ochoa's group have very recently isolated a protein which stimulates the activity of eIF-2 (eIF-2 stimulating protein ESP) and have found that phosphorylation abolishes the interaction of eIF-2 with ESP so that the initiation factor is no longer active at the low concentrations found in lysates (de Haro *et al.*, 1978; de Haro and Ochoa, 1978). Thus it now seems very likely that the basis of inhibition of initiation in this case is phosphorylation of eIF-2.

Similar/

Similar relationships have not yet been demonstrated for other factors but four initiation factor polypeptides, including eIF-4B, can be phosphorylated *in vivo* and an analogous role is possible (Benne *et al.*, 1978).

Lodish (1974) has derived a kinetic rate equation for initiation and elongation of polypeptide chains which predicts that any reduction in the rate of initiation at or before binding of mRNA will lead to preferential inhibition of translation of mRNAs with lower initiation rate constants. Hence, control might be exerted by non-specific lowering of initiation rates which would favour the translation of "better" mRNAs. Studies on inhibition of initiation have shown that such an effect can occur (see Lodish, 1976; Koch *et al.*, 1976).

However, more recent experiments using purified initiation factors suggest that this is an oversimplification. Addition of purified eIF-4A and eIF-4B has been shown to change the translation of α globin mRNA relative to β globin mRNA without increasing the overall rate of translation. Thus the effect could not be ascribed to an increase in the concentration of a rate limiting factor (Kabat and Chappell, 1977). Other anomalous results have been reviewed by Revel and Groner (1978) who have proposed that contrary to Lodish's hypothesis, mRNAs do not have a uniqueinitiation constant. They suggest that the efficiency of mRNA recognition by the ribosomal machinery is due to the overall kinetic parameters of the initiation system. Thus, changes in the concentration of factors modify the relative affinity of different mRNAs for the ribosomal machinery. In one sense then, the population of initiation factors has message specificity.

A more specific effect of initiation factors is proposed by Heywood and coworkers who have reported a mRNA specific activity in partially purified eIF-3 preparations. They find that although translation of globin/

globin and myosin mRNAs is stimulated by reticulocyte eIF-3 as might occur if the concentration of this factor were limiting, preferential stimulation of myosin mRNA translation is brought about by muscle eIF-3 (Kennedy and Heywood, 1976a). A similar result was found for tubulin mRNA (Gilmore-Hebert and Heywood, 1976). They suggest that modulator molecules may be associated with eIF-3 (Kennedy and Heywood, 1976a,b). Further work is necessary to characterise these modulators.

In summary, it is clear that initiation factors have a role in translational control but at present this is not completely understood.

1.7.2.4. Elongation Factors.

It is generally assumed that elongation and termination of all polypeptides occur at the same rate. This implies an absence of differential control but general control of translation may operate at this level. An example of such control is the inhibition of protein synthesis by diptheria toxin. This protein catalyses ADP-ribosylation of EF-2 thus rendering it non-functional and inhibiting translation of all mRNAs. (Pappenheimer, 1977).

1.7.2.5. Ribosomes.

There is no evidence that synthesis of ribosomes is an important method of short term control. Studies of inactivation are complicated by the association of factors with ribosomes. Phosphorylation of a ribosomal protein is associated with dsRNA-induced inhibition if protein synthesis (Farrell et al., 1977) but as this is not the only effect of dsRNA its importance is unknown. Phosphorylation of other ribosomal proteins has been demonstrated but as yet these events have not been correlated/

correlated with control.

1.7.2.6. Other Factors.

Low molecular weight U-rich oligonucleotides (tcRNA) which inhibit protein synthesis *in vitro* have been isolated from muscle (Kennedy *et al.*, 1978), *Artemia salina* (Lee-Huang *et al.*, 1977) and rat calvaria (Zeichner and Breitreutz, 1978). Lee-Huang and coworkers have shown that their tcRNA blocks peptide chain elongation by interfering with the EF-1 dependent binding of aminoacyl tRNA. The tcRNA isolated by Heywood and coworkers binds specifically to myosin mRNA and found in mRNP particles. They suggest that in addition to non-specific regulation, tcRNAs may have high affinities for specific mRNAs and may be involved in sequestering mRNA in non-translatable mRNPs (Kennedy and Heywood, 1976a; Kennedy *et al.*, 1978 and see 1.7.2.2.).

1.7.2.7. Summary.

Most of the controls described in this section can be defined as initiation or elongation specific. Most of what is known concerns control at initiation and advances are being made in determining the mechanism involved in regulation. Several models for control of initiation have been proposed. These will be considered in detail in Chapter 8. Control of elongation is less frequently observed and in many cases (see, for example, Orlowski and Sypherd, 1978) no knowledge of the mechanism is available.

1.7.3. The Interferon System.

Interferon induced translational control has been considered separately because it operates at several levels and its mode of action may/

may illustrate a unifying principle. The subject has been reviewed recently (Revel & Groner, 1978) and can be summarised as follows:

Exposure of sensitive cells to interferon induces an antiviral state which at least in part results from inhibition of viral mRNA translation. The way in which discrimination between viral and cellular mRNAs takes place is not known and this discrimination is lost when treated cells are homogenised to prepare cell free translation systems. However, such studies have yielded information on the translational controls operative in those systems.

Extracts from interferon treated cells contain a dominant inhibitor of translation. The activity of this factor is potentiated if the extracts are incubated with dsRNA and ATP prior to addition to cell free systems. Activation requires ATP hydrolysis and is associated with protein phosphorylation and the formation of a small nucleotidic inhibitor of translation.

Three principal phosphorylations take place. A ribosomal protein of molecular weight 67,000 is phosphorylated; eIF-2 is phosphorylated; and a protein kinase specific for arginine rich histones has been identified. The relationships between these kinase activities has not been fully characterised. The nucleotidic inhibitor has been identified as ppA (2')p(5')A(2')p(5')A and has been shown to activate an endonuclease that cleaves mRNA (Clemens and Williams, 1978).

Translation is inhibited at both the initiation and elongation steps. Protein phosphorylation is likely to be involved in the inhibition of initiation and in particular the inactivation of eIF-2 is similar to that occurring in haem deficient reticulocytes (Farrell et al., 1977). There is evidence also that the interferon induced translational control is mediated by tRNA. Changes in the isoaccepting

tRNA/

tRNA levels (Zilberstein et al., 1976) may affect elongation and a deacylase activity which might induce deacylated tRNA suppression of initiation (see 1.7.2.1.) has been detected.

In summary, there appear to be a number of translational controls operative in interferon treated cells and these encompass many of those discussed in the previous section.

1.7.4. Translational Control in PrV Infected Cells.

The applicability of the controls discussed in 1.7.2. and 1.7.3. to the translational controls operative in PrV infected cells can be discussed. The length of the replicative cycle for PrV is short (1.5) and inhibition of host protein synthesis is an early event (1.6.3.). Hence induction of interferon and interferon-induced controls are unlikely to be important in cell culture although they undoubtedly have a role in vivo. For the same reason changes in the energy status of the cell are unlikely to have any effect. No changes in the tRNA population in herpesvirus-infected cells have been detected (Ben-Porat and Kaplan, 1973).

It is probable, therefore, that controls act on the mRNA populations and on ribosomes and factors. Degradation of cellular mRNA requires an enzyme which recognises differences in the mRNA structure. There are at present no known structural differences between viral and cellular mRNA. Both mRNAs contain caps and poly-(A) tracts (1.5.3.2.); hence recognition of these features cannot be important. It is possible, however, that differences in late viral mRNA methylation (1.5.3.2.) may have a role in control. Nothing is known about factors and ribosomes in infected cells and except for the conclusion that a change in cap recognition is not important, no evidence is available.

In summary, then, very little is known about translational controls operative/

operative in PrV-infected cells. In particular, proposed models for control have not been examined in this system. The controls operating in infected cells are likely to be similar to those in uninfected cells and hence the general applicability of these mechanisms is important. The work described in this thesis was undertaken to examine the application of models for translational control of protein synthesis in PrV-infected cells and to extend the general knowledge in this field.

Chapter 2

Isolation and Characterisation of Poly-(A) Containing RNA.

2.1. Introduction.

Poly-(A) containing RNA was isolated from polysomes for two reasons. Firstly, cellular mRNA molecules are found in herpesvirus infected cells late in infection but most of the mRNA present on polysomes is viral (see Chapter 1). Therefore isolation of polysomes provides a means of separating viral and cellular mRNA. Secondly there is some evidence that *in vitro* translation systems are inhibited by excess RNA (Sonenstein & Brawerman 1976). Only about 2% of HeLa cell polysomal RNA is mRNA and it is necessary to remove other RNA species so that sufficient mRNA can be added without inhibition by excess quantities of RNA. Most mRNA molecules synthesised in eukaryotic cells including those specified by herpesviruses contain 3' poly-(A) tracts (see Chapter 1). Similar poly-(A) sequences have not been found in other RNA species and so isolation of poly-(A) containing RNA is a convenient method of isolating mRNA.

2.2. Isolation of Polysomes.

Polysomes were isolated from exponentially growing HeLa cells 5h after infection with PrV and from mock-infected cells as described in the Materials and Methods. The yield of polysomes obtained was usually of the order for 60 A_{260} units per 10^9 cells but was dependent on variations in cell growth. Good yields of polysomes and higher polysome to monosome ratios were more consistently obtained when heparin was added to buffers as an RNAase inhibitor.

Profiles of polysomes spread on sucrose gradients are shown in Figure 2.2.1. In agreement with earlier results (see 1.5.4.1.) PrV infected/

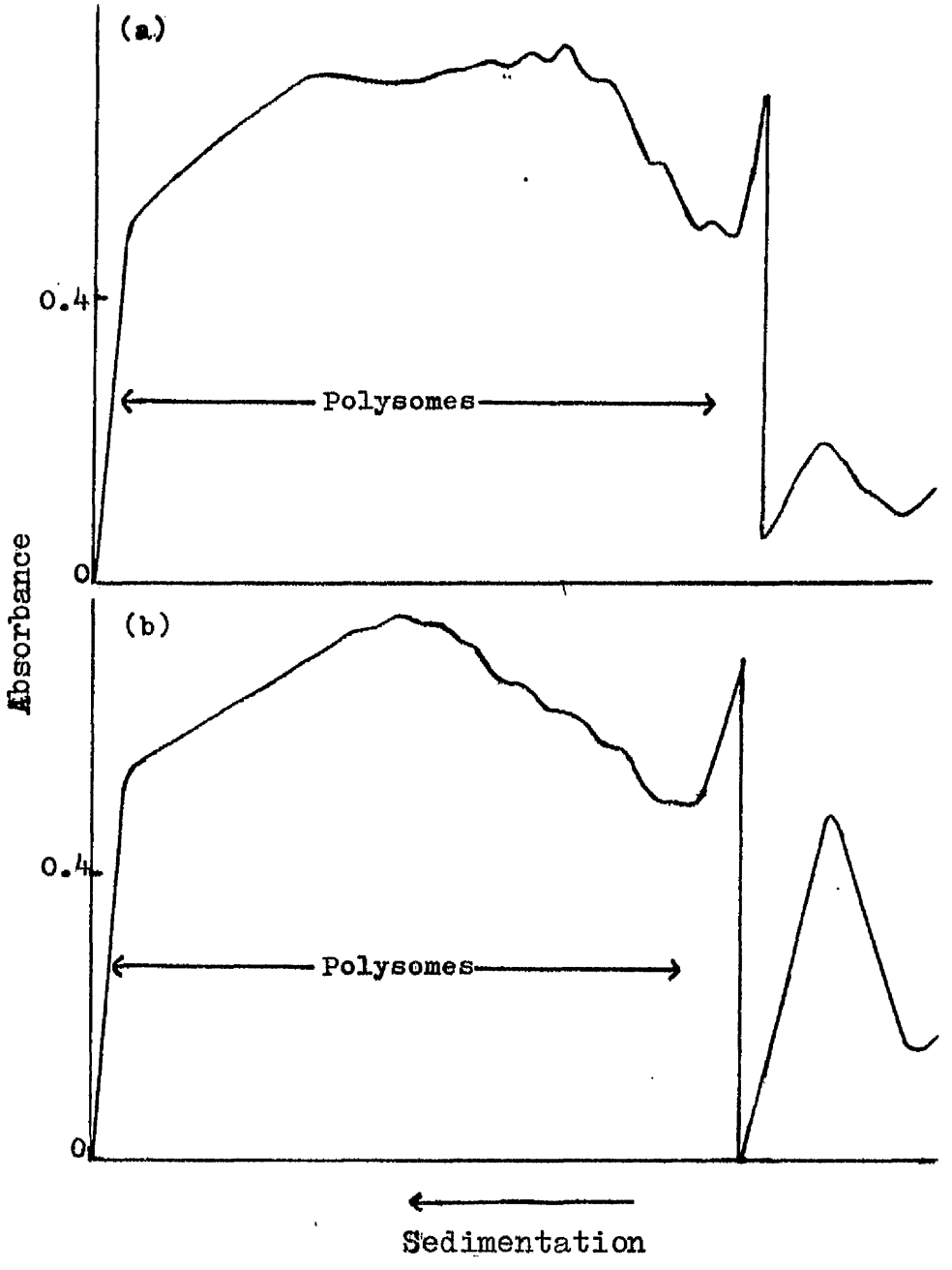
FIG. 2.2.1. POLYSOME PROFILES IN PrV-INFECTED AND MOCK-INFECTED HeLa CELLS

Exponentially growing HeLa cells were mock-infected or infected with 20pfu/cell PrV. After 5h the cells were harvested mechanically, lysed and the cytoplasmic extracts were layered onto 15-30% sucrose gradients. The gradients were centrifuged for 110 min at 27,000g in a SW27 rotor and collected by pumping from the bottom of the tube through a Gilford 2000 recording spectrophotometer which monitored the absorbance at 260nm.

The polysome regions are as indicated. The slow sedimenting peak contains monosomes and subunits.

a) Mock-infected cell polysomes

b) PrV-infected cell polysomes


(a)
0.4
Polysomes
0
Absorbance
(b)
0.4
Polysomes
0
Sedimentation

infected cells were found to show a higher proportion of rapidly sedimenting polysomes and a higher monosome to polysome ratio than mock-infected cells.

2.3. Preparation of Poly-(A) Containing RNA.

2.3.1. Extraction of RNA from Polysomes.

RNA was isolated from polysomes which showed the characteristic virus-infected or mock-infected profiles on sucrose gradients as described in the Materials and Methods. 90% of the A_{260} units in the polysomes could be recovered by this method.

2.3.2. Affinity Chromatography.

Poly-(A) containing RNA can be isolated on the basis of the ability of poly-(A) tracts to form hybrids with poly-(U) bound to Sepharose (Lindbergh and Persson, 1972) or oligo-(dT) bound to cellulose (Aviv and Leder, 1972). Hybrids are readily dissociated with buffers of low ionic strength.

Poly-(A) containing RNA was isolated from rabbit reticulocyte RNA by affinity chromatography on poly-(U) Sepharose as described in the Materials and Methods and was found to stimulate incorporation of radioactive amino acids into Trichloroacetic acid (TCA) precipitable material in cell-free translation systems. Poly-(U) Sepharose chromatography of polysomal RNA isolated from infected and mock-infected HeLa cells did not, however, yield a similarly active fraction although bound material with measurable RNA content was recovered from the column. The bound fraction was precipitated several times from ethanol at $-20^{o}C$ to remove any inhibitory substances but stimulatory material could not be recovered.

Oligo/

Oligo-(dT) cellulose chromatography of rabbit reticulocyte RNA and polysomal RNA was carried out as described in the Materials and Methods. An elution profile of the polysomal RNA washed from the column is shown in Figure 2.3.2.1. Most of the polysomal RNA was not bound and passed straight through the column. Loosely bound material, probably containing short poly-(A) tracts was washed off by the intermediate buffer which is of lower ionic strength than the starting buffer. Bound material was rapidly eluted with elution buffer. Recovery of RNA from oligo-(dT) cellulose columns is shown in Table 2.3.2.1. 1.1 - 2.6% of the input RNA was bound.

The oligo-(dT) cellulose fractions were precipitated twice with ethanol at $-20^{\circ}C$ and material from rabbit reticulocytes and mock-infected or infected HeLa cells was shown to have mRNA activity in cell-free systems. Some mRNA activity could also be demonstrated in the unbound fraction but the stimulation was considerably less than that observed in the bound fraction.

2.4. RNA-DNA Hybridisation.

Poly-(A) containing RNA from PrV-infected cells was assayed for the presence of viral coded sequences by hybridisation to PrV DNA bound to nitrocellulose filters. Polysomal RNA was prepared from mock-infected and PrV-infected HeLa cells 5h post-infection labelled with $\{^{3}H\}$-uridine for 3h prior to harvesting as described previously. Poly-(A) containing RNA was prepared by oligo-(dT) cellulose chromatography as previously described, except that ethanol precipitation was replaced by gel filtration through a Sephadex G25 (coarse) column (0.5 x 6cm) and lyophilisation. This was necessary because to obtain as high a specific activity as possible without

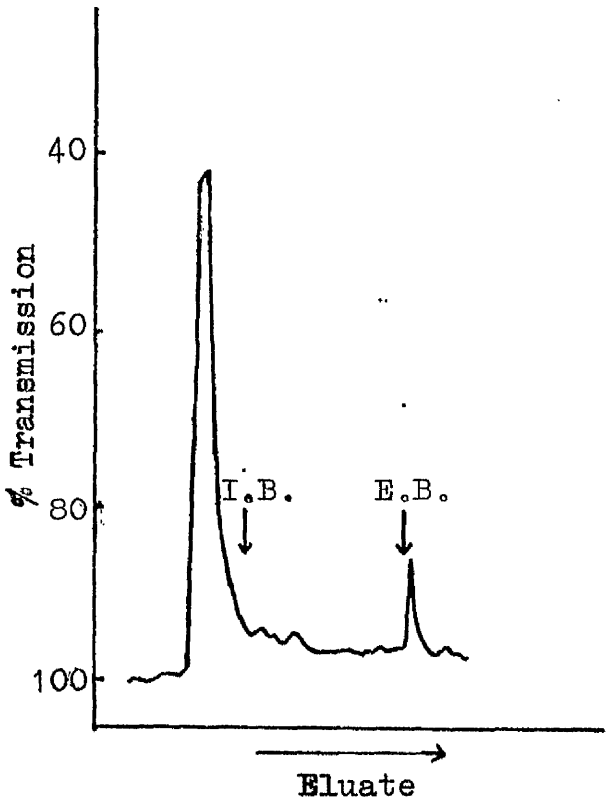


FIG. 2.3.2.1. ELUTION PROFILE OF POLYSOMAL RNA FROM OLIGO-(dT) CELLULOSE AFFINITY COLUMN

Polysomal RNA from mock-infected HeLa cells was dissolved in starting buffer and applied to an oligo-(dT) cellulose mini-column. The eluate was pumped through an LKB 8300A Uvicord II which recorded the percentage transmission at 260nm. The column was washed initially with starting buffer, then intermediate buffer (I.B) and elution buffer (E.B) were applied sequentially as shown.

TABLE 2.3.2.1.

Binding of Polysomal RNA to Oligo-(dT) Cellulose Affinity Chromatography Columns

Polysomes	µg Polysomal RNA Applied	µg RNA Bound	% Bound
Mock-infected	1650	32	2.0
"	3000	34	1.1
PrV-infected	1260	29	2.3
"	1900	29	1.5
"	2340	61	2.6

using excessive quantities of isotope only three burlers of mock-infected or infected cells, one of which was labelled with 20μCi/ml {^{3}H}-uridine were used and the amount of bound RNA thus obtained was small and subject to considerable loss in ethanol precipitation.

Poly-(A) containing RNA from infected cells was dissolved in 0.1 x SSC and samples containing 0.1 - 0.3μg were hybridised to filters containing 5μg PrV DNA as described in the Materials and Methods. Mock-infected cell poly-(A) containing RNA was also hybridised to estimate the extent of non-specific binding. Over 50% of the poly-(A) containing RNA from infected cells bound to the filters while less than 2% of the cellular RNA was bound (Table 2.4.1.).

2.5. Discussion.

Poly-(A) containing RNA was isolated from polysomal RNA rather than total cytoplasmic RNA because this provided a means of selecting viral RNA. An additional advantage was that the characteristic changes in polysome profiles provided a means of monitoring viral infection. Infected cells were harvested during the eclipse phase of growth when no measure of virus production could be made and morphological changes were not apparent. Variations in the growth of tissue culture cells occur from week to week and can cause variation in the infective process. Poor infections were apparent when sucrose gradients were scanned and the preparations were discarded.

Poly-(U) Sepharose chromatography has been reported to remove non-poly-(A) containing RNA more efficiently than oligo-(dT) cellulose chromatography (Bishop et al., 1974; Vass, 1975). W. Schuch (personal communication) has found that this method of oligo-(dT) cellulose chromatography yields preparations contaminated with rRNA. However/

TABLE 2.4.1.

Hybridisation of Poly-(A) containing polysomal RNA from PrV-infected and mock-infected HeLa cells to PrV DNA bound to nitrocellulose filters.

Poly-(A) containing RNA	Input		Bound	% Bound
	µg	cpm		
PrV - infected	0.1	340	189	54.7
Mock-infected	0.3	866	16	1.8

However, for the purpose of translation pure poly-(A) containing RNA is not necessary and this method yielded sufficiently enriched material.

There are several possible reasons why polysomal RNA fractions with mRNA activity could not be obtained by poly-(U) Sepharose chromatography of polysomal RNA. Poly-(U) Sepharose elution buffer contains SDS and is of higher ionic strength than oligo-(dT) cellulose elution buffer and while the quantity of reticulocyte poly-(A) containing RNA is sufficient that it can be dissolved in a large enough volume to dilute out these contaminants to insignificant levels, similar quantities cannot be obtained from cultured cells. Contaminants may be removed by reprecipitation but recovery of small quantities of RNA is poor and it is likely that equivalent losses of RNA and contaminants occur so that the final precipitates still contain too high a ratio of contaminant to RNA.

Secondly, it is likely that lack of experience in the techniques contributed to the failure as these experiments were carried out during the early part of the work and have not been repeated. Again, the large quantities of reticulocyte RNA would account for the success of the technique with this RNA.

RNA-DNA hybridisation showed that the poly-(A) containing RNA from the polysomes of PrV-infected cells contained appreciable amounts of virus specific RNA. This RNA species was therefore termed <u>viral mRNA</u> although cellular mRNA species and rRNA are probably present. Poly-(A) containing RNAs from mock-infected HeLa cells and from rabbit reticulocytes were similarly termed <u>cellular</u> and <u>reticulocyte</u> mRNAs respectively.

Chapter 3

Characterisation of the Wheat Germ Cell-free System.

3.1. Introduction.

Eucaryotic cell-free translation systems capable of translating exogenous mRNAs have been prepared from many sources including rabbit reticulocytes (Hunt & Jackson, 1974), Krebs II Ascites cells, (Mathews and Korner, 1970), HeLa cells (Weber et al., 1976), Xenopus oocytes (Gurdon et al., 1971) and wheat germ (Roberts and Paterson, 1973; Davies and Kaesberg, 1973; Marcu and Dudock, 1974). The usefulness of some of these systems is limited by the low efficiency of translation observed in vitro and by the high endogenous levels of protein synthesis which obscure products made by exogenous mRNAs. The wheat germ cell-free system does not have these disadvantages. The efficiency is high compared to other systems and endogenous protein synthesis is low (Roberts and Paterson, 1973). The system is capable of translating a variety of heterologous mRNAs ranging from Qβ bacteriophage (Davies & Kaesberg, 1973) to pro α collagen (Benveniste et al., 1976) as well as many relatively homologous mRNAs from plant viruses (Davies and Kaesberg, 1974). In addition, large quantities of extract may be rapidly prepared from readily available material.

This system was therefore investigated for ability to translate viral and cellular mRNAs. Translation of reticulocyte mRNA in the system has been demonstrated (Roberts and Paterson, 1973) and was used for comparison.

3.2./

3.2. mRNA Activity of Viral, Cellular and Reticulocyte mRNAs.

mRNA activity is the ability to serve as a template for protein synthesis. For the purpose of this chapter mRNA activity is defined as the ability to stimulate the incorporation of radio-active amino acids into TCA precipitable material. This definition does not imply that the mRNA directs synthesis of the complete polypeptides found *in vivo*. There are several reasons why this may not be so and these will be considered in this chapter where appropriate and more fully in Chapter 4.

Viral, cellular and reticulocyte mRNA were assayed for mRNA activity in the wheat germ cell-free system. Wheat germ extract was prepared and assays set up as described in the Materials and Methods. Assays containing 75mM K^+, 1.5mM Mg^{++}, 0.2mM spermidine were incubated for 90 min at 25°C. All three mRNAs were found to have significant mRNA activity under these conditions (Table 3.2.1.). The mRNA activity of the polysomal RNA which was not bound to the oligo-(dT) cellulose column is shown for comparison.

3.3. Characterisation of Optimum Conditions.

Considerable variations in the optimum ionic concentrations for translation of mRNA in the wheat germ cell free system have been described. Some of these are due to slightly different methods of preparation of the extract and others are peculiar to the mRNA species. Hence, the optimum conditions were characterised for the mRNA species and wheat germ investigated in this study.

3.3.1/

TABLE 3.2.1.

Stimulation of the Wheat Germ Cell-free System by Exogenous RNA.

RNA Species	RNA µg/ml	Stimulation *
Viral	8.8	16
Cellular	6.8	13
Reticulocyte	16.6	19
Viral polysomal unbound	70	5
Cellular polysomal unbound	86	6

* Times endogenous (1240 cpm)

3.3.1. Optimum K^+ Concentrations.

The optimum concentration of K^+ for mRNA activity of the three mRNA species under investigation was determined by varying the amount of KCl added to assays. In these experiments the assays contained 2.5mM Mg^{++} but no polyamine and TCA precipitation was carried out on filters. Figure 3.3.1.1. shows the amount of $\{^3H\}$-leu incorporated at various K^+ concentrations. The optimum concentrations are in the range 60-76mM for viral and cellular mRNAs and 74-81mM for reticulocyte mRNA. Similar optima were found for several different mRNA and wheat germ preparations.

Weber et al., (1977) reported that the apparent inhibitory effects of high $\{K^+\}$ on cell-free protein synthesising systems are due not to K^+ but to concentrations of Cl^- ions exceeding those normally found in vivo. A breakdown of the sources of 'endogenous' K^+ in the standard wheat germ assay mix (Table 3.3.1.1.) shows that the 'endogenous' Cl^- concentration is 36mM. Hence addition of K^+ as KCl to a final concentration of 100mM leads to a Cl^- concentration of 80mM which is the upper limit of the physiological range (Berstein, 1954). The K^+ optimum for viral and cellular mRNA was therefore examined using CH_3COOK as the source of K^+. The results are shown in Figure 3.3.1.2. The optima are not significantly higher than those found with KCl but the range of incorporation is wider.

3.3.2. Optimum Mg^{++} Concentration.

The Mg^{++} optima observed for translation of various mRNAs in the wheat germ system in the absence of other polyvalent cations are in the range 2-4mM. The concentration of Mg^{++} for maximum mRNA activity/

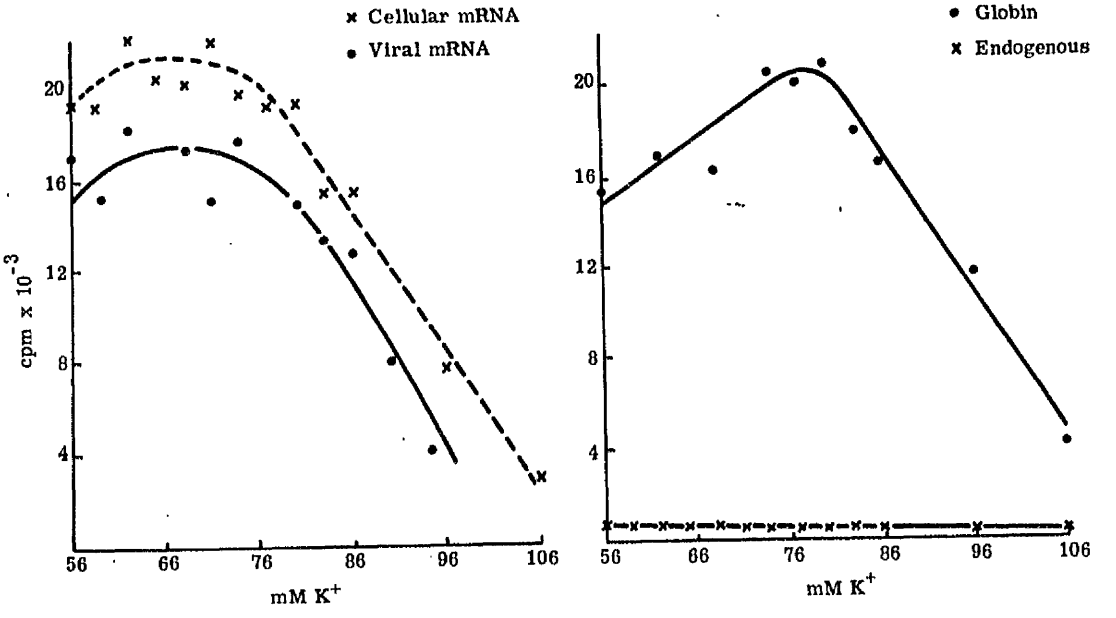


FIG. 3.3.1.1. THE EFFECT OF $\{K^+\}$ ON SYNTHESIS OF TCA PRECIPITABLE MATERIAL IN THE WHEAT GERM SYSTEM, I

The $\{K^+\}$ in wheat germ assay mixes (A3.4.11.) containing 12.2µg/ml viral (PrV) mRNA, 11.2µg/ml cellular (HeLa) mRNA, 17.3µg ml globin (rabbit reticlocyte) mRNA or no added mRNA was varied by addition of KCl. Assay mixes contained 50µCi/ml $\{^3H\}$-leucine (53Ci/mmol). The $\{Mg^{++}\}$was 2.5mM and no polyamine was added. After incubation the synthesis of TCA precipitable radioactivity (cpm/5µl) was measured by precipitation on filter discs and scintillation counting.

TABLE 3.3.11.

Contributions to the K^+ Concentration in Wheat Germ Assay Mixes.

Source of K^+	Contributing Compound	Final $\{K^+\}$ in assay
HEPES buffer	KOH	13.6mM
Energy Mix	KOH	2.3mM
Wheat Germ Extract	KOH	3.7mM
Wheat Germ Extract	KCl	36.0mM
Total		55.6mM

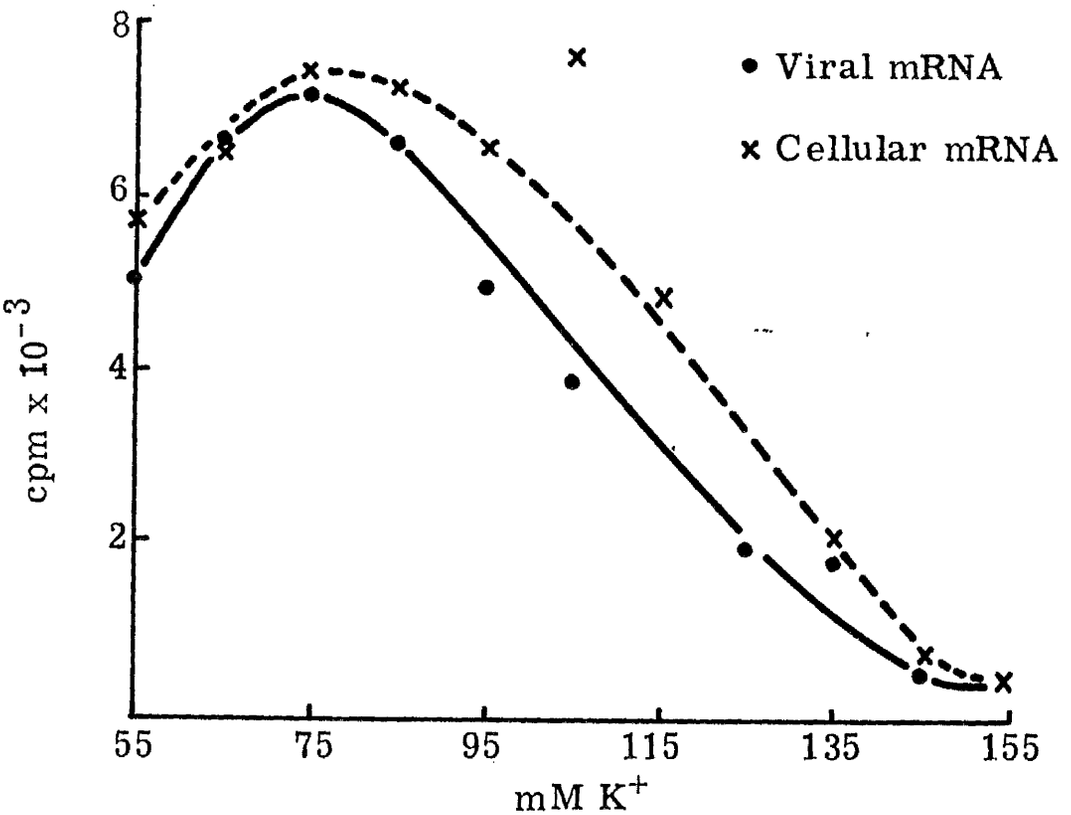


FIG. 3.3.1.2. THE EFFECT OF $\{K^+\}$ ON THE SYNTHESIS OF TCA PRECIPITABLE MATERIAL IN THE WHEAT GERM SYSTEM, II

The $\{K^+\}$ in wheat germ assay mixes (A3.4.11.) containing 28µg/ml viral (PrV) mRNA or 5.6µg/ml cellular (HeLa) mRNA and 50µCi/ml $\{^3H\}$-leucine (53Ci/mmol) was varied by addition of CH_3COOK. Assay mixes contained 2.5mM Mg^{++} and no added polyamine. After incubation the synthesis of TCA precipitable material (cpm/5µl) was determined by precipitation on filter discs and scintillation counting.

activity of the three mRNAs under investigation was determined in wheat germ assays containing 75mM K^+ and no polyamine. The effect of added $Mg(CH_3COO)_2$ is shown in Figure 3.3.2.1. Amino acid incorporation was maximum at 2.5mM Mg^{++}.

3.3.3. Optimum Polyamine Concentration.

Polyamines are polyvalent cations found in all procaryotic and eucaryotic cells. The polyamines found in eucaryotic cells are putrescine, spermidine and spermine, the biologically active species being the latter two. Their roles in cellular growth and metabolism are many and diverse and they are implicated in transcription and translation in all cells. Their effect is maximal at suboptimal concentrations of Mg^{++} but they cannot completely replace Mg^{++} (Cohen, 1971).

The optimum concentration of spermidine for mRNA activity in the wheat germ cell-free system at suboptimal Mg^{++} (1.5mM) was therefore investigated. Figure 3.3.3.1. shows the incorporation of amino acids at various spermidine concentrations in assays containing 75mM K^+. The optimum concentration of spermidine is 0.2mM for viral and cellular mRNA and 0.3mM for reticulocyte mRNA. The enhancement of incorporation by spermidine over that observed at optimum $\{Mg^{++}\}$ is shown in Table 3.3.3.1. Addition of spermidine at suboptimal Mg^{++} concentrations leads to a 1.6-fold increase in the incorporation observed at optimal $\{Mg^{++}\}$.

3.3.4. Definition of Standard Conditions.

The optimum concentrations of K^+ for translation of the three mRNAs vary slightly but a common region of high activity is observed. Hence/

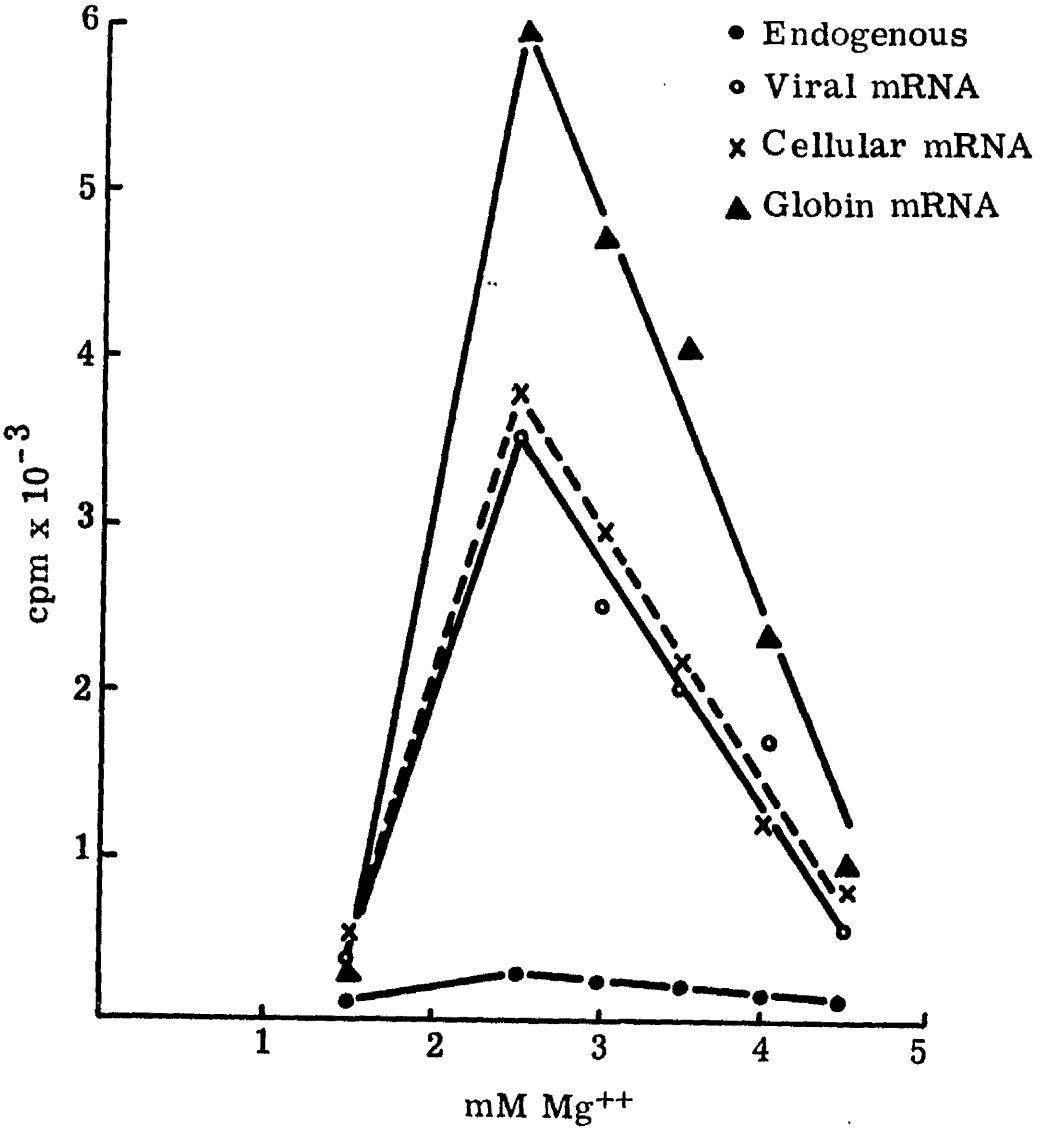


FIG. 3.3.2.1. THE EFFECT OF $\{Mg^{++}\}$ ON THE SYNTHESIS OF TCA PRECIPITABLE MATERIAL IN THE WHEAT GERM SYSTEM

The $\{Mg^{++}\}$ in wheat germ assay mixes (A3.4.11.) containing 16µg/ml viral (PrV) mRNA, 20.8µg/ml cellular (HeLa) mRNA, 16.5µg/ml globin (rabbit reticulocyte) mRNA or no added RNA was varied by addition of $Mg(CH_3COO)_2$. Assay mixes contained 50µCi/ml $\{^3H\}$-leucine (53Ci/mmol). No polyamine was added. After incubation the synthesis of TCA precipitable material (cpm/5µl) was measured by precipitation on filter discs and scintillation counting.

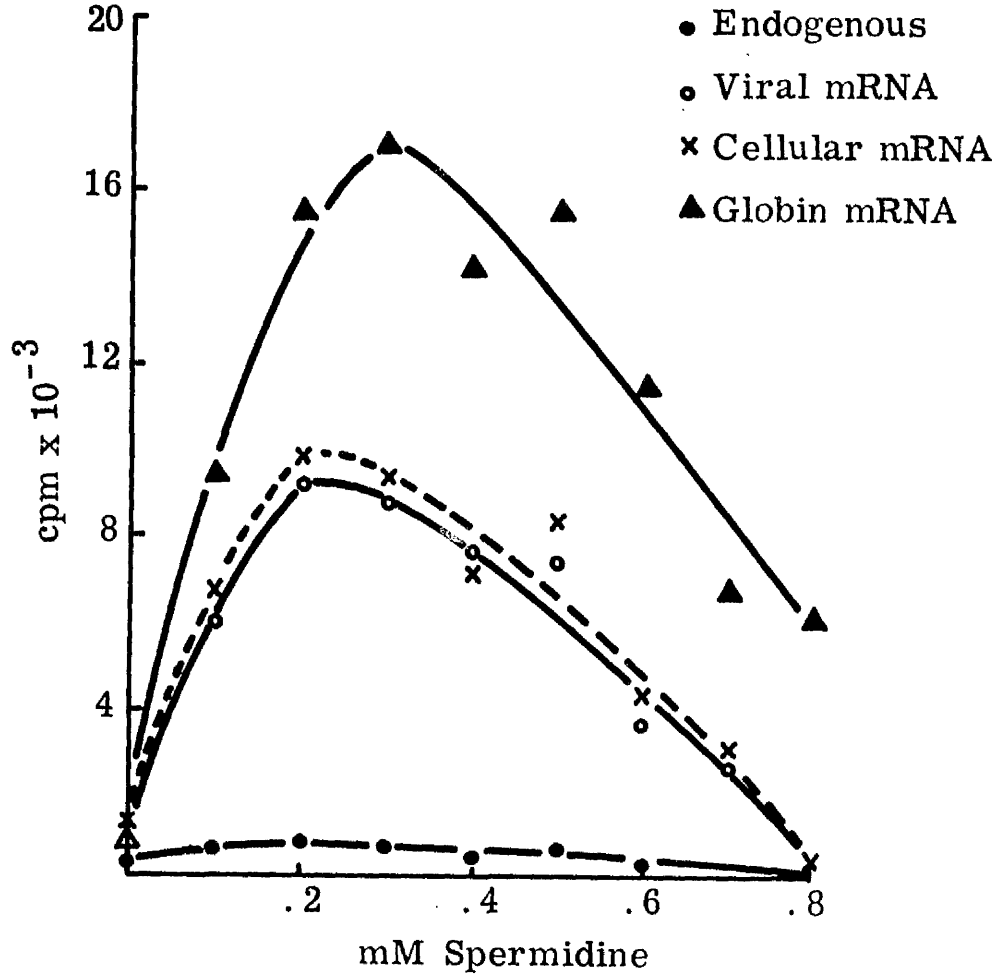


FIG. 3.3.3.1. THE EFFECT OF ADDED POLYAMINE ON SYNTHESIS OF TCA PRECIPITABLE MATERIAL IN THE WHEAT GERM SYSTEM

The effect of polyamine on wheat germ translation assays was investigated by addition of spermidine to assay mixes (A3.4.11.) containing viral (PrV, 16µg/ml) cellular (HeLa, 20.8µg/ml), or globin (rabbit reticulocyte, 16.5µg/ml) mRNAs or no added mRNA. The $\{Mg^{++}\}$ was 1.5mM and assays contained 50µCi/ml $\{^{3}H\}$-leucine (53Ci/mmol). After incubation the synthesis of TCA precipitable radioactivity (cpm/5µl) was measured by precipitation on filter discs and scintillation counting.

TABLE 3.3.3.1.

Enhancement of mRNA Stimulated {^{3}H}-leucine incorporation in Wheat Germ Extracts by Spermidine.

Exogenous mRNA	{Mg^{++}}	{Spermidine}	TCA precipitable cpm/5µl
Cellular	1.5mM		528
Cellular	2.5mM		38 43
Cellular	1.5mM	0.3mM	6121

Hence a standard $\{K^+\}$ of 75mM was chosen. The optimum concentration of Mg^{++} was found to be 2.5mM, however, addition of 0.2mM spermidine at 1.5mM Mg^{++} gave enhanced incorporation over that observed with Mg^{++} alone. Thus the standard salt conditions chosen were 75mM K^+, 1.5mM Mg^{++} and 0.2mM spermidine.

3.4. Endogenous Amino Acids in Wheat Germ Extracts.

Marcu and Dudock (1974) found that the stimulation of labelled amino acid incorporation above background by exogenous RNA in the wheat germ system was reduced by only 47% if amino acids were omitted from assays. Roberts and Paterson (1973) also suggest that there may be significant amounts of endogenous amino acids present after gel filtration. This has been confirmed by H.H. Singer (personal communication). Incorporation in mRNA stimulated assays with or without exogenous amino acids was therefore examined. Table 3.4.1. shows that reticulocyte mRNA stimulated incorporation is not significantly decreased in the absence of exogenous aas. This showed that endogenous amino acids were present in sufficient concentration to support the maximum amount of protein synthesis in this system.

The concentration of endogenous methionine and leucine was then estimated as follows. Standard assays containing no exogenous unlabelled amino acids were set up and incubated with increasing amounts of $\{^3H\}$-leucine or $\{^{35}S\}$-methionine. Trace amounts (0.02µCi/assay) of the other labelled amino acid were added to ensure that protein synthesis was constant. Incorporation into TCA precipitable material was determined by precipitating assay mix proteins in the presence of carrier and counting in solution (see Materials and Methods). This method was chosen because it was possible to include a tRNA deacylation step/

TABLE 3.4.1.

The Effect of Exogenous Amino Acids on RNA Stimulated Incorporation in Wheat Germ Extracts.

RNA	Assay mix	$\{^3H\}$-leu incorporated cpm/5µl
Endogenous	No exogenous aas	260
	19aas at 20µM + 1µM leu	250
Reticulocyte mRNA	No exogenous aas	27,190
	19aas at 20µM + 1µM leu	30,300

step, and because double-labelled samples are counted more efficiently in solution. The percentage of total label incorporated was calculated and plotted against the amount of added amino acid (Figure 3.4.1.). The incorporation of the tracer amino acid was found to be constant in both cases.

The decrease in incorporation declines exponentially and the endogenous amino acid content can be calculated in the following way: The amount of exogenous amino acid necessary to reduce the percentage incorporation at any point on the curve to half its value at that point is equal to the amount of endogenous amino acid plus the amount of exogenous amino acid already added at that point. However, in neither case was sufficient exogenous amino acid added to reduce the incorporation by 50% and since the decrease is not linear it was necessary to calculate the endogenous amino acid content as follows: Because the amount of protein synthesis is constant the incorporation of amino acid is a constant K pmol/assay. If the percentage incorporation when x pmol are added to the assay is Ix, then

$$Ix = \frac{K}{E+x} \times 100$$

where E = number pmol of endogenous amino acid. Similarly when y pmol are added

$$Iy = \frac{K}{E+y} \times 100$$

Rearranging

$$E = \frac{Iy(y) - Ix(x)}{Ix - Iy}$$

For leucine, if x = 20pmol and y = 80pmol, then 15µl wheat germ extract contains 83 pmol lew. Similarly, for methionine, if x = 10pmol and y = 30, 15µl extract contains 57 pmol mel. Hence, for these points the endogenous

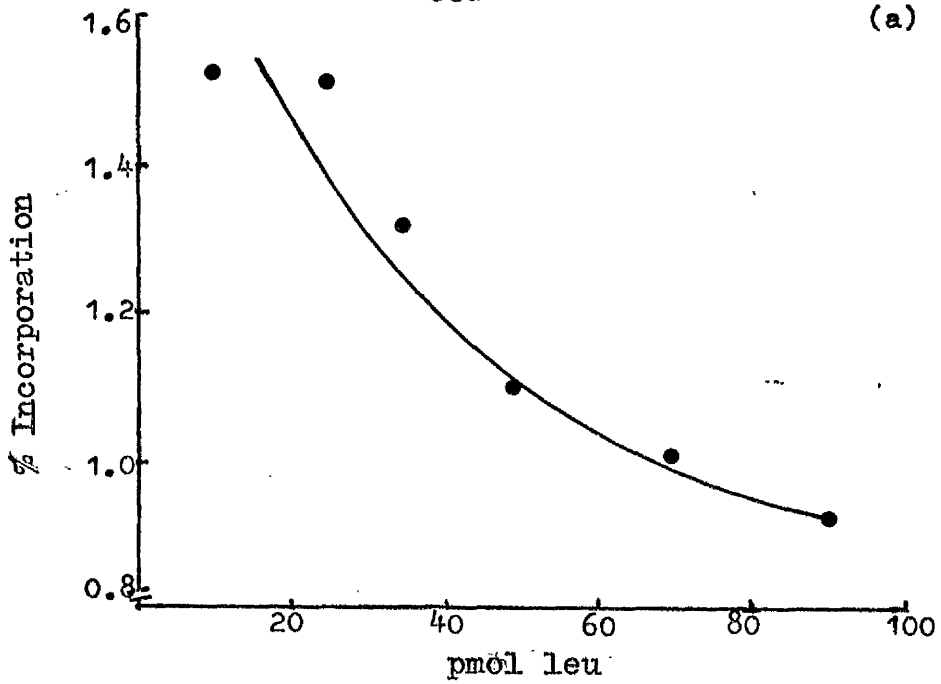
FIG. 3.4.1. PERCENTAGE INCORPORATION OF EXOGENOUS AMINO ACIDS IN THE WHEAT GERM SYSTEM AS A FUNCTION OF EXOGENOUS AMINO ACID CONCENTRATION

The concentration of amino acid in standard wheat germ assay mixes (A3.4.11.) containing 3.3µg/ml viral mRNA was varied by addition of different amounts of {^{3}H}-leucine or {^{35}S}-methionine. Assay mixes were incubated as described (A10.2.) and the synthesis of TCA precipitable radioactivity was measured by precipitation of assay mixes in the presence of carrier and scintillation counting. The percentage of added labelled amino acid incorporated into polypeptide was calculated.

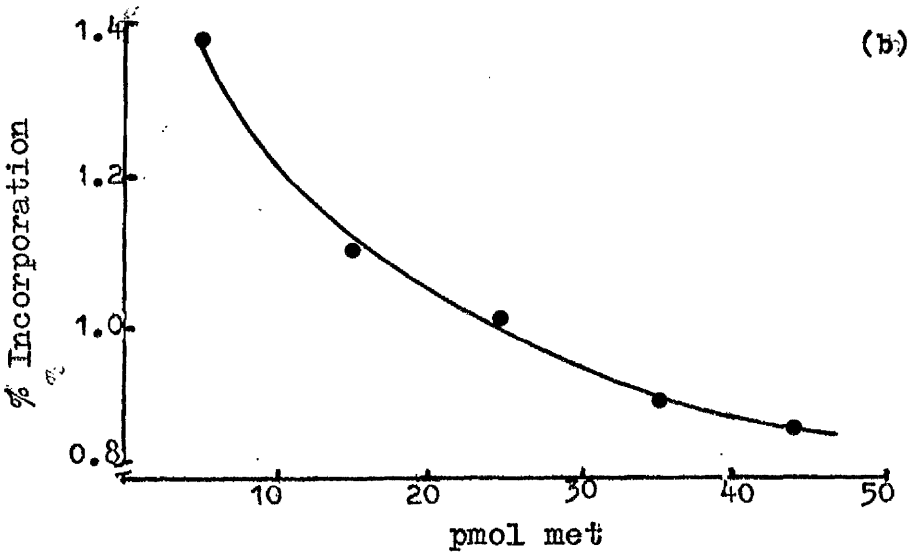
(a) % added {^{3}H}-leucine incorporated as a function of added leucine

(b) % added {^{35}S}-methionine incorporated as a function of added methionine

(a)


1.6
1.4
1.2
1.0
0.8
% Incorporation
20
40
60
80
100
pmol leu

(b)


1.4
1.2
1.0
0.8
% Incorporation
10
20
30
40
50
pmol met

concentrations of methionine and leucine are 3.8μM and 5.5μM respectively.

3.5. Time Course of the Wheat Germ System.

The time course of in vitro protein synthesis was characterised in two ways:

The incorporation of radioactive amino acids into TCA precipitable material in response to added viral mRNA was measured as a function of time. Standard assays labelled with 400μCi/ml {^{35}S}-methionine were incubated at 25^{o}C for the appropriate time. Incorporation was stopped by rapid addition of SDS and β-mercaptoethanol to final concentrations of 2% and 5% respectively and immediate incubation in a boiling water bath for 2 min. Duplicate 5μl aliquots were removed and the TCA precipitable activity determined by the filter method. The time course of the assay is shown in Figure 3.5.1. The incorporation was found to increase linearly for 45 min. No lag phase was detected.

The remainder of the assay material was used to examine the polypeptides synthesised as a function of time. 15μl aliquots of the samples were adjusted to 20% glycerol fractionated on 12% polyacrylamide gels and the in vitro products were detected by fluorography as described in the Materials and Methods. A fluorograph of viral mRNA products is shown in Figure 3.5.2. Products were detectable after 15 min incubation and some high molecular weight polypeptides were detected after 30 min. Maximum synthesis of large products was not achieved until after 60 min incubation although incorporation plateaued after 45 min.

3.6/

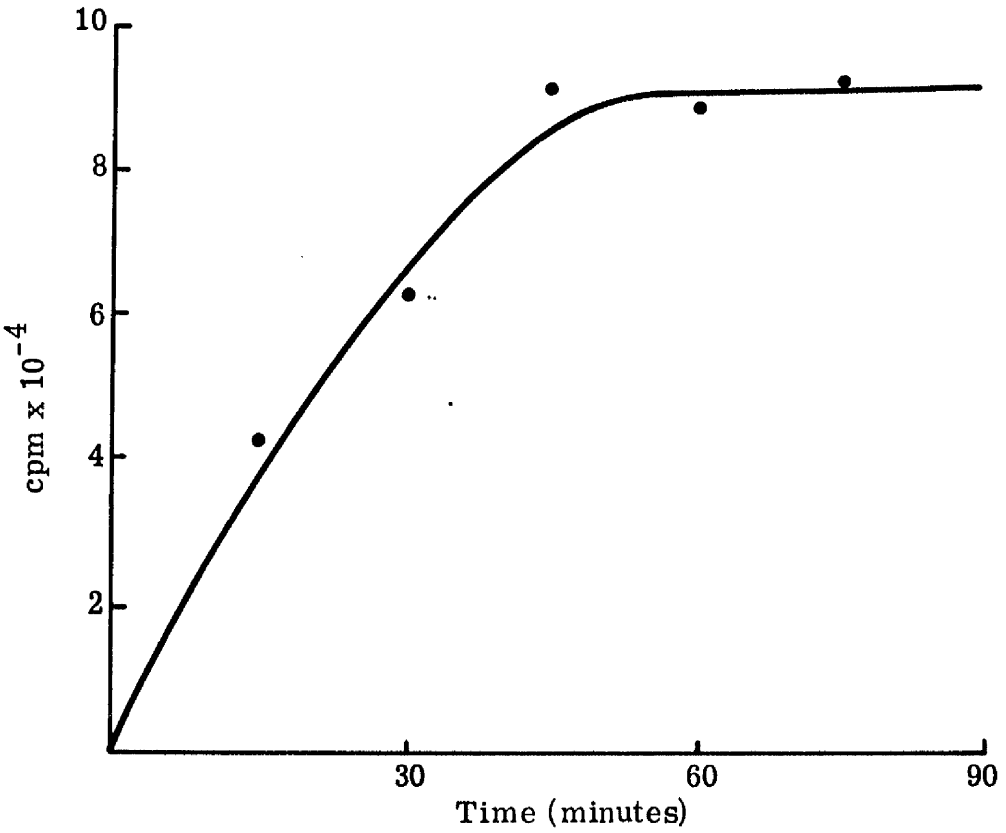
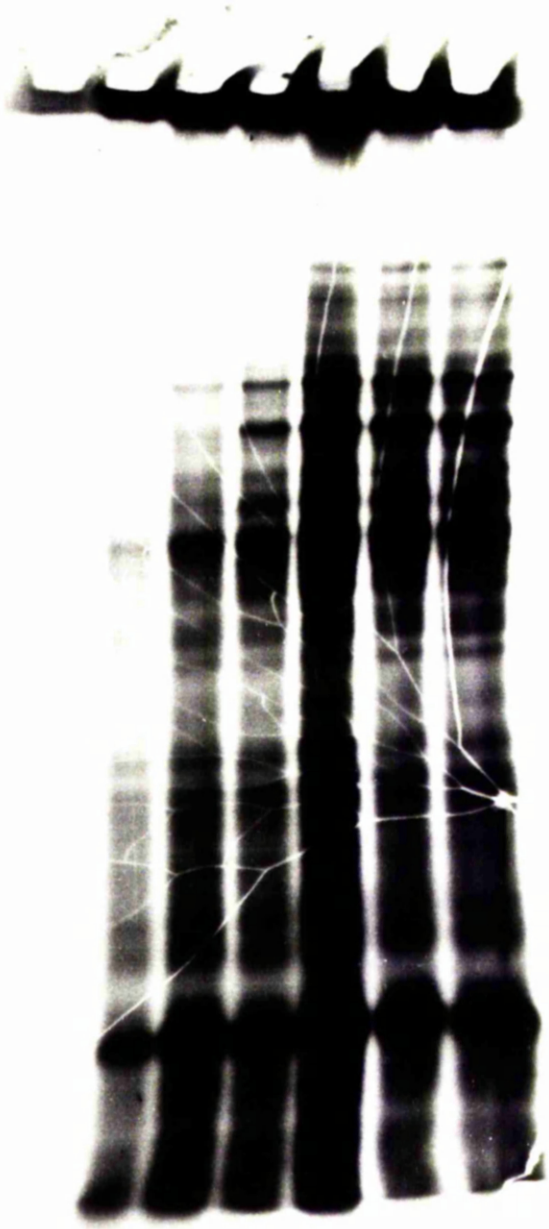


FIG. 3.5.1. TIME COURSE OF SYNTHESIS OF TCA PRECIPITABLE MATERIAL IN THE WHEAT GERM SYSTEM

Assay mixes containing PrV mRNA (14.4µg/ml) and 460µCi/ml {^{35}S}-methionine (1035Ci/mmol) were incubated for the appropriate time. Incorporation was stopped by denaturing in SDS and β-mercaptoethanol as described (A10.2) and the synthesis of TCA precipitable radioactivity (cpm/3µl) was measured by precipitation on filter discs and scintillation counting.

FIG. 3.5.2. TIME COURSE OF POLYPEPTIDE SYNTHESIS IN THE WHEAT GERM SYSTEM

The relationship between the time of incubation and the polypeptide species detectable in wheat germ assays was examined by polyacrylamide gel electrophoresis of aliquots of assay mixes incubated with PrV mRNA for different lengths of time (see previous Figure, 3.5.1.). Aliquots containing approximately 1/4 of the original assay mix were made 20% in glycerol and the polypeptides were separated on a 12% polyacrylamide gel. The gel was processed for fluorography and exposed to X-ray film.



0 15 30 45 60 75 90

Minutes

3.6. Dose Dependence of the Wheat Germ System.

The dose response of the wheat germ system to exogenous RNAs was characterised in the same way as the time course.

The incorporation of {^{35}S}-methionine into TCA precipitable activity was determined by the filter method in wheat germ assays programmed with 0.8-35µg/ml exogenous mRNA. The dose response for viral and cellular mRNA is shown in Figure 3.6.1. The maximum stimulation of incorporation was observed with RNA concentrations of approximately 8µg/ml. Maximum stimulation was 9-10 times the endogenous incorporation and represented incorporation of 5 pmol methionine.

The effect of high concentrations of RNA was examined using polysomal RNA not bound to oligo-(dT) cellulose. This material was precipitated three times to remove SDS. The stimulation of incorporation is shown in Table 3.6.1. Increasing the concentration inhibited the mRNA activity.

The polypeptides synthesised *in vitro* in response to various concentrations of mRNA were examined by polyacrylamide gel electrophoresis and fluorography. Fluorographs of the *in vitro* products of viral and cellular mRNA are shown in Figures 3.6.2.(a) and 3.6.2.(b). There is an increase in synthesis of high molecular weight polypeptides as the mRNA concentration is raised to 6-8µg/ml. This corresponds to the maximum stimulation of incorporation of {^{35}S}-methionine into TCA precipitable material (Figure 3.6.1.). Further increasing the concentration of exogenous mRNA decreases the proportion of high molecular weight products.

3.7. Discussion.

mRNA was defined as RNA which bound to poly-(U) Sepharose or oligo/

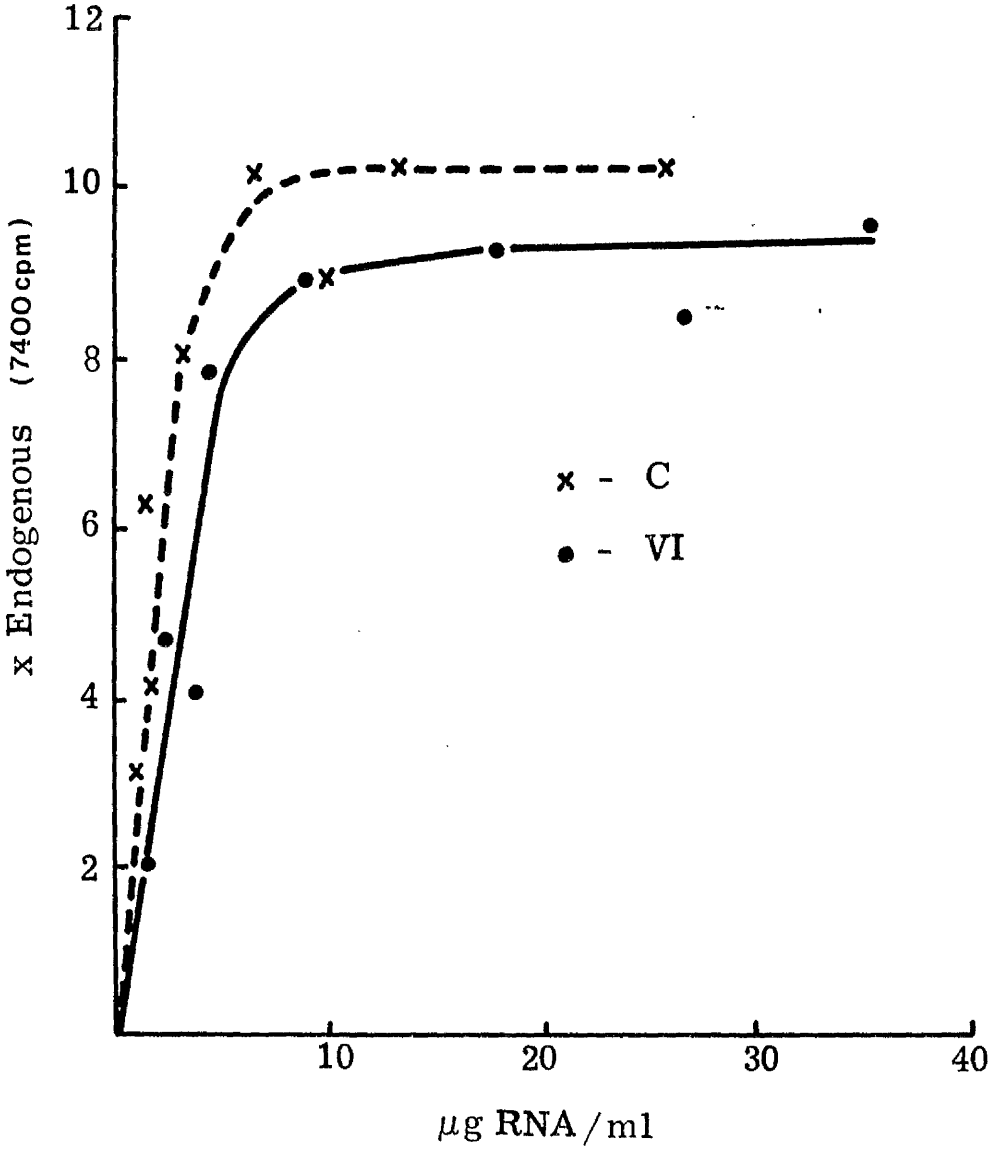


FIG. 3.6.1. DOSE DEPENDENCE OF SYNTHESIS OF TCA PRECIPITABLE MATERIAL IN THE WHEAT GERM SYSTEM

Wheat germ translation assays in which the concentration of exogenous mRNA was varied from 0 - 35μg/ml were carried out as described in the Materials and Methods (A10.2.). Assay mixes contained 460μCi/ml {^{35}S}-methionine (1080Ci/mmol). Synthesis of TCA precipitable radioactivity was measured by precipitation on filters and scintillation counting. The stimulation of endogenous incorporation (no exogenous mRNA) for cellular (C) and viral (VI) mRNA was calculated.

TABLE 3.6.1.

Stimulation of Amino Acid Incorporation in Wheat Germ Assays by Polysomal RNA not bound to Oligo-(dT) Cellulose Columns.

RNA Species	RNA Concentration µg/ml	Stimulation*
Cellular unbound	86	4.1
" "	156	2.0
Viral unbound	70	4.5
" "	138	1.0

* Times endogenous (980cpm)

FIG. 3.6.2. DOSE DEPENDENCE OF POLYPEPTIDE SYNTHESIS IN THE WHEAT GERM SYSTEM

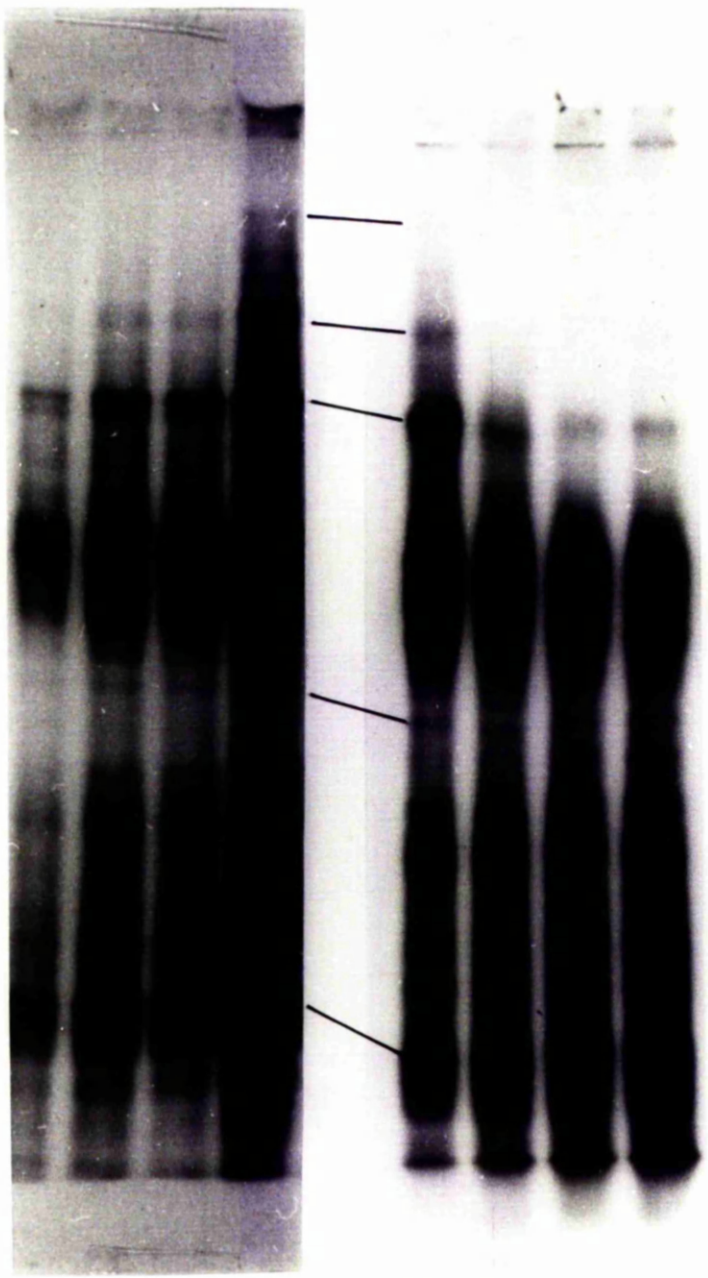
The relationship between the concentration of mRNA and the polypeptide species detectable in wheat germ assays was examined by polyacrylamide gel electrophoresis of aliquots of wheat germ translation assays incubated with a range of viral (PrV) or cellular (HeLa) mRNAs (see Fig. 3.6.1.). Aliquots containing approximately 1/4 of the assay mixes were treated for electrophoresis and the polypeptides were separated on 12% polyacrylamide gels. The gels were processed for fluorography and exposed to X-ray film.

Different gels were used for the upper and lower ranges of mRNA concentration and the migration distances were different. The connecting lines indicate bands with the same relative mobility.

(a) viral mRNA stimulated assays

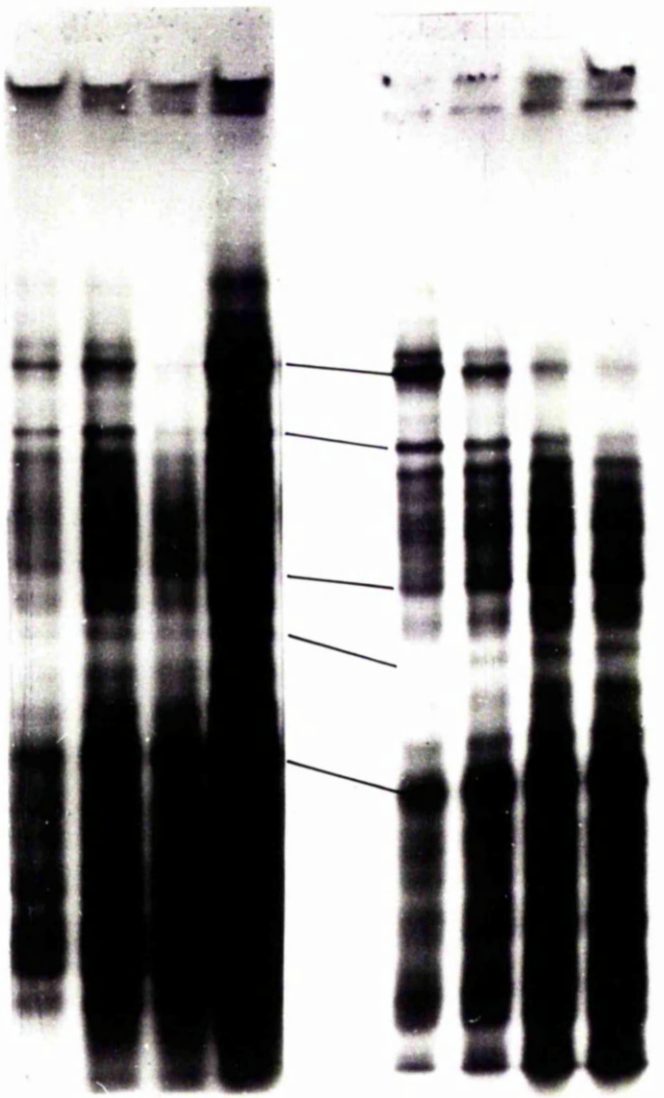
(b) cellular mRNA stimulated assays

Dose Response - VI mRNA



(a)

Dose Response - C mRNA



0.6 1.2 2.0 3.2 6.4 12.8 19.2 25.6

µg RNA/ml

(b)

oligo-(dT) cellulose. This definition was verified in this chapter by the finding that "mRNA" stimulated incorporation of amino acids into TCA precipitable material.

No differences were observed in the optimum concentration of cations for translation of viral and cellular mRNA whereas reticulocyte mRNA exhibited a slightly higher $\{K^{+}\}$ optimum. These results are important in relation to models for translational control and will be discussed further.

The reticulocyte mRNA requirements for K^{+} and Mg^{++} differed from those previously described for this messenger (Roberts & Paterson, 1973) emphasising the necessity of determining these conditions for individual mRNA and wheat germ preparations.

In agreement with other reports (Hunter et al., 1977; Marcu and Dudock, 1974; Konecki et al., 1975; Atkins et al., 1975; Ricciardi et al., 1978), addition of polyamines enhanced the maximum incorporation observed with Mg^{++} alone. Polyamines increase the elongation rate (Hunter et al., 1977) and there is also evidence that they have a role in initiation (Konecki et al., 1975). tRNA charging is enhanced by polyamines and this could increase the incorporation of limiting amino acids (Tabor & Tabor, 1972).

The presence of endogenous amino acids in the wheat germ extract is not surprising because the combination of coarse grade Sephadex and a high flow rate leads to large zone broadening. Endogenous amino acids lower the specific activity of the labeled amino acid. Therefore in order to obtain as high a specific activity as possible in assays where the in vitro products were to be examined relatively large amounts (500μCi/ml) of high specific activity (>800Ci/mmol) $\{^{35}S\}$-methionine were used. In other studies lower specific activities have been used to obtain equivalent incorporation

(Roberts and Paterson, 1973; Prives et al., 1974; Hunter et al., 1977). It is possible that their extracts contained lower amounts of endogenous amino acids.

Incorporation of amino acids into TCA precipitable material was determined either by hot TCA precipitation on filters or by TCA precipitation from solution following alkali treatment to discharge tRNAs. tRNA is TCA precipitable (Soffer, 1974) and hence precipitation of charged tRNAs may overestimate the amount of amino acid incorporated into polypeptides. For most purposes this is not important because the amount will be constant between assays. However, precipitation of charged tRNAs may be important where the amount of labelled amino acid was varied as in the determination of endogenous amino acids. Therefore in this case tRNAs were discharged by alkali treatment.

Incorporation of {^{35}S}-methionine into TCA precipitable material increased linearly for 45 min. The absence of the lag phase noted by others (Roberts and Paterson, 1973; Marcu and Dudock, 1974) may be due to polyamine induced suppression of the lag phase (Thang et al., 1976) or to the fact that the first time point was 15 min. Synthesis of the highest molecular weight product was not achieved until after 60 min incubation. It is probable that after 45 min the rate of protein synthesis had slowed considerably so increases in incorporation were insignificant in terms of the total incorporation but elongation of high molecular weight polypeptides was not completed until after 60 min incubation.

Excess polysomal RNA was found to inhibit incorporation in the wheat germ system. The reason for this is not known. Sonenshein and Brawerman (1976), have suggested that polysomal RNA contains an inhibitor of translation but this has not been identified. Although increasing the concentration of mRNA to 35µg/ml did not inhibit incorporation, the/

the maximum sized products were not detectable at mRNA concentrations exceeding 10µg/ml. A similar observation has been reported for Brome Mosaic Virus (Zagorski, 1977). One possible explanation for this is that by increasing the RNA concentration the number of mRNAs initiated increases and the average polysome size decreases. If mRNA is protected from endonucleolytic cleavage by ribosomes, then fewer ribosomes per mRNA would lead to an increased rate of degradation and a decrease in the number of high molecular weight products.

CHAPTER 4

Characterisation of Cell-Free Translation Products.

4.1. Introduction.

The experiments described in the preceeding chapter showed that the wheat germ cell-free system is efficient in synthesis of acid precipitable polypeptides in response to mRNA isolated from PrV infected and mock-infected HeLa cells and reticulocytes. The size of the products was shown to depend on the time of incubation and the concentration of mRNA. This chapter describes attempts to correlate the *in vitro* products with the polypeptides found *in vivo*.

Before carrying out classification, the synthesis of high molecular weight polypeptides in the wheat germ system was examined. Most of the proteins whose synthesis in wheat germ extracts has been verified have molecular weights less than 100,000 and difficulties have been experienced in synthesising larger products (Anderson *et al.*, 1974). mRNA from PrV-infected and mock-infected HeLa cells should code for a number of polypeptides with molecular weights greater than 100,000 and hence a system capable of translating these was necessary.

4.2. Cell-Free Synthesis of Globin.

Globin is a small polypeptide having a molecular weight of 17,000. Reticulocyte mRNA, which contains 90% globin mRNA (Lodish & Desalu, 1973), or the 9S globin mRNA prepared from reticulocyte mRNA has frequently been used to examine the ability of cell-free systems to translate exogenous RNA and globin has been identified by tryptic digestion and peptide mapping (Roberts & Paterson, 1973; Mathews, 1972) or by ion-exchange chromatography (Gurdon *et al.*, 1973).

Reticulocyte/

Reticulocyte mRNA was prepared as described in Materials & Methods and translated in the wheat germ system with exogenous polyamine completely replaced by 2.5mM Mg^{++}. The assay products were separated on an 18% polyacrylamide gel and detected by fluorography. Chymotrypsinogen A and cytochrome C were used as standards to estimate molecular weights. The fluorograph is shown in Figure 4.2.1. Two mRNA preparations were translated and in both cases the major product had an estimated molecular weight of 17,000. Hence, it was concluded that complete globin chains were being synthesised.

4.3. Synthesis of High Molecular Weight Polypeptides.

Fluorographs of the products of viral and cellular mRNA translation were shown in 3.5 and 3.6. The optimum concentration of mRNA and the minimum time of incubation for synthesis of the largest polypeptides were defined. The maximum size of polypeptides is further examined in this section in order to clarify the role of monovalent (K^+) and polyvalent (polyamine) cations.

Mathews and Osborn (1974) measured the elongation rate for the Krebs II Ascites system programmed with encephalomyocarditis (EMC) virus mRNA and found that it was 2.3 times higher at 150mM K^+ than at 50mM, although the overall amino acid incorporation was maximum at 100mM. Harwood et al., (1975) and Benveniste et al., (1976) described synthesis of pro α collagen (molecular weight 155,000) in wheat germ extracts of 150mM K^+. At lower $\{K^+\}$, incomplete polypeptides were synthesised although the total incorporation was higher. This K^+ requirement for synthesis of high molecular weight polypeptides may be due to the fact that the increased elongation rate should allow complete translation before the mRNA is degraded by endogenous nucleases/

FIG. 4.2.1. PRODUCTS OF RABBIT RETICULOCYTE mRNA TRANSLATION IN THE WHEAT GERM SYSTEM

Two preparations of rabbit reticulocyte mRNA were added to wheat germ assay mixes (A3.4.11.) which contained 2.5mM Mg^{++}, no added polyamine, and 150μCi/ml {^{35}S}-methionine (920Ci/mmol). Assay mixes were incubated as described (A10.2.). 10μl aliquots were treated for electrophoresis and the polypeptides were separated on an 18% polyacrylamide gel. Chymotrypsinogen A and cytochrome C were run in parallel slots as standards. The gel was stained with Coomassie Brilliant Blue, processed for fluorography and exposed to X-ray film.

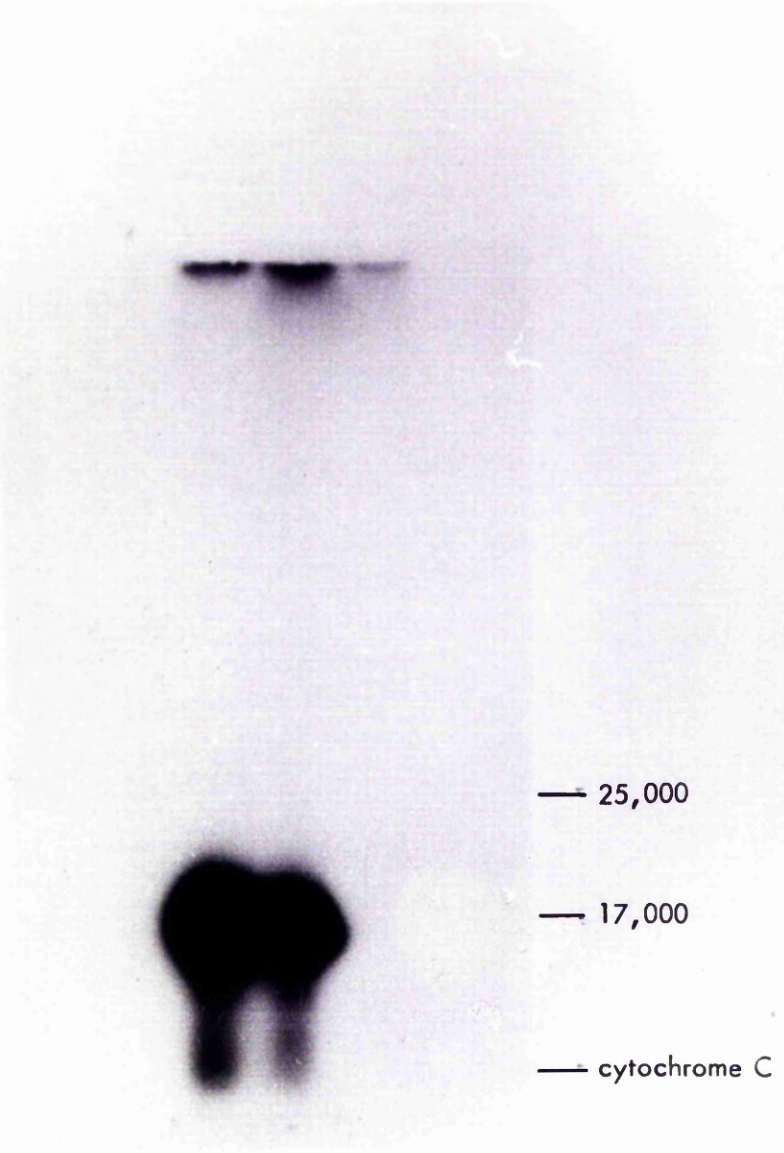
Molecular weights calculated from the standards are shown.

Track:

A reticulocyte mRNA I (3.2μg/ml)

B reticulocyte mRNA II (37.4μg/ml)

C no added mRNA


25,000
17,000
cytochrome C

nucleases or to an absolute requirement for K^+ to stabilise mRNA-ribosome-elongation factor interactions. The translation products of viral mRNA were examined at 4 concentrations of K^+ (Figure 4.3.1.). At 130mM K^+ there is no detectable polypeptide synthesis. At 100mM K^+ there is much less incorporation than at 75mM K^+ but no significant change in the maximum size of the products synthesised. However, one effect is evident. Differential suppression of polypeptide synthesis can be detected. Visual inspection of the fluorograph shows that some polypeptides are more resistent to high $\{K^+\}$ than others. Polypeptides a-g are synthesised in higher proportions at 100mM K^+ while the synthesis of polypeptide A is much more suppressed than the synthesis of other polypeptides.

An alternative method of increasing the size of products was used by Villa-Komaroff et al., (1975). They showed that in a HeLa cell free system, the size of products is increased by raising the $\{K^+\}$ from 90mM to 155mM after 15 min and continuing incubation at the increased $\{K^+\}$ for a further period. The effect of such a "salt shift" on translation of PrV-infected and mock-infected HeLa cell mRNA in wheat germ was examined by incubating assays for 15 min at 75mM K^+ and then raising the $\{K^+\}$to 148mM by addition of 1/25 volume of 1.9M KCl and incubating for a further 75 min. The inhibition of synthesis brought about by salt shift is less than that observed when assays are incubated with high $\{K^+\}$ from the start of the assay (Table 4.3.1.). The effect of salt shift on the size of products of viral mRNA is shown in Figure 4.3.2. Under salt shift conditions both the maximum molecular weight detected and the proportion of high molecular weight material synthesised are increased. A polypeptide with the same electrophoretic mobility as the major capsid protein of PrV was synthesised in salt shift assays

but/

FIG. 4.3.1. THE EFFECT OF $\{K^+\}$ ON POLYPEPTIDES SYNTHESISED IN THE WHEAT GERM SYSTEM

Viral (PrV, 8.8µg/ml) was added to wheat germ assay mixes, (A3.4.11.) in which the $\{K^+\}$ was varied by addition of KCl. Assay mixes contained 570µCi/ml $\{^{35}S\}$-methionine (1080Ci/mmol). After incubation, 10µl aliquots were withdrawn, treated for electrophoresis and separated on 12% polyacrylamide gel. The gel was processed for fluorography and exposed to X-ray film.

The tracks showing products synthesised at 100 and 130 mM K^+ were exposed to 2½ times as long as the other two tracks. Polypeptides designated a-g were synthesised preferentially at 100mM K^+ and polypeptide A was suppressed preferentially at 100mM K^+.

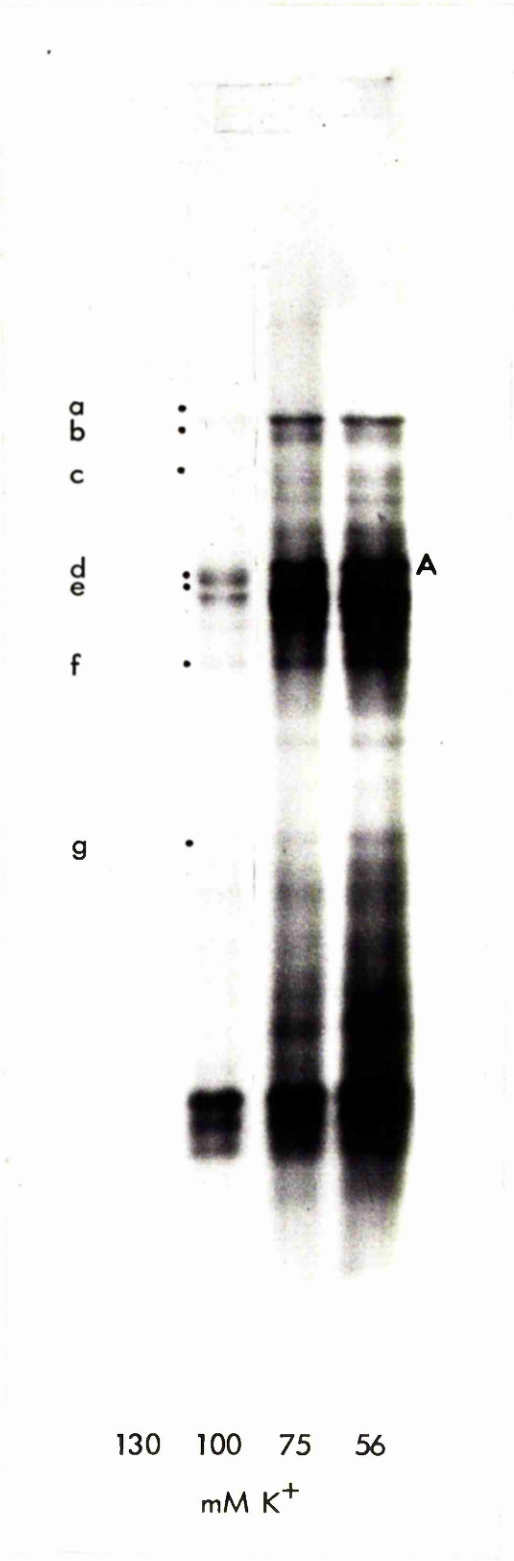

a
b
c
d
e
f
g
A
130
100
75
56
mM K+

TABLE 4.3.1.

Inhibition of protein synthesis in wheat germ extracts brought about by variations in the concentration of K^+.

Exogenous mRNA	Initial $\{K^+\}$	Final $\{K^+\}$	cpm/µl	% Inhibition
Viral	75mM	75mM	50,300	
	75mM	148mM	47,700	5.2
Viral	75mM	75mM	56,400	
	75mM	148mM	41,200	27.9
Viral	74mM	74mM	32,800	
	144mM	144mM	9,100	72.2
Viral	75mM	75mM	155,400	
	130mM	130mM	29,500	81.0

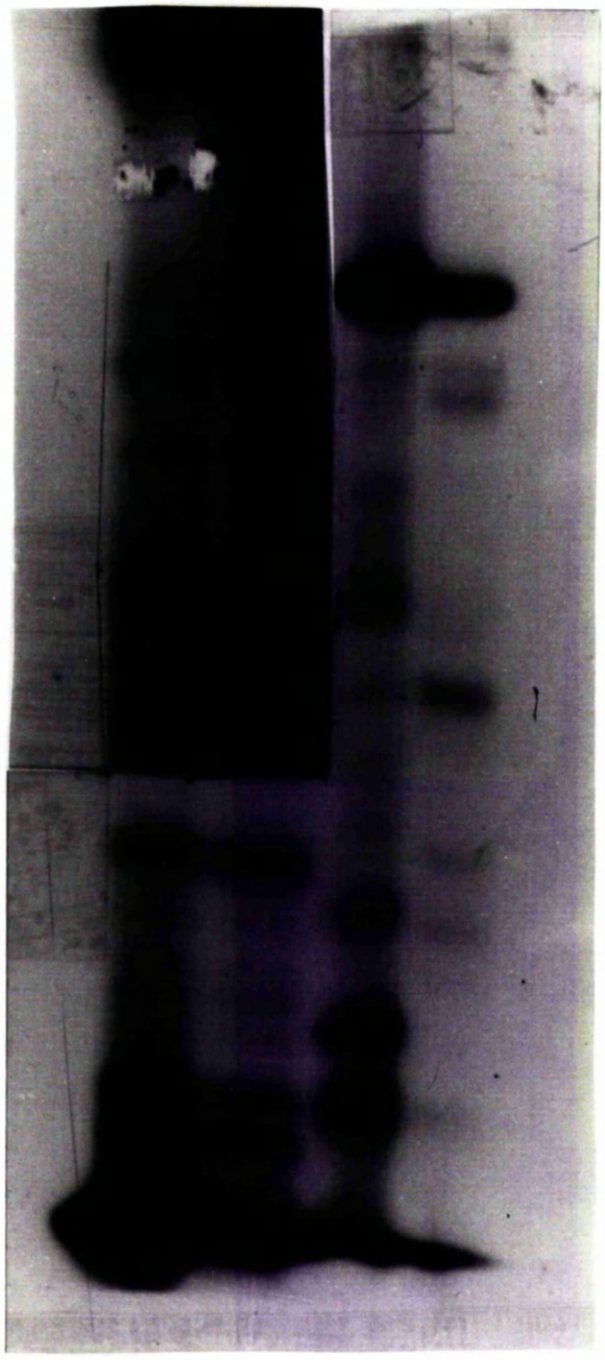
FIG. 4.3.2. THE EFFECT OF INCREASING THE $\{K^+\}$ DURING INCUBATION OF THE POLYPEPTIDES SYNTHESISED IN WHEAT GERM ASSAYS

Wheat germ translation assays of viral (PrV, 38µg/ml mRNA were carried out under constant salt conditions (75mM K^+, 2.5mM Mg^{++}) or salt shift conditions where the initial cation concentrations were the same as for constant salt assays but the $\{K^+\}$ was raised to 145mM by addition of 2µl 1.9mM KCl after 15 min incubation. Assays were labelled with 125µCi/ml $\{^{35}S\}$-methionine (920Ci/mmol). 20µl aliquots were treated for electrophoresis and coelectrophoresed in a 10% polyacrylamide 0.5% DATD gel with PrV virion and capsid (A9). The gel was processed for fluorography and exposed to X-ray film. A longer exposure time was used to detect faint bands.

V: viral mRNA, constant conditions

V+K^+: viral mRNA, salt shift

The top left portion of the gel was exposed for 14 days and the remainder for 2 days, because the high molecular weight <u>in vitro</u> products were present in low amounts.



MAJOR CAPSID

V V+ K+ Capsid Virion

but was not detectable under low salt conditions.

Hunter et al., (1977) examined the role of polyamines in the wheat germ system. They found that addition of spermine and spermidine increased the elongation rate and enhanced the synthesis of high molecular weight products. Increases in the size of products in the presence of polyamines have been reported by several groups (Fritsch et al., 1977; Thang et al., 1976). Figure 4.3.3. shows that the maximum molecular weight of products synthesised in the presence of spermidine (Tracks A & B) is greater than that of the polypeptides synthesised in low K^+ concentrations (Tracks G & H).

Qualitatively the increase in molecular weight observed when spermidine is added to the assay system is similar to that brought about by increasing the K^+ concentration (Tracks D & E). However, spermidine also enhances the total incorporation and hence translation in the presence of spermidine was considered to be a more efficient method of increasing molecular weights and was used in the following characterisation of products.

4.4. Classification of in vitro products by co-migration studies.

Preliminary classification of the polypeptides synthesised in vitro as viral or cellular coded was carried out by comparison of electrophoretic mobilities in SDS polyacrylamide gels. A fluorograph of the in vitro products together with cell lysates prepared from PrV-infected and mock-infected HeLa cells labelled with $\{^{35}S\}$-methionine from 4.5 to 5.5 h post-infection and purified PrV capsid proteins is shown in Figure 4.4.1. Polypeptide molecular weights were calculated using albumin, trypsin inhibitor, chymotrypsinogen A, ovalbumin, β galactosidase and RNA polymerase as standards. The largest poly-

peptide/

FIG. 4.3.3. POLYPEPTIDES SYNTHESISED UNDER DIFFERENT CATIONIC CONDITIONS IN THE WHEAT GERM SYSTEM

Wheat germ translation assays of viral (PrV, 8.9µg/ml), cellular (HeLa, 12.8µg/ml) and endogenous mRNA were carried out under standard salt conditions (0.2mM spermidine, 75mM K^+, 1.5mM Mg^{++}, see A3.4.11.) without added polyamine at optimum $\{Mg^{++}\}$ (75mM K^+, 2.5mM Mg^{++}), or under salt shift conditions where the initial cationic conditions were 75mM K^+, 2.5mM Mg^{++} but were altered after 15 min incubation by addition of 2µl 1.9M KCl so that the final $\{K^+\}$ was 148mM. Assays contained 570µCi $\{^{35}S\}$-methionine (1080Ci/mmol). 10µl aliquots of assay mixes were treated for electrophoresis and separated on a 12% polyacrylamide gel. The gel was processed for fluorography and exposed to X-ray film.

Track		cpm/track
A	Cellular mRNA, standard salt.	3,300
B	Viral mRNA " "	11,400
C	Endogenous mRNA, " "	13,200
D	Cellular mRNA without added polyamine	7,000
E	Viral mRNA " " "	11,500
F	Endogenous mRNA " " "	12,000
G	Cellular mRNA salt shift	2,700
H	Viral mRNA " "	9,800
I	Endogenous mRNA " "	4,000

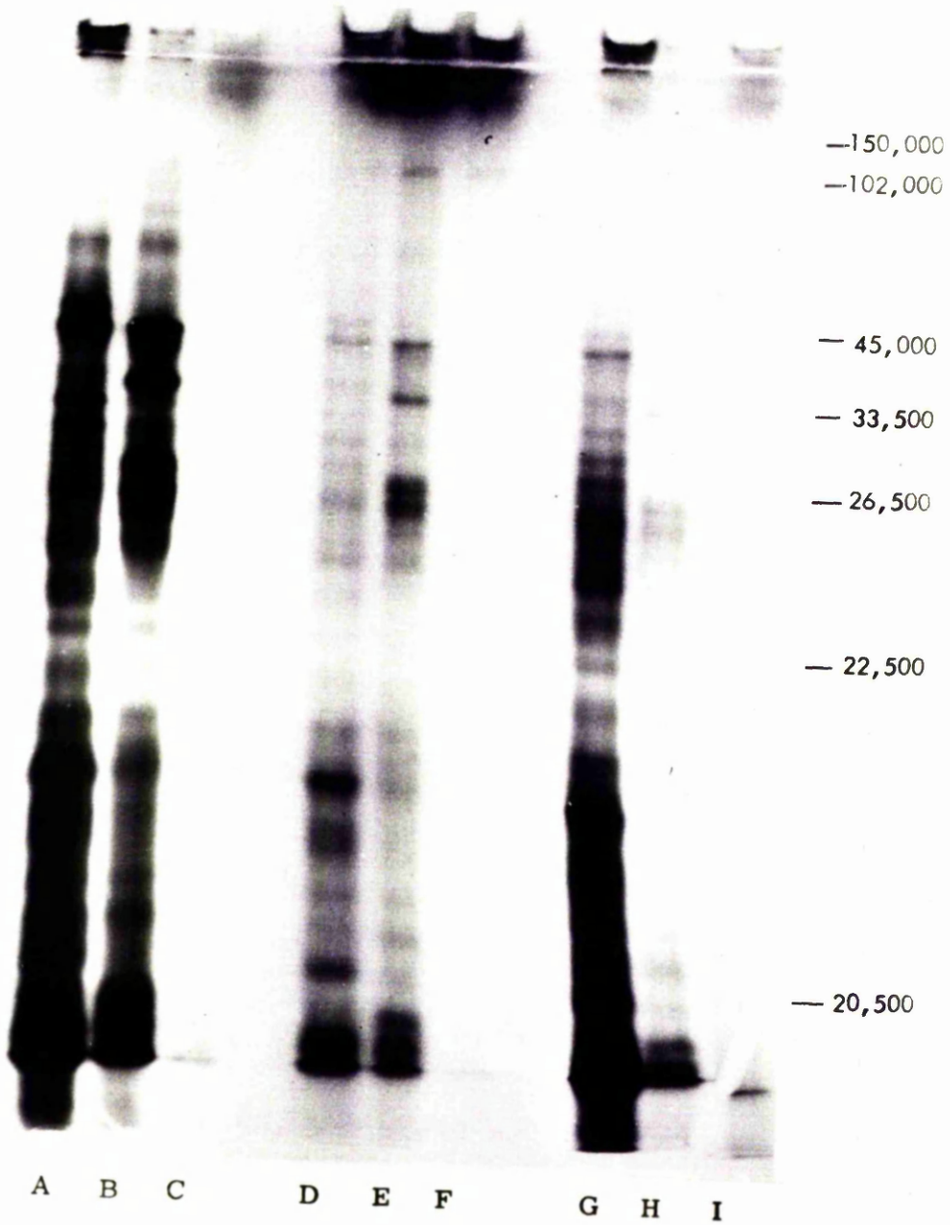
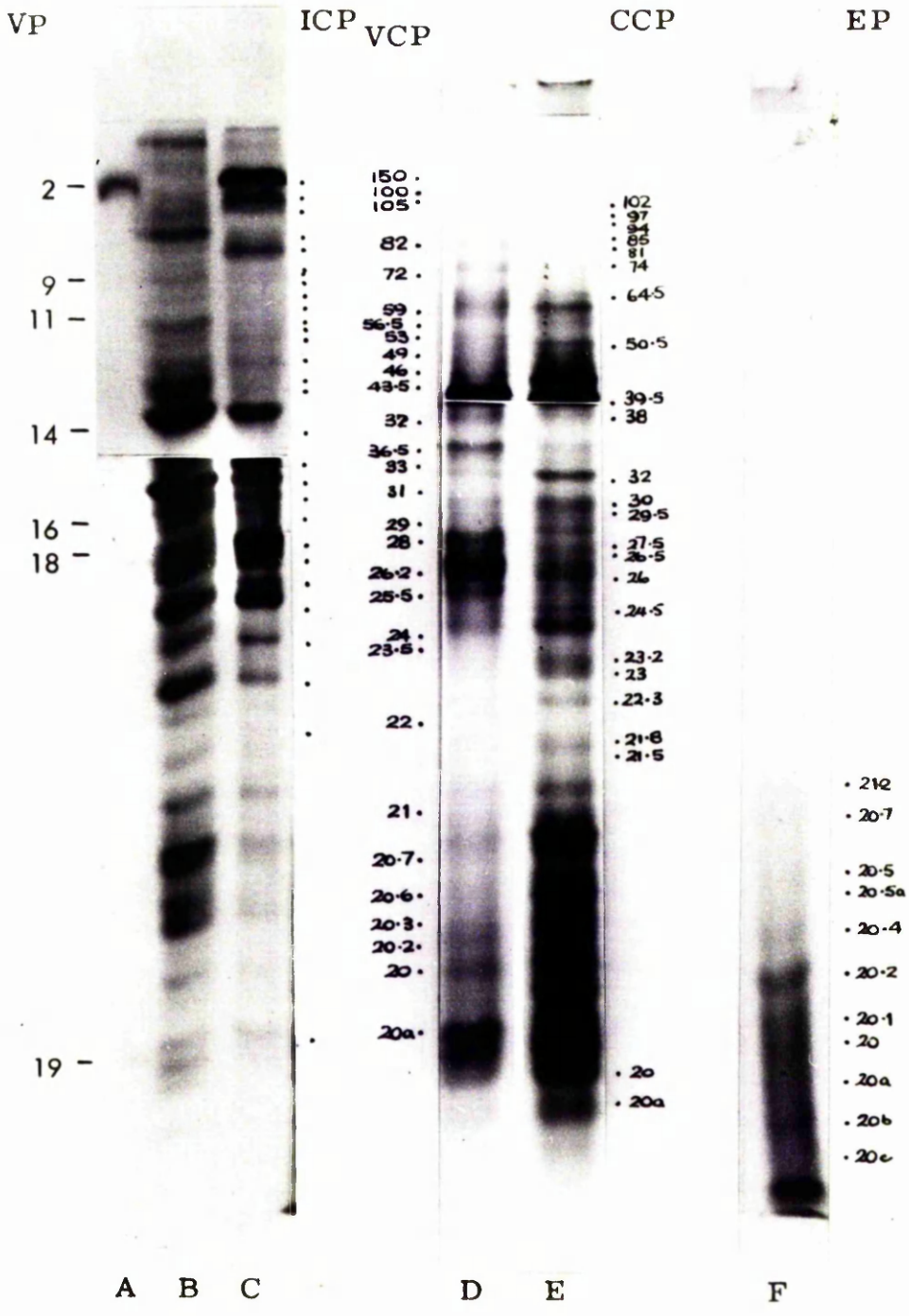

—150,000
—102,000
— 45,000
— 33,500
— 26,500
— 22,500
— 20,500
A B C D E F G H I

FIG. 4.4.1. CLASSIFICATION OF PRODUCTS OF PrV AND HeLa CELL mRNA TRANSLATED IN THE WHEAT GERM SYSTEM BY COMIGRATION

Wheat germ translation assays of viral (PrV, 6.0µg/ml) cellular (HeLa 3.2µg/ml) and wheat germ endogenous mRNA were carried out as described in the Materials and Methods (A10). Assay mixes contained 500µCi/ml {^{35}S}-methionine (1080Ci/mmol). 10µl aliquots from the assays were denatured for electrophoresis and separated on a 12% polyacrylamide gel in parallel with {^{35}S} methionine-labelled PrV capsid (A9) and cell lysates prepared from PrV-infected and mock-infected HeLa cells labelled with {^{35}S}-methionine so that the in vivo polypeptides correspond to those coded by the viral and cellular mRNAs (A9).

PrV capsid proteins are labelled in accordance with the virion polypeptides (VP) described by Stevely (1975); polypeptides detectable in infected but not mock-infected cell lysates are labelled infected cell polypeptides (ICP); polypeptides detectable in in vitro products of viral but not cellular mRNA are labelled viral coded polypeptides (VCP); polypeptides detectable in in vitro products of both viral and cellular mRNA are labelled cellular coded polypeptides (CCP) and the polypeptides detectable in wheat germ assays in the absence of exogenous mRNA are labelled endogenous polypeptides (EP). The various polypeptides are designated by their molecular weights x 10^{-3}.

Track		cpm/track
A	PrV capsid	35,000
B	Mock-infected HeLa cell lysate	205,000
C	PrV-infected HeLa cell lysate	198,000
D	Viral mRNA products	307,000
E	Cellular mRNA products	280,000
F	Endogenous products	40,000



The upper portion of tracks A, B, C was exposed for 44h and the lower portion for 168h. The upper portion of tracks D, E was exposed for 65h and the lower portion for 24h. Track E was exposed for 65h.

peptide synthesised in viral mRNA assays had a molecular weight of 150,000 while the largest cellular product was 102,000.

The proportion of low molecular weight products synthesised *in vitro* is much higher than is found *in vivo*. The synthesis of a high proportion of low molecular weight products is a recognised feature of the wheat germ system. Schechter & Burstein (1976) showed that some small polypeptides synthesised in wheat germ are premature termination products. Thus, it is difficult to classify polypeptides with molecular weights less than 20,000.

Comparison of the migration distances of viral and cellular mRNA *in vitro* products showed that at least 29 polypeptides were synthesised only in assays programmed with viral mRNA. A further 35 polypeptides present in viral mRNA assays co-migrated with products of cellular mRNA. After the exposure time used to develop this fluorograph, no high molecular weight endogenous wheat germ proteins were detectable, but after longer exposure a polypeptide with a molecular weight of 94,000 was identified. Eleven polypeptides with molecular weights between 20,000 and 22,000 could be detected. These endogenous products accounted for 11 of the 35 co-migrating viral and cellular mRNA products. Hence, a total of 53 products of exogenous mRNA could be detected in viral mRNA programmed assays.

Viral and cellular coded proteins present in the products of infected cell mRNA were further differentiated by examining the relative proportions in co-migrating bands. Cellular coded protein 39.5 (CCP 39.5) was synthesised in approximately the same concentrations in assays programmed with both mRNA species and was present in a similar concentration in cell lysates. Most of the other CCPs were present in reduced amounts in the viral mRNA products.

Comparison/

Comparison of the proportions synthesised *in vitro* was used to aid classification of polypeptides co-migrating with endogenous protein 20.1 and 20 (E 20.1 and E20). The ratio of E 20.1 to E 20 was much lower in the cellular mRNA stimulated assays than in the viral mRNA assay. This suggested that although the products have the same apparent molecular weights, they might not be identical. The band co-migrating with E 20.1 could contain a viral coded protein (VCP). Alternatively, a cellular protein which was present in reduced amounts in infected cells might be co-migrating with E 20 and thus increasing the relative proportions in the products of mock-infected cell mRNA. Comparison of the relative amounts of E 20 and CCP 20.2 suggested that the first explanation was unlikely and hence the band co-migrating with E 20 was designated VCP 20a.

Co-migration studies were further used to examine the relationship of polypeptides synthesised *in vitro* to those found in mock-infected and PrV-infected cell lysates. 22 of the polypeptides synthesised *in vitro* designated VCPs co-migrated with polypeptides found only in infected cell lysates. VCP 150 co-migrated with the major capsid protein VP2 which has a molecular weight of 150,000. This polypeptide was present only in infected cell lysates and *in vitro* assays programmed with viral mRNA. Other polypeptides closely co-migrating with it were not identified. Hence, it was classified as VP 2. VCP which co-migrated with VP 9, VP 11, VP 14, VP 16 and VP 18, VP 19 were also identified but their classification must be considered less absolute because CCP with similar electrophoretic mobilities were identified.

4.5. Immune Precipitation of *in vitro* Products.

Although most of the mRNA on polysomes of 5 h post-infection is virus coded, it is likely that some cellular mRNA species are present and therefore, "viral" mRNA may code for cellular proteins (see Chapter 1). Comparison of the *in vitro* products separated on polyacrylamide gels showed that some polypeptides are synthesised exclusively by viral mRNA. By this means the major capsid protein VP 2 can be identified. However, a more definitive means of identification is desirable for the following reasons:

(i) polypeptides synthesised in response to infected cell mRNA may represent virus-induced cellular polypeptides.

(ii) the total number of polypeptides synthesised in exponentially growing HeLa cells and PrV-infected cells is large and different proteins with similar molecular weights are not well resolved.

Precipitation with the appropriate antisera is known to be an extremely specific method of identifying polypeptides. Therefore, antisera to the major capsid protein and infected cell lysates were raised.

4.5.1. Preparation and Characterisation of Antibodies to VP 2.

Infection with PrV is lethal to rabbits, hence it was necessary to ensure that no infectious particles were inoculated into the animals. Purified PrV capsids were denatured by boiling in SDS and electrophoresed into a polyacrylamide slab gel. A section of the gel was stained to locate the major capsid protein and the band

containing/

containing this protein was cut out of the gel, crushed and used to inoculate rabbits. Antiserum was collected at intervals and assayed for the presence of antibodies by the Ouchterlony double diffusion method as described in the Materials and Methods.

Sera were tested against purified PrV capsid proteins solubilised in 2% (w/v) SDS, PrV-infected and mock-infected total HeLa cell lysates boiled in 2% (w/v) SDS and cytoplasm prepared from infected and mock-infected cells in the absence of SDS. The serum from immunised rabbits reacted with purified capsid proteins (Figure 4.5.1.1(b)). It also showed formation of precipitin lines with the SDS treated cell lysates (Figure 4.5.1.1(a)) but did not react with the cytoplasms in the absence of SDS, although 5 h infected cell cytoplasm contains VP 2. The antiserum was absorbed with SDS treated mock-infected cell lysate to remove cross-reacting material. The resultant absorbed antiserum did not react with capsid proteins or the SDS treated cell lysates (data not shown).

It was therefore concluded that there was antibody activity in the serum directed against SDS-protein complexes. However, it was not obvious whether there were antibodies to VP 2 because although the serum did not react with infected cell cytoplasm, it was possible that there were antibodies to antigenic sites on the VP 2 which were not exposed unless the protein was denatured by SDS.

The specificity of the antiserum was further characterised by indirect immune precipitation. SDS treated cell lysates labelled with{^{35}S}methionine were diluted with TKM-det buffer, incubated with rabbit serum for 15 min and then the total rabbit immunoglobulin G (IgG) was precipitated with goat-antirabbit-IgG serum (GAR). A higher percentage of the input radioactivity was precipitated from PrV-infected/

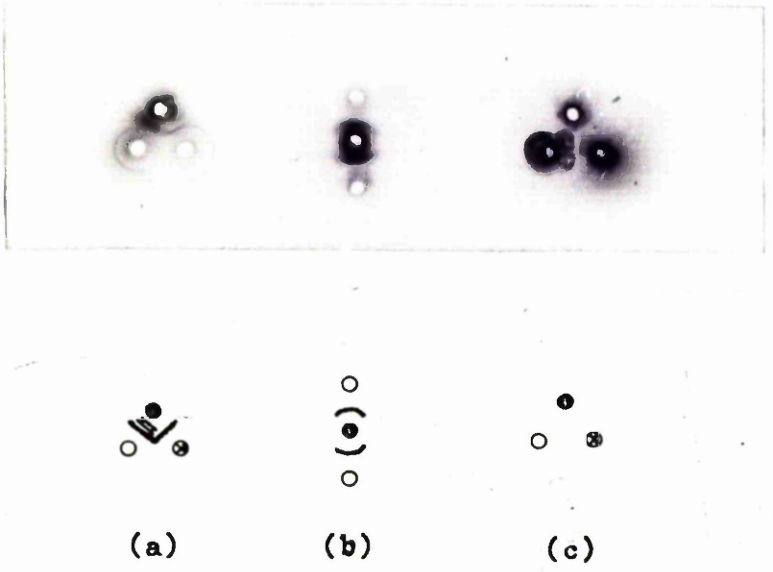


FIG. 4.5.1.1. ANTIBODY ACTIVITY OF ANTISERUM RAISED AGAINST SDS-DENATURED PrV MAJOR CAPSID PROTEIN

The antibody activity of antiserum raised by inoculation of rabbits with SDS-denatured PrV major capsid protein against SDS treated PrV-infected or mock-infected HeLa cell lysates, against PrV-infected or mock-infected HeLa cell cytoplasms and against SDS-denatured PrV capsid was examined by double diffusion in agarose/polyethylene glycol gels as described in the Materials and Methods (A.14.2.). The gel was stained with Coomassie Brilliant Blue and dried before photographing.

(a) ● antiserum
o SDS-PrV-infected HeLa cell lysate
⊗ SDS-mock-infected HeLa cell lysate

(b) ● Antiserum
o SDS-PrV capsid

(c) ● antiserum
o PrV-infected HeLa cell cytoplasm
⊗ Mock-infected HeLa cell cytoplasm

Staining was necessary because the cell lysates were too dilute to give good precipitin lines. Blotching was caused by the stain and overfilling wells

infected cell lysate than from mock-infected cell lysate (Table 4.5.1.1.). The precipitates were solubilised and examined by polyacrylamide gel electrophoresis. A single polypeptide which co-migrated with VP 2 was precipitated from the infected cell lysate and no detectable polypeptides were precipitated from the mock-infected cell lysate (Figure 4.5.1.2.). There may have been several reasons for the failure to precipitate polypeptides from the mock-infected cell lysate. It is possible that the antigen was present in such excess that the equivalence point was surpassed and the rabbit anti-SDS immunoglobulins did not have antigenic sites accessible to the goat antiserum. Alternatively, the fact that the Ouchterlony test is more sensitive than coprecipitation (Gill, 1972) may account for it.

However, the indirect immune precipitation made it possible to conclude that although the serum had a general reactivity against SDS treated polypeptides, it also had a specific antibody to VP 2.

4.5.2. Precipitation of material synthesised *in vitro*.

IgG was prepared from rabbit anticapsid serum and used in immune precipitation studies of polypeptides synthesised in the wheat germ cell free system stimulated by viral and cellular mRNAs. The antibody activity of the purified IgG was characterised by double diffusion assays (Figure 4.5.2.1.). The cross reactivity was found to be the same as that of the serum.

In vitro wheat germ assays were diluted with PBS-det and treated with rabbit anticapsid IgG and GAR. The percentage of the total TCA precipitable material polymerised *in vitro* which was precipitated by the anticapsid antibody is shown in Table 4.5.2.1. A higher percentage of the input material was precipitated from the viral/

TABLE 4.5.1.1.

Precipitation of cell lysate polypeptides with Rabbit Anti-capsid serum.

Material treated with antiserum	cpm input	cpm in precipitation	% cpm precipitated
SDS-PrV-infected HeLa cell lysate	15,730	3,720	.24
SDS-Mock-infected HeLa cell lysate	23,950	640	.3

FIG. 4.5.1.2. POLYPEPTIDES PRECIPITATED FROM PrV-INFECTED AND MOCK-INFECTED CELL LYSATES WITH ANTISERUM TO PrV CAPSID

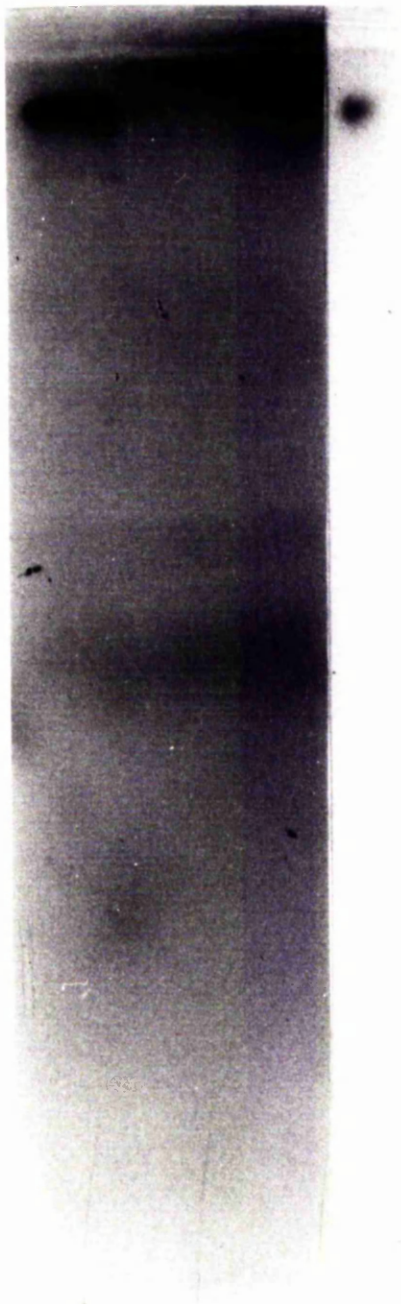
5µlaliquots of SDS-denatured PrV-infected and mock-infected HeLa cell lysates (A9.1.) were diluted with 200µl of TKM-det buffer (A3.8.4.) and treated with 5µl rabbit anti-PrV capsid serum and 150µl goat anti-rabbit IgG serum as described in the Materials and Methods (A14.5.). The immune precipitates were coelectrophoresed with PrV capsid proteins in a 12% polyacrylamide gel. The gel was processed for fluorography and exposed to X-ray film.

ICL Immune precipitate from PrV-infected cell lysate

CCL Immune precipitate from mock-infected cell lysate

Capsid PrV capsid showing the major capsid protein VP2

Fogging in the left-hand tracks is caused by the use of old film.



ICL CCL Capsid

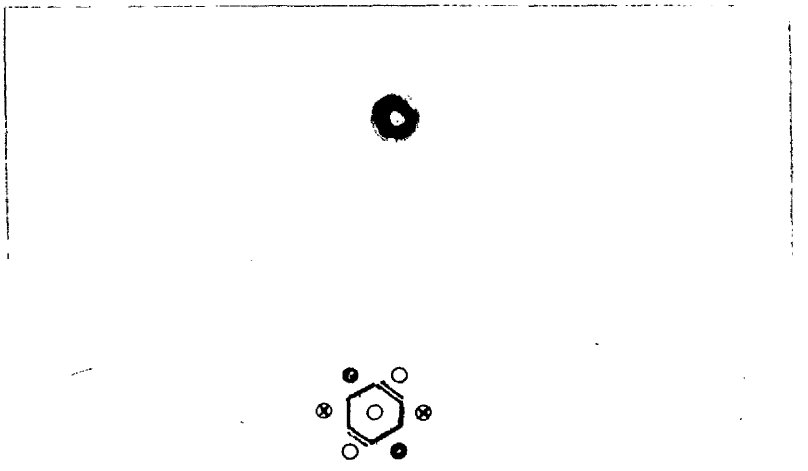


FIG. 4.5.2.1. ANTIBODY ACTIVITY OF IgG PREPARED FROM ANTISERUM RAISED AGAINST SDS-DENATURED PrV MAJOR CAPSID PROTEIN

The antibody activity of IgG prepared from antiserum raised by inoculation of rabbits with SDS-denatured PrV major capsid protein against SDS-denatured infected or mock-infected HeLa cell lysates and SDS-denatured PrV capsid was examined by double diffusion in agarose/polyethylene glycol gels as described in the Materials and Methods (A.14.2.). The gel was stained with Coomassie Brilliant Blue and dried before photographing.

The centre well contained IgG and antigen solutions in the outside wells were as follows:

- ⊙ PrV capsid
- o SDS-PrV-infected HeLa cell lysate
- ⊗ SDS-mock-infected HeLa cell lysate

Precipitin lines are shown in a line diagram for clarity.

Staining was necessary because the precipitin lines were weak. This caused the extensive blotching around the centre well.

TABLE 4.5.2.1.

Precipitation of polypeptides synthesised in vitro with Rabbit Anticapsid IgG.

Material treated with IgG	cpm input	cpm precipitated	% cpm precipitated
Wheat germ assay stimulated with viral mRNA	392,990	18,830	4.9
Wheat germ assay stimulated with cellular mRNA	981,870	9,690	1.0

viral mRNA stimulated assay. In the absence of SDS, no immune precipitation of the polypeptides synthesised in vitro in response to either mRNA was detected.

Examination of the products (Figure 4.5.2.2.) showed that a band which co-migrated with the major capsid protein was precipitated by anticapsid IgG. These studies allowed unequivocal identification of the major capsid protein VP 2.

4.5.3. Preparation and characterisation of antibodies to Infected cell proteins.

Antibodies were raised in rabbits against 5 h PrV-infected BHK/21 (C13A) cell lysates by a similar method to that used to raise antibodies to VP 2. SDS-treated cell lysates were electrophoresed a few millimetres into a 15% (w/v) polyacrylamide gel. The band containing the total polypeptides was excised from the gel and used to inoculate the rabbits. Sera were tested for antibody activity by the Ouchterlony diffusion method. Figure 4.5.3.1. shows that the serum contains antibodies to SDS treated PrV-infected and mock-infected HeLa cell lysates. The cross reaction between antibodies to BHK proteins and HeLa cell proteins was expected because proteins which have the same function will be present in both cell types and will show very little species diversity. In addition, it was likely that some of the antibody to SDS would be present.

The antiserum was treated with mock-infected HeLa cell lysates to absorb out cross reacting antibodies in the absence of SDS. Serum treated three times (Figure 4.5.3.1.) showed reduced reactivity with SDS-treated infected cell lysates and very little cross reaction with SDS treated mock-infected HeLa cell lysates.

Attempts/

FIG. 4.5.2.2. POLYPEPTIDES PRECIPITATED FROM IN VITRO TRANSLATION PRODUCTS WITH ANTISERUM TO PrV CAPSID

20µl aliquots containing approximately 4 x 10^5 and 10^6 cpm were withdrawn from wheat germ assay mixes which had been incubated with viral (PrV) and cellular (HeLa) mRNA respectively. They were diluted with 200µl PBS-det (A3.8.3.) and treated with 5µ rabbit anticapsid IgG and 105µl goat antirabbit IgG serum as described in the Materials and Methods (A14.5.). The immune precipitates were coelectrophoresed with unprecipitated aliquots of viral and cellular mRNA stimulated wheat germ assay mixes on a 10% polyacrylamide gel.

Track:

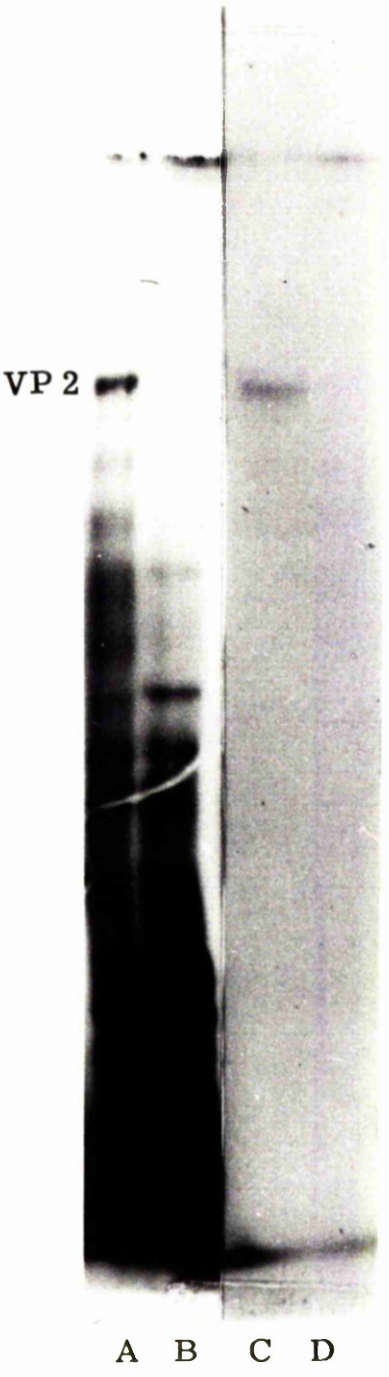
A Viral mRNA products

B Cellular mRNA products

C Immune precipitate from viral mRNA stimulated assay

D Immune precipitate from cellular mRNA stimulated assay.

The position of VP2 was determined by coelectrophoresis of standards.


VP 2
A
B
C
D

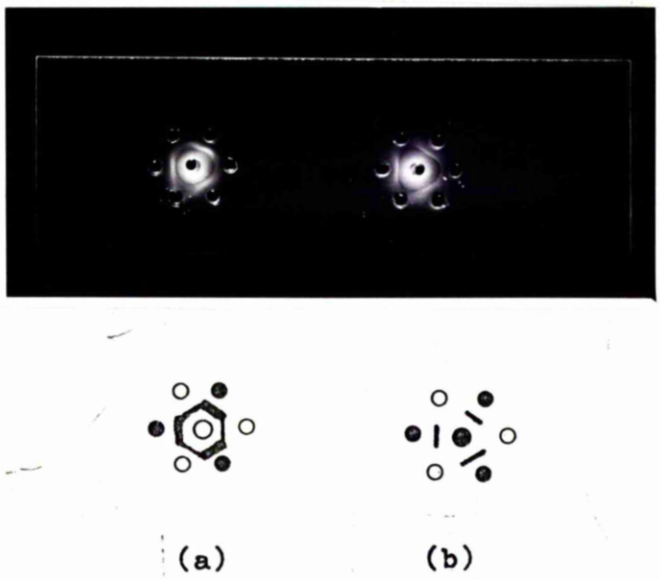


FIG. 4.5.3.1. ANTIBODY ACTIVITY OF ANTISERUM RAISED AGAINST PrV-INFECTED BHK/21 (C13A) SDS-DENATURED CELL LYSATE

Antiserum raised by inoculation of rabbits with PrV-infected BHK/21 (C13A) SDS-treated cell lysates was absorbed with mock-infected HeLa cell lysates. The antibody activity of the absorbed serum against SDS-denatured PrV-infected or mock-infected HeLa cell lysates was compared with that of untreated serum by double diffusion in agarose/polyethylene glycol gels (see Materials and Methods, A14.2.). The gel was photographed unstained.

(a) Centre well, SDS-PrV-infected HeLa cell lysate

(b) Centre well, SDS-mock-infected HeLa cell lysate

- o absorbed serum
- ● untreated serum

Attempts to further characterise the specificity of the absorbed antiserum by precipitation of radioactive antigen from solution by double precipitation with GAR were unsuccessful. It was not possible to precipitate a higher percentage of the TCA precipitable radioactivity from viral mRNA stimulated assays than was precipitated from cellular mRNA stimulated assays.

Examination of the products on gels was hampered by two problems. Firstly the total radioactivity precipitated was very small, probably because of the low antibody activity of the serum, and secondly, precipitation of rabbit IgG with GAR leads to large precipitates which overloaded the gel causing distortion of the polypeptide bands with molecular weights less than 100,000. This problem was also encountered with anticapsid serum but to a much lesser extent because the IgG protein chains (molecular weights 23,000 and 50,000) migrated ahead of VP 2.

For these reasons it was not possible to show precipitation from viral mRNA stimulated wheat germ assays with antiserum to infected cell proteins.

4.6. Hybrid arrested translation.

Immunoprecipitation studies did not prove useful in identification of polypeptides synthesised *in vitro* other than VP 2 because of the non-specificity of the antisera. Therefore, an alternative method of classification was necessary.

Paterson *et al.*, (1977) found that mRNA when hybridised to its complementary DNA did not direct cell-free synthesis of complete polypeptides. Translational activity of the mRNA was recovered upon the heat melting of the hybrid. The technique, which is termed/

termed hybrid arrested translation (HART), has been used to investigate the polypeptides coded by different abundance classes of mRNA in chick myoblast (Paterson & Bishop, 1977) and mouse liver (Hastie & Held, 1975).

The technique can be extended to the detection of genes in double stranded DNA by hybridisation in high concentrations of formamide which favour RNA-DNA hybridisations. In this way the specific protein coding regions in restriction fragments of Adenovirus 2 DNA were mapped (Paterson et al., 1978). HART has also been used to identify the polypeptide products of the eight RNA molecules of fowl influenza A virus (Inglis et al., 1977).

The technique was used to identify the viral coded polypeptides in the in vitro translation products of "viral" mRNA. PrV DNA was hybridised to viral mRNA as described in the Materials and Methods and identical samples either melted by heating at 100°C for 60 s followed by quick chilling or retained in hybrid form. The precipitated nucleic acids were then translated in the wheat germ system. The translational activity of the treated mRNA was less than that of the starting material. The reduction may have been caused by loss of mRNA or inhibition of the system by the increased concentration of nucleic acid. However, significant stimulation of activity was shown by both the intact and the melted hybrids (Table 4.6.1.). The translational activity was reduced by 50.6

The in vitro products of melted and hybridised mRNA are shown in Figure 4.6.1. Comparison of tracks B and C showed that synthesis of some proteins was suppressed in the hybrid. The relative intensity of bands in these tracks was measured on a microdensitometer and reductions in peak heights were quantitated by/

TABLE 4.6.1.

Incorporation of TCA precipitable material in hybrid stimulated wheat germ assays.

mRNA	cpm incorporated /50ul	* % reduction
Endogenous	128,530	
Viral mRNA-PrV DNA hybrid	262,720	50.6
Viral mRNA-PrV DNA treated hybrid	400,230	

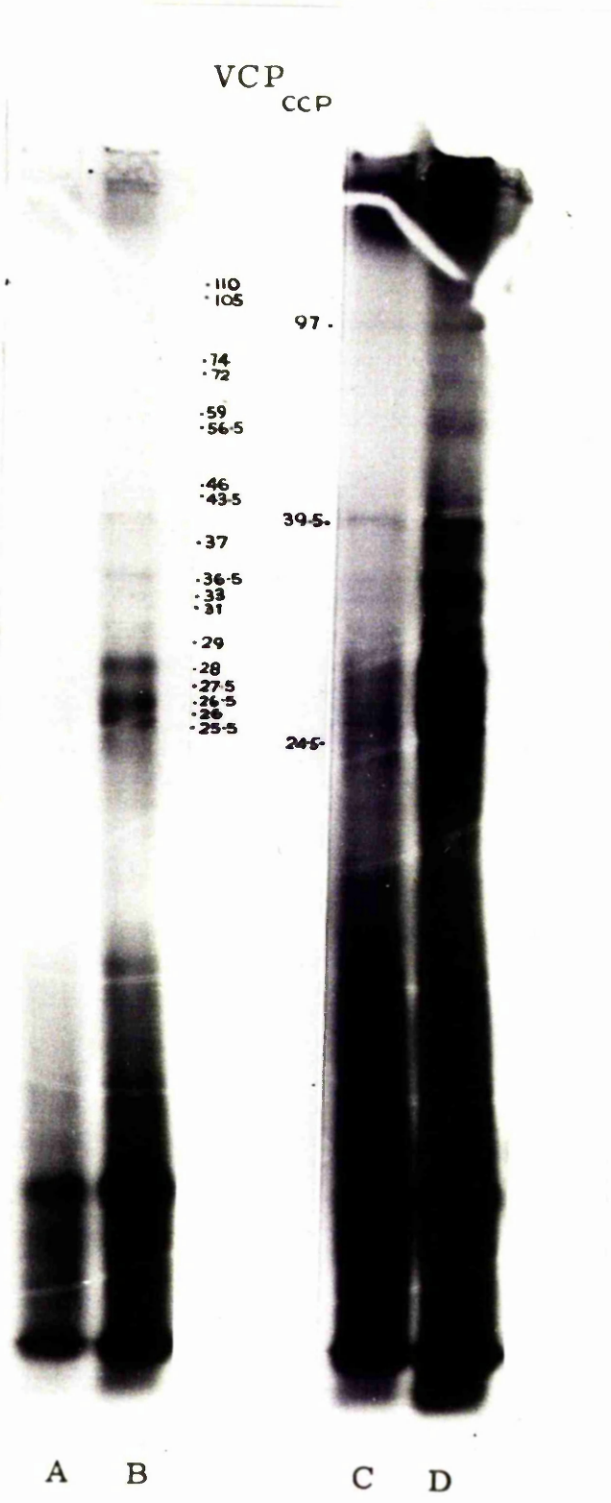
*The percentage decrease was calculated in the following way: The endogenous incorporation was subtracted from the cpm incorporated in exogenous mRNA stimulated assays; the corrected cpm for the undenatured mRNA-DNA hybrid stimulated assay (134, 190) was subtracted from the corrected cpm for the melted hybrid assay (271,700) and the difference (137,510) is expressed as a percentage of the corrected cpm incorporated in the melted hybrid assay.

FIG. 4.6.1. PRODUCTS OF HYBRID ARRESTED TRANSLATION

0.4µg viral (PrV) mRNA was hybridised to a tenfold excess of PrV DNA as described in the Materials and Methods (A8.2). One half of the sample was heat denatured and both hybrid and melt were added to wheat germ assay mixes (A3.4.11.). The assay mixes contained 400µCi/ml {^{35}S}-methionine (820Ci/mmol) and were incubated as described (A10.2.). 10µl aliquots were treated for electrophoresis and separated on 12% polyacrylamide gels. Gels were processed for fluorography and exposed to X-ray film. Two exposure times of the gel are shown. Viral coded polypeptides (VCP) and cellular coded polypeptides (CCP) are labelled in accordance with Fig. 4.4.1.

		cpm/track
Tracks A and C	mRNA -DNA hybrid products	105,000
Tracks B and D	"melted" mRNA-DNA products	160,000

Tracks A,B were exposed for 72h; tracks C,D for 120h.


VCP
CCP
110
105
97
74
72
59
56·5
46
43·5
39·5
37
36·5
33
31
29
28
27·5
26·5
26
25·5
24·5
A
B
C
D

by cutting out and weighing several clearly defined peaks. The ratios of the peaks relative to CCP 39.5, which was of approximately the same intensity in both tracks, were calculated (Table 4.6.2.). Seven polypeptides which had been putatively classified as viral by co-migration were found to show suppressed synthesis in the mRNA-DNA hybrids. This confirmed the viral origin of these polypeptides.

4.7. Classification of polypeptides preferentially synthesised at elevated K^+ concentrations.

The studies on the effect of elevated K^+ concentrations on polypeptide synthesis *in vitro* (4.3.) showed that some viral mRNA products were preferentially synthesised at 100mM K^+. The identity of these polypeptides can now be considered in the light of attempts to classify the *in vitro* products described in the preceeding three sections.

The products of viral and cellular mRNA synthesised under standard conditions together with the products of viral mRNA synthesised at 100mM K^+ and the products of mRNA extracted from infected cells treated with cycloheximide from the start of infection are shown in Figure 4.7.1. The "viral" mRNA species contains late viral mRNAs and the mRNA extracted from cycloheximide treated cells should contain only immediate-early viral mRNA (Ben-Porat & Kaplan, 1973). Classification of the polypeptides is shown in Table 4.7.1. Three of the seven polypeptides whose synthesis is elevated at 100mM K^+ have been classified as viral coded by co-migration studies. Polypeptide A is classified as viral by co-migration and HART. Comparison of electrophoretic mobilities suggested evidence that VCP 43.5 might be an immediate-early protein.

4.8./

TABLE 4.6.2.

Ratio of viral mRNA products synthesised in HART assays relative to cellular coded polypeptide 39.5 (CCP 39.5).

Polypeptide	Melted hybrid	Unmelted hybrid
CCP 97	.88	1.0
CCP 39.5	1.0	1.0
VCP 105	0.94	ND *
37	1.4	0.5
36.5	1.6	0.94
28	4.8	2.2
26.5	3.3	1.6
26	3.9	1.7
25.5	3.5	1.8

* ND not detectable

FIG. 4.7.1. POLYPEPTIDES SYNTHESISED AT DIFFERENT $\{K^+\}$ IN THE WHEAT GERM SYSTEM

Cellular (HeLa, 6.4µg/ml), late viral (PrV, 8.8µg/ml) and immediate early viral (PrV, 5µg/ml) mRNAs were translated in the standard wheat germ assays (A3.4.11.). Late viral mRNA was also translated in an assay mix where the $\{K^+\}$ was increased to 100mM K^+ by addition of KCl. Assay mixes contained 570µCi/ml $\{^{35}S\}$-methionine (1080Ci/mmol). PrV immediate early mRNA was prepared by oligo-(dT) cellulose affinity chromatography of polysomal RNA extracted from PrV-infected which were treated with cycloheximide from the time of infection until 15 min prior to harvesting and was a generous gift from Mr.M. Chowdhury. 10µl aliquots of assay mixes were treated for electrophoresis and the polypeptides separated on a 12% polyacrylamide gel. The gel was processed for fluorography and exposed to X-ray film.

The viral mRNA products which show enhanced synthesis of 100mM K^+ are designated a-g and one whose synthesis is suppressed is designated A.

Late V 75: late PrV mRNA, 75mM K^+

Late V 100: late PrV mRNA, 100mM K^+

IEV 75: immediate early PrV mRNA, 75mM K^+

C 75: cellular mRNA, 75mM K^+

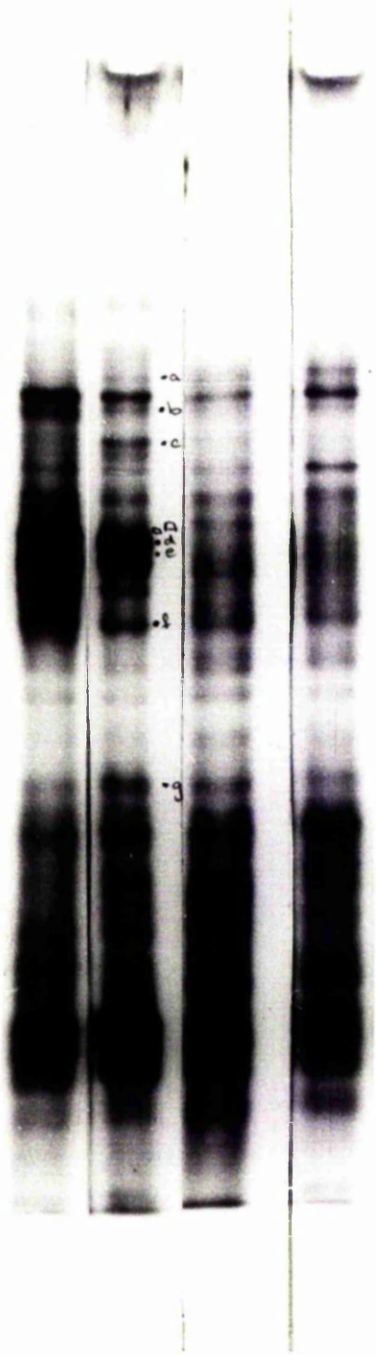

late V
75
late V
100
IE V
75
C
75

TABLE 4.7.1.

Classification of polypeptides showing altered patterns of synthesis at 100mM K^+

Polypeptide stimulated at 100mM K^+	Polypeptide inhibited at 100mM K^+	Classification
a		VCP 43.5
b		CCP 38
c		VCP 36.5
d		CCP 27.5
e		CCP 26.5
f		VCP 24
g		EP 21.2
	A	VCP 28

4.8. Discussion.

These studies show that synthesis of polypeptides with molecular weights greater than 100,000 is possible in the wheat germ cell free system. However, the population of polypeptides synthesised *in vitro* differs from that found *in vivo*. The proportion of total radioactive label incorporated into high molecular weight material is much lower *in vitro* and the number of polypeptides with molecular weights less than 25,000 synthesised *in vitro* is higher than is found *in vivo*. It has been suggested that premature termination will be found to be a feature of all *in vitro* systems (Boime and Leder, 1972). Such incomplete polypeptide synthesis may occur because of mRNA degradation (Hunter *et al.*, 1977) or may be due to limiting features of cell free systems.

One interesting point is that the low molecular weight polypeptides are of discrete sizes and the banding patterns are reproducible. This suggests that synthesis of low molecular weight polypeptides is not a random event.

Co-migration studies have identified twenty-nine polypeptides present in assays programmed with viral mRNA which are not synthesised in response to cellular mRNA. A further twentythree viral mRNA products have the same electrophoretic mobility as cellular mRNA products. Most of the polypeptides are found in reduced amounts in viral mRNA stimulated assays suggesting that at least some are of cellular origin. However, a definitive classification cannot be made on the basis of co-migration studies alone. An alternative classification is based on the suggestion that addition of mRNA to wheat germ extracts stimulates endogenous synthesis. Thus, some of the polypeptides could be endogenous proteins which were not detected on the absence of exogenous mRNA. However, the synthesis of high molecular products is not stimulated/

stimulated by reticulocyte mRNA.

The *in vitro* products of both mRNA species contained polypeptides which had no detectable counterpart in cell lysates. There are three possible explanations for this. Firstly some of the *in vitro* polypeptides may be incomplete products due to degraded mRNA or incompleted translation. Secondly, post-translational processing of polypeptides occurring in a heterologous cell free system is not analagous to *in vivo* processing. Cleavage of precursor moleculas (Maurer *et al.*, 1976) and glycosylation (Katz *et al.*, 1977; Inglis *et al.*, 1977) do not occur. Finally, classifications such as these are subject to the limitations of the technique. Polypeptides present in small amounts are not well resolved in one dimensional gels and there are a large number of proteins present; hence, the failure to detect co-migrating bands does not preclude their existence.

Further investigation of the origin of the *in vitro* polypeptides by immunoprecipitation confirmed the identity of VCP 150 which was classified as VP 2 by co-migration. Failure to verify the viral origin of other polypeptides by this method probably occurred for two reasons. Firstly, immunoprecipitation from the wheat germ system has been found to be difficult and high backgrounds have been observed (Nakanishi *et al.*, 1977). This has been attributed partly to the presence of incomplete polypeptides chains (Schmeckpeper *et al.*, 1974). Secondly, problems arose because of the non-specific nature of the anti-serum.

Preparation of antiserum to SDS-treated viral polypeptides by a similar method to that used here has been described (Johnson *et al.*, 1972; McMillan and Consigli, 1977). The antibody activity of the serum has been shown to be directed against the dissociated proteins and antisera did not react with intact virions. The antigens on whole virus/

virus were assumed to differ from those on the individual viral polypeptides. This was confirmed by these studies which showed that it was necessary to treat proteins with SDS to demonstrate antibody activity in antisera to SDS denatured proteins.

In addition, these studies showed a non-specific activity against SDS denatured proteins not previously noted. This prevented absorption of anti-HeLa cell protein antibodies from antisera to infected cell lysates because in order to do this efficiently it would have been necessary to use SDS treated HeLa lysates and this would cause non-specific precipitation of viral antibodies. Treating anticapsid serum with SDS denatured HeLa cell lysates removed all antibody activity. Some reduction of anti-HeLa cell protein activity was achieved using non-SDS denatured HeLa cell lysates but several precipitations were necessary and the reduction may have been simply due to loss of IgG. Hence, it was concluded that this method of raising antisera was unsuitable for these purposes.

HART proved to be an efficient means of classifying polypeptides synthesised *in vitro*. The failure to identify more polypeptides by this method is probably due to the reduction in synthesis observed in HART assays. It seems likely that further application of the technique would yield more information.

The experiments described here showed that preferential synthesis of some polypeptides occurred at elevated K^+ *in vitro*. K^+ resistant species were found in all three mRNA populations - viral, cellular and endogenous. Similar observations have been made for the synthesis of rat liver albumin (Tse and Taylor, 1977) and polyoma virus polypeptides (Wheeler *et al.*, 1977). These studies are of interest with regard to the hypothesis that monovalent cations have a role in viral-induced shut/

shut-off of cellular protein synthesis (Carrasco, 1977) and will be discussed further.

Chapter 5

Comparative Studies in Three Cell Free Systems.

5.1. Introduction.

A common feature of a number of virus infections is the diversion of the protein synthetic apparatus from translation of host cell mRNA to an almost exclusive translation of viral mRNA. There is evidence that translational control has a role in the process and mechanisms for preferential translation of viral mRNA have been proposed. One hypothesis attributes the preferential translation of viral mRNA to a virus-induced elevation of the intracellular monovalent cation concentration to a level which is inhibitory to cellular but not viral mRNA translation (Carrasco, 1977). Support for this model comes from the observation that EMC virus RNA exhibits mRNA activity at a 25mM higher K^+ concentration than reticulocyte mRNA (Mathews, 1972) or total mouse cell poly(A)-containing RNA (Carrasco and Smith, 1976) in cell free system prepared from Krebs II ascites cells.

Studies on translation conditions described in the preceding two chapters have shown that there is no difference in the translation characteristics of HeLa cell and late PrV mRNA in wheat germ extracts. Thus the model does not seem to be applicable to herpes viruses. It remains possible, however, that differences are masked in such a heterologous translation system and therefore the translation of EMC virus RNA and PrV viral mRNA was compared in the wheat germ system and the translation of these and other mRNAs was investigated in other cell free protein synthesising systems.

A second reason for investigating translation in other cell free systems was to provide a check on the authenticity of "exogenous mRNA products/

products" detected in the wheat germ system.

5.2. Translation of EMC Virus RNA in the Wheat Germ Cell Free System.

5.2.1. Isolation and Characterisation of EMC Virus RNA.

EMC virus RNA was extracted from purified virus by the phenol: chloroform:isoamyl alcohol technique at pH 9.0 as described in the Materials and Methods for extraction of RNA from polysomes. The RNA was precipitated three times to remove SDS and assayed for mRNA activity under standard conditions in the wheat germ cell free system. As noted by Daggleman and Beard (1976) EMC virus RNA was found to be a comparatively inefficient messenger in wheat germ assays. The maximum stimulation of endogenous activity observed at an RNA concentration of 62µg/ml was four fold.

The mRNA activity was verified by examination of the *in vitro* translation products by polyacrylamide gel electrophoresis and fluorography. At least fifteen polypeptides with molecular weights ranging from less than 20,000 to 105,000 which did not comigrate with products of endogenous or other exogenous mRNA were synthesised (data not shown).

5.2.2. The Optimum $\{K^+\}$ Concentration for Translation of EMC Virus RNA in Wheat Germ Extracts.

The incorporation of $\{^3H\}$-leucine into TCA precipitable material in wheat germ assays stimulated by EMC virus RNA at varied $\{K^+\}$ was investigated by addition of KCl to standard assay mixes. The result of such an experiment is shown in Figure 5.2.2.1. The effect of variations in $\{K^+\}$ on PrV mRNA activity (from figure 3.3.1.1.) is shown for comparison. The optimum concentration of K^+ was similar for both RNAs but the messenger activity/

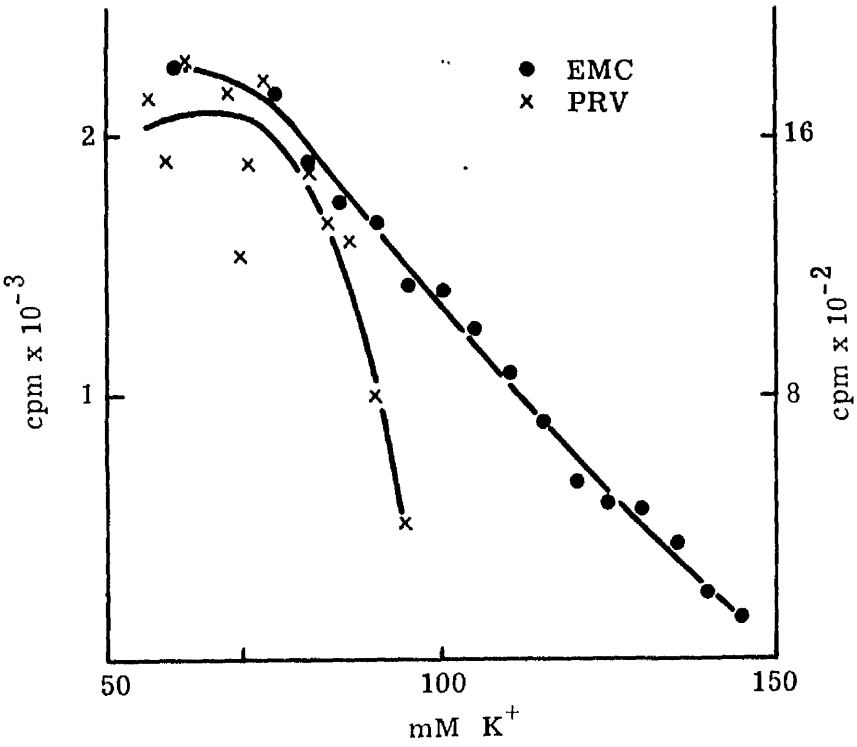


FIG. 5.2.2.1. THE EFFECT OF $\{K^+\}$ ON EMC VIRUS RNA AND PrV mRNA STIMULATED SYNTHETIC ACTIVITY IN THE WHEAT GERM SYSTEM

The $\{K^+\}$ in wheat germ assay mixes (A3.4.11.) containing 96µg/ml EMC virus RNA was varied by addition of KCl. Assay mixes contained 50µCi/ml $\{^3H\}$-leucine (56Ci/mmol). After incubation the synthesis of TCA precipitable material was determined by precipitation on filter discs and scintillation counting.

The PrV mRNA stimulated synthesis as a function of $\{K^+\}$ (from Fig. 3.2.2.1.) is shown for comparison. The left-hand scale refers to EMC virus RNA stimulated incorporation, the right-hand scale to PrV mRNA stimulated incorporation.

activity of EMC virus RNA was present over a wider range of $\{K^+\}$ than that of Pr viral mRNA. Pr viral mRNA-stimulated incorporation drops to endogenous as the $\{K^+\}$ approaches 100mM while EMC RNA maintains stimulation to 120mM K^+.

5.3. Krebs II Ascites Cell Free Translation.

5.3.1. mRNA Activity of Exogenous RNAs in Ascites Extracts.

S30 Krebs II ascites cell extracts were prepared and preincubated as described in the Materials and Methods. The mRNA activity of EMC virus RNA and reticulocyte, Pr viral and cellular mRNAs was determined in preincubated S30 containing 71mM K^+ and 100μCi/ml $\{^3H\}$-leucine. Assays were incubated at 37°C for 60 min and the incorporation into TCA precipitable material was determined by the filter method. Similar assays were also carried out with unincubated S30 where the $\{K^+\}$ was 77mM and assays contained 80μCi/ml $\{^3H\}$-leucine. The total incorporation and the stimulation of endogenous incorporation are shown in Table 5.3.1.

EMC virus RNA and reticulocyte mRNA gave equivalent stimulation of incorporation in preincubated extracts. However Pr viral and cellular mRNAs were found to be poor messengers under these conditions and variation of RNA, K^+ or Mg^{++} concentrations did not increase the stimulatory activity. For these mRNAs stimulation was only observed at a concentration of 0.8μg/ml. This is good stimulation per μg.

The translation of EMC virus RNA and Pr viral mRNA was compared in unincubated ascites extracts because it has been noted that the preincubation step preferentially curtails the ability of the system to translate heterologous mRNAs (Mathews, 1972). In this case equivalent stimulation was obtained with both RNAs but because the level of endogenous/

TABLE 5.3.1.

mRNA Activity of Exogenous RNAs in Krebs II Ascites Extracts.

Ascites S30	Exogenous RNA species	RNA concentration µg/ml	{^{3}H}- leucine incorporated cpm/ 5µl	Stimulation*
unincubated	-	-	10,380	
	viral	0.8	17,820	1.7
	EMC	60	20,160	1.9
preincubated	-	-	2,820	
	viral	1.6	4,510	1.6
preincubated	-	-	2,260	
	EMC	62	25,700	11.4
preincubated	-	-	1,900	
	reticulocyte	37	21,670	11.4
	reticulocyte	74	20,460	10.8

* Times endogenous

genous protein synthesis is high in these extracts and the stimulation low, translation in unincubated extracts was not considered useful.

5.3.2. The Effect of Variations in the $\{K^+\}$ on mRNA Activity in Ascites Extracts.

The effect of variations in the $\{K^+\}$ on mRNA activity in the ascites cell free system was investigated by addition of KCl to assays. The $\{K^+\}$ dependence of stimulation of incorporation by EMC virus RNA and Pr viral and reticulocyte mRNAs is shown in Figure 5.3.2.1. EMC virus RNA shows optimum stimulation in the range 95-110mM which is similar to that described by others. The optimum for reticulocyte and viral mRNAs is approximately 50mM.

As discussed previously, (see 3.3.1.) there is evidence that the chloride ion may be involved in inhibition of translation. The ascites cell free system is buffered by 25mM Tris HCl pH7.5 so before addition of KCl the $\{Cl^-\}$ is 20mM. Addition of 60mM KCl raises the $\{Cl^-\}$ to 80mM which is the upper limit of the physiological range. It is therefore possible that translation may be affected by excess Cl^-.

5.4. Rabbit Reticulocyte Cell Free Translation.

The unfractionated reticulocyte lysate is a highly efficient cell free protein synthesising system with *in vitro* translation rates close to those observed in intact cells (Hunt & Jackson, 1974; Mathews & Osborn, 1974). However, although exogenous mRNA can be translated in the system, the endogenous activity is sufficiently high that stimulation of amino acid incorporation by exogenous mRNA cannot be detected. Thus it is necessary to use mRNAs whose products can be readily identified. When this project was started the unfractionated reticulocyte lysate did not/

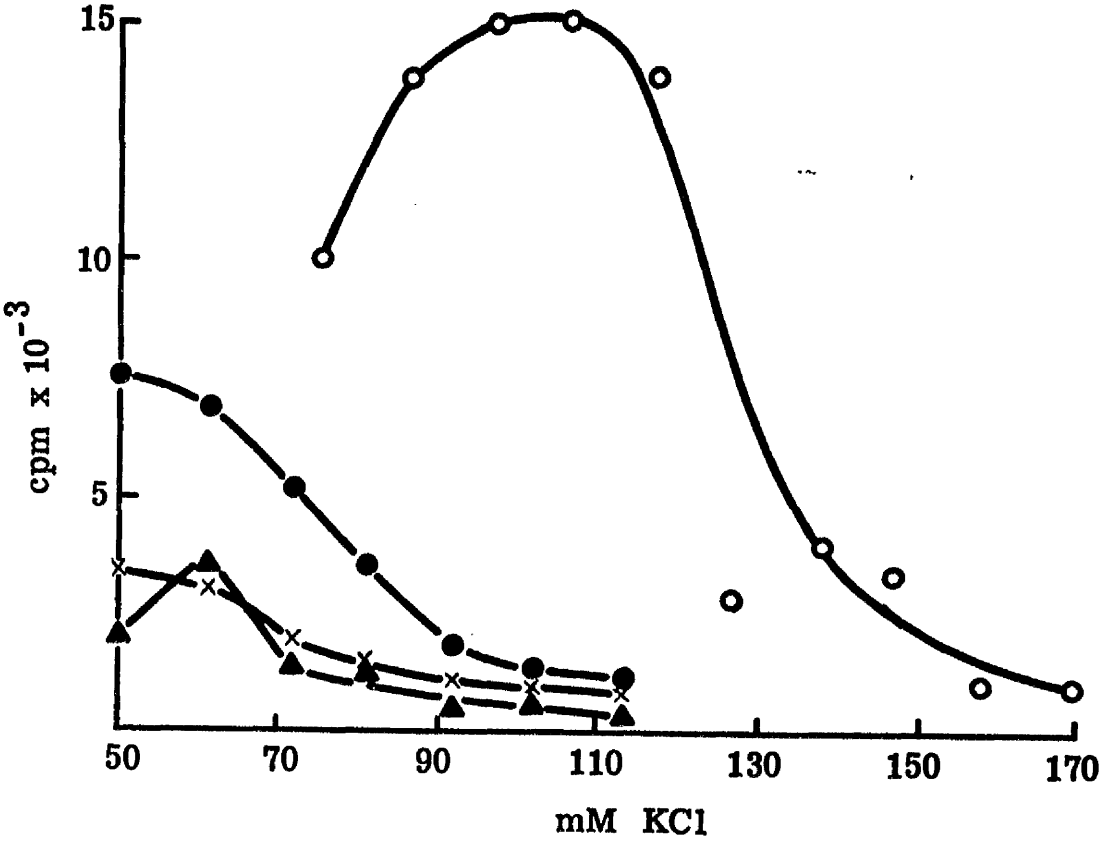


FIG. 5.3.2.1. THE EFFECT OF $\{K^+\}$ ON SYNTHESIS OF TCA PRECIPITABLE MATERIAL IN THE KREBS II ASCITES SYSTEM

The $\{K^+\}$ in Krebs II ascites assay mixes (A3.6.14.) containing 1.5µg/ml PrV mRNA, 4µg/ml globin (Rabbit reticlocyte) mRNA, 24µg/ml EMC virus RNA or no added RNA was varied by addition of KCl. Assay mixes contained 57µCi/ml $\{^3H\}$-leucine (52Ci/mmol). After incubation the synthesis of TCA precipitable radioactivity (cpm/5µl) was measured by precipitation on filter discs and scintillation counting.

- o EMC virus RNA
- ● Globin mRNA
- x Viral (PrV) mRNA
- ▲ No added RNA

not seem to be useful for investigating the stimulatory activity of the heterologous mRNAs of mock-infected and PrV-infected HeLa cells.

However, a mRNA dependant reticulocyte lysate (MDL) which does not have this disadvantage has since been developed (Pelham & Jackson, 1976). An unfractionated reticulocyte lysate is treated with micrococcal nuclease to degrade endogenous mRNA and after an appropriate time, nuclease activity is arrested by chelating calcium ions which are required for this nuclease activity. The treated lysate has low endogenous activity, no detectable nucleases and translates exogenous mRNA almost as efficiently as the untreated lysate.

5.4.1. mRNA Activity of Exogenous RNAs in the MDL.

Pr viral and cellular mRNAs and EMC virus RNA were assayed for mRNA activity in a MDL prepared as described in the Materials and Methods. Assay mixes containing 50µl of MDL and mRNA and {^{35}S}-methionine in a final volume of 57.5µl were incubated at 30^{o}C for 90 min. Table 5.4.1.1. shows the exogenous mRNA stimulated incorporation of {^{35}S}-methionine into TCA precipitable material in the system.

The products of exogenous RNAs in the MDL were examined by polyacrylamide gel electrophoresis and fluorography (Figure 5.4.1.1.). Aliquots of the assay mix were treated with SDS and β-mercaptoethanol as described in the Materials and Methods and applied directly to the gel. The high concentration of globin leads to some distortion of the ion front but did not affect the separation of high molecular weight polypeptides. The polypeptides were not rigorously characterised but a polypeptide with a molecular weight of 150,000 which comigrated with the major capsid protein of PrV was readily detected in Pr viral mRNA programmed assays.

The/

TABLE 5.4.1.1.

mRNA Activity of Exogenous RNAs in the Messenger Dependent Lysate Cell-free System

RNA Species	Concentration mRNA (µg/ml)	{^{35}S}-methionine incorporated cpm/3µl	Stimulation *
Endogenous mRNA		7,200	
Viral mRNA	7.3	20,800	2.9
Cellular mRNA	10.8	26,000	3.6
EMC virus RNA	41.7	15,000	2.1

* Times endogenous

FIG. 5.4.1.1. PRODUCTS OF EXOGENOUS mRNA TRANSLATION IN THE MESSENGER DEPENDENT RETICULOCYTE LYSATE

Messenger dependent reticulocyte lysate (MDL) translation assays of PrV mRNA (7.0µg/ml), HeLa cell mRNA (12.8µg/ml) and EMC virus RNA (49.2µg/ml) were carried out as described in the Materials and Methods (A11.4.). Assays were labelled with {^{35}S}-methionine (420µCi/ml, 1080Ci/mmol). Assay mixes were treated for electrophoresis and the polypeptides were separated on a 12% polyacrylamide gel. The gel was processed for fluorography and exposed to X-ray film.

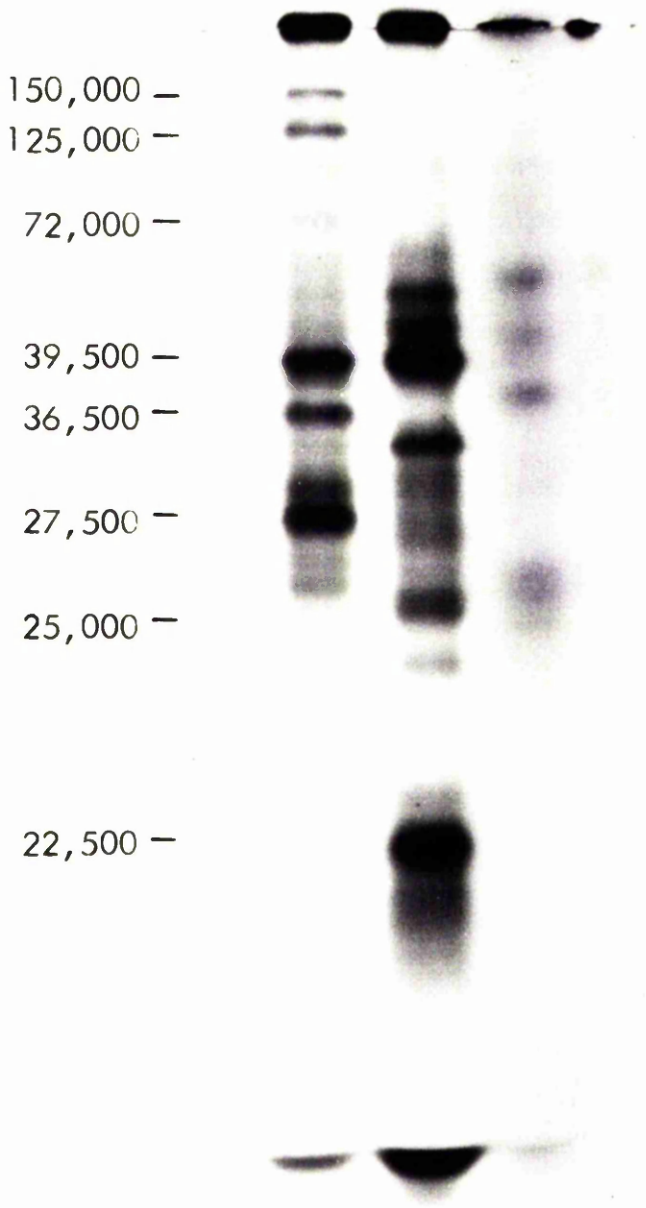
Molecular weights were estimated by comparison with Figure 4.4.1.

VI, PrV mRNA

C, HeLa cell mRNA

EMC, EMC virus RNA

E, No added mRNA


150,000
125,000
72,000
39,500
36,500
27,500
25,000
22,500
VI
C
EMC
E

The Pr viral and cellular mRNAs used in this experiment were the same preparations as those used to programme the wheat germ assays described in 4.4. Comparison of the polypeptides synthesised in the MDL (Figure 5.4.1.1.) with those detected in the wheat germ system (Figure 4.4.1.) showed that the major polypeptides synthesised are the same in both systems. The proportion of high molecular weight products synthesised in the MDL, however, was higher than that in the wheat germ and longer exposure times were not necessary to detect polypeptides with molecular weights greater than 40,000.

5.4.2. The Effect of Variations in the $\{K^+\}$ on mRNA Activity in the MDL.

The MDL used in these experiments had been prepared by the batch method described by Pelham and Jackson (1976). The concentration of added K^+ present in the MDL is 102mM and because the lysate is not fractionated by gel filtration endogenous K^+ is present. Therefore it was not possible to investigate fully the optimum K^+ for translation of exogenous RNAs. Instead the translation activities were compared at $\{K^+\}$ from 102 to 157mM.

The $\{K^+\}$ was varied by freeze drying concentrated solutions of KCl in assay tubes prior to addition of MDL, mRNA and $\{^{35}S\}$-methionine because of the limited volume which can be added to assay mixes. Assays were incubated as previously described and the total radioactivity incorporated was determined by TCA precipitation on filter discs. The results of such an experiment are shown in Figure 5.4.2.1. No species of RNA showed significant resistance to the increased concentration of KCl.

5.5. Discussion/

FIG. 5.4.2.3. THE EFFECT OF $\{K^+\}$ ON SYNTHESIS OF TCA PRECIPITABLE MATERIAL IN THE MESSENGER DEPENDENT RETICULOCYTE LYSATE

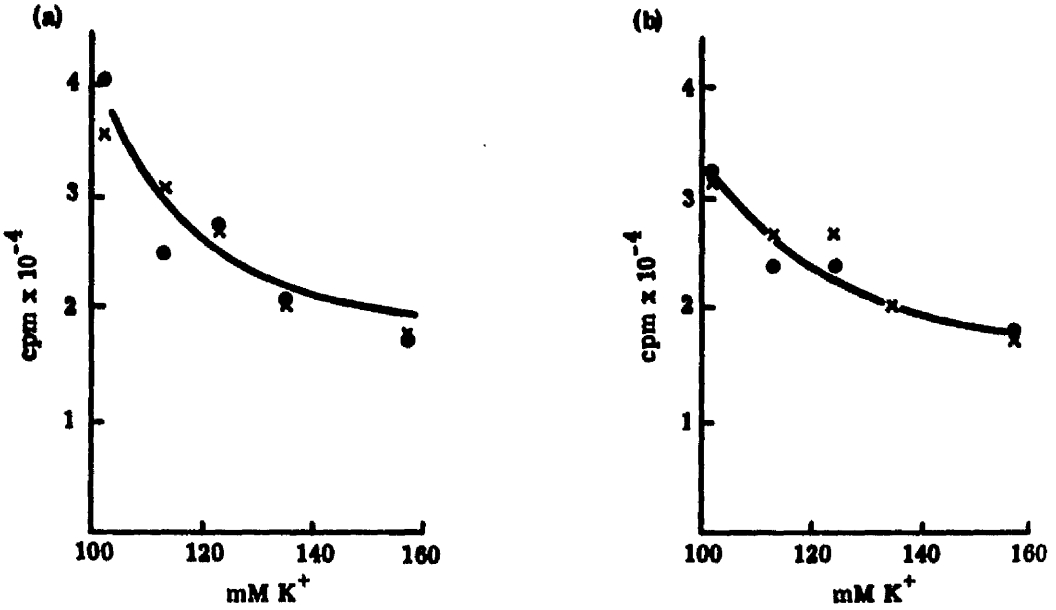
The $\{K^+\}$ in messenger dependent reticulocyte lysate (MDL) assay mixes containing 11µg/ml PrV mRNA, 4µg/ml cellular (HeLa) mRNA, 22µg/ml EMC virus RNA or 18µg/ml rabbit reticulocyte mRNA was varied by freeze drying concentrated KCl solutions in assay tubes before addition of the MDL (A11.3.) and RNA. Assay mixes were labelled with 250µCi/ml $\{^{35}S\}$-methionine (820Ci/mmol). They were incubated as described (A11.4.) and the synthesis of TCA precipitable material (cpm/5µl) was determined by precipitation of aliquots on filter discs and scintillation counting.

(a) ● PrV mRNA

x Cellular mRNA

(b) ● EMC virus RNA

x Rabbit reticulocyte mRNA


(a)
(b)
cpm x 10^{-4}
mM K^+
100
120
140
160
1
2
3
4

5.5. Discussion.

EMC virus RNA was found to be a poor mRNA in the wheat germ cell free system but was translated well in the ascites system. This result, together with the observation that discrete sized products are synthesised in EMC virus RNA programmed wheat germ assays, provides evidence that the EMC virus RNA used in these studies was functional.

The mRNA activity demonstrated for Pr viral and cellular mRNAs in the wheat germ system could not be reproduced in preincubated ascites extracts and the activity observed was considerably less than that of EMC virus RNA and reticulocyte mRNA. The reason for this is unknown but the possibility remains that a more vigorous characterisation of translation conditions would yield better results. It is clear, however, that there are inherent differences in the translation characteristics of mRNAs in different systems.

The optimum $\{K^+\}$ for EMC virus RNA stimulated incorporation in wheat germ extracts was similar to that observed for viral (PrV) mRNA but was less critical in that activity was still observed at relatively high $\{K^+\}$. In ascites extracts, however, this RNA exhibited a requirement for a considerably higher $\{K^+\}$ for maximum mRNA activity than viral (PrV), or reticulocyte mRNAs. Thus, although differences in $\{K^+\}$ optima are less obvious in the wheat germ system than in the ascites system, these results agree with the previous observation (see 3.3.1.) that late PrV mRNA does not have different $\{K^+\}$ requirements for *in vitro* translation from those of HeLa cell mRNA.

Pr viral and cellular mRNAs and EMC virus RNA all showed mRNA activity in the MDL cell free system. The stimulation observed in this system was less than that found in the wheat germ system (cf Table 3.2.1.) but the dose dependence was not determined and it is possible that these concentrations/

concentrations which saturated the wheat germ system were suboptimal in the MDL. In addition, the difference may be related to the use of $\{^{35}S\}$-methionine in MDL assays instead of $\{^{3}H\}$-leucine as in the wheat germ assays.

Comparison of the Pr viral and cellular mRNA products synthesised in the MDL with those synthesised in the wheat germ system confirmed the fidelity of the wheat germ system. The MDL, however, was more efficient in synthesis of high molecular weight products than the wheat germ system. These results suggest that the MDL will prove a useful system for translation of PrV and HeLa cell mRNAs.

CHAPTER 6

The Effect of Hypertonic Conditions on Protein Synthesis In Vivo

6.1. Introduction.

Protein synthesis in exponentially growing HeLa cells is rapidly inhibited when the cells are subjected to hypertonic medium. The lesion is at initiation and is accompanied by complete breakdown of polysomes (Saborio et al., 1974). However, protein synthesis in HeLa cells infected with poliovirus, vesicular stomatitis virus and vaccinia virus is relatively resistant to hypertonic medium and the polypeptides synthesised in the presence of a hypertonic initiation block have been shown to be predominantly of viral origin (Nuss et al., 1975; Oppermann and Koch, 1976a). Growth in hypertonic medium has also been used to unmask synthesis of viral proteins in cells where host protein synthesis is not inhibited after virus infection (Oppermann and Koch, 1976b).

On the basis of these results a model for translational control which attributes the virus-induced shut-off of host cell protein synthesis to an overall decrease in the initiation rate such that only virus mRNA which has a higher initiation rate than most cellular mRNAs is translated (Nuss et al., 1975). The model is supported by studies using other inhibitors of initiation such as dimethyl sulphoxide and ethanol (Koch et al., 1976).

Until recently, (see Gupta and Rapp, 1978) experiments of this nature had not been reported for herpesvirus-infected cells. It was therefore of interest to examine the effect of the hypertonic initiation block on protein synthesis in PrV-infected cells.

6.2/

6.2. The Effect of hypertonic media on protein synthesis.

Protein synthesis in infected and mock-infected HeLa cells was compared by determining the incorporation of {^{3}H}-methionine into TCA precipitable material at appropriate times after infection as described in the Materials and Methods. The total incorporation per coverslip at 2 h and 6 h after infection or mock-infection is shown in Table 6.2.1. At 6 h protein synthesis was decreased in virus-infected cells.

The effect of hypertonic media on protein synthesis was examined by adding NaCl to the medium 30 min prior to harvesting and determining incorporation as previously described. Results of such experiments at 2 h and 6 h after infection or mock-infection are shown in Figure 6.2.1. To allow readier comparison of the relative sensitivities to hypertonicity the incorporation at elevated NaCl concentrations is plotted as a percentage of that in normal medium. This was particularly important at 6 h post-infection because protein synthesis in infected cells was already considerably depressed and a similar percentage decrease represented a smaller decrease in the number of counts incorporated. Plotting the results in this way shows that protein synthesis in infected cells at high levels of added NaCl is inhibited as much as that in uninfected cells. At low levels of added NaCl infected cells show an increased sensitivity to the hypertonic treatment.

6.3. The effect of hypertonic media on polysomes.

The effect of hypertonic media on mock-infected and PrV-infected HeLa cell polysomes was investigated by appropriately adjusting the NaCl concentration in the medium 15 min prior to harvesting. Cytoplasmic extracts were centrifuged through sucrose gradients and the polysomes profiles recorded as previously described. In accordance with/

TABLE 6.2.1.

Incorporation of {^{3}H} methionine into normal and infected HeLa cells

Time after infection or mock-infection	{^{3}H} methionine (cpm/coverslip)	
	Mock-infected	Infected
2 h	4423	4594
6 h	4644	2190

HeLa cells were grown on glass coverslips in 50mm Petri dishes and infected with PrV or mock-infected, 15 min before harvesting {^{3}H}-methionine (15µCi/dish) was added. Coverslips were removed from the dishes, washed 3 times with 5% (w/v) TCA, once in ethanol and the radioactivity measured by scintillation counting.

Incorporation in isotonic medium:

	2h	6h
mock-infected	4423	4594
PrV-infected	4644	2190

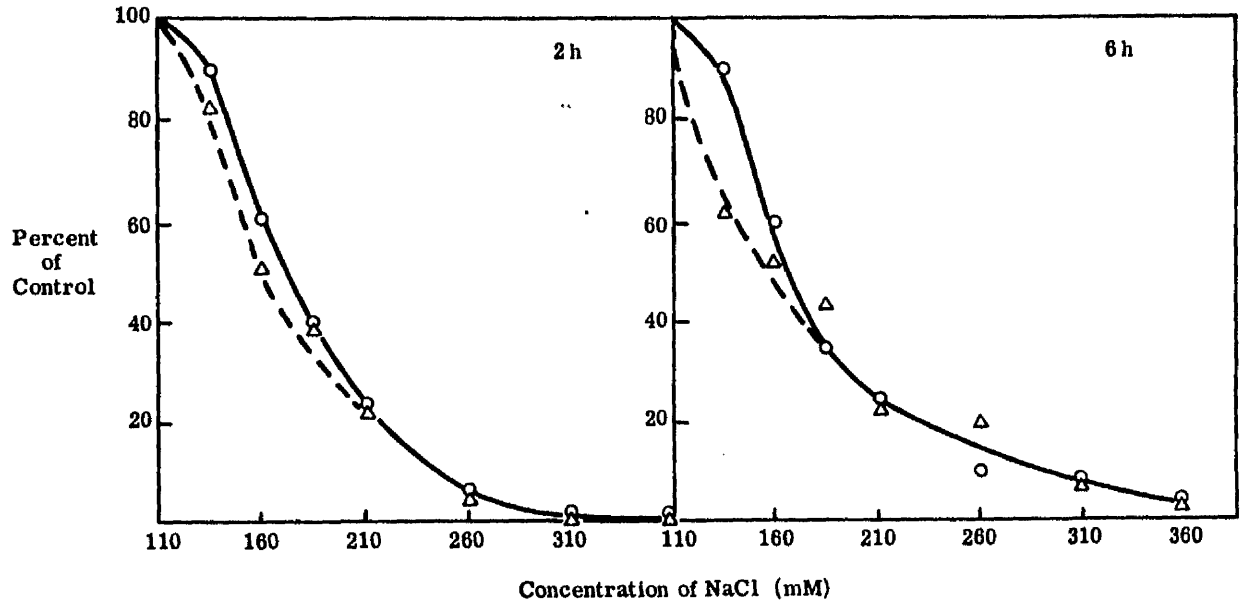


FIG. 6.2.1. EFFECT OF HYPERTONIC MEDIUM ON PROTEIN SYNTHESIS IN PrV-INFECTED AND MOCK-INFECTED HeLa CELLS

HeLa cells were grown on glass coverslips in 50mM Petri dishes and infected with PrV (Δ) or mock-infected (o). 30 min before harvesting the medium was replaced with normal (isotonic) medium which contained 110mM NaCl or medium made hypertonic by the addition of NaCl. {^{3}H}-methionine (15μCi/dish) was added to the medium 15min before harvesting. Coverslips were removed from the dishes, washed three times with 5% (w/v) TCA, once in ethanol and the radioactivity was measured by scintillation counting. The incorporation into acid-insoluble material is expressed as a percentage of that occurring in isotonic medium.

Incorporation in isotonic medium:

	2h	6h
mock-infected	4423	4594
PrV-infected	4644	2190

with previous reports, growth in hypertonic media caused disaggregation of polysomes in uninfected HeLa cells (Figure 6.3.1. a-c). A similar decrease in the number of polysome was found in PrV-infected cells (Figure 6.3.1. d-f). In both cases no polysomes could be detected when cells were incubated in media containing 260mM NaCl. The reduction in the number of polysomes was quantitated by calculating the polysome to monosome ratio at various degrees of hypertonicity. The amounts were calculated by cutting out and weighing the areas under the curve obtained from the chart recording. Figure 6.3.2. shows the ratios in mock-infected and infected cells at differing NaCl concentrations. Although the percentage reduction is less because of virus induced disaggregation of polysomes, polysomes in infected cells are no more resistant to hypertonicity than those in uninfected cells.

6.4. Discussion.

Both sets of experiments showed that protein synthesis in herpesvirus infected cells is no more resistant to growth in hypertonic media than protein synthesis in uninfected cells. At low levels of hypertonicity infected cells show an increased sensitivity to the hypertonic treatment. This may reflect some effect of the virus on the cell membrane which facilitates equilibration of extracellular and intracellular ion concentrations. These results will be considered further in Chapter 8.

FIG. 6.3.1. THE EFFECT OF HYPERTONIC MEDIUM ON POLYSOME PROFILES

Exponentially growing HeLa cells were mock-infected or infected with 20pfu/cell PrV. After 4h 45min the medium was replaced with normal (isotonic) medium or medium made hypertonic by addition of NaCl. 15min later (5h post-infection) the cells were harvested. Cytoplasmic extracts were prepared and layered onto 15-30% sucrose gradients. Gradients were centrifuged for 110min at 27.000g in a SW27 rotor and collected by pumping from the bottom of the tube through a Gilford 2000 recording spectrophotometer which monitored the absorbance at 260nm.

(a) mock-infected cells, isotonic medium, 110mM NaCl

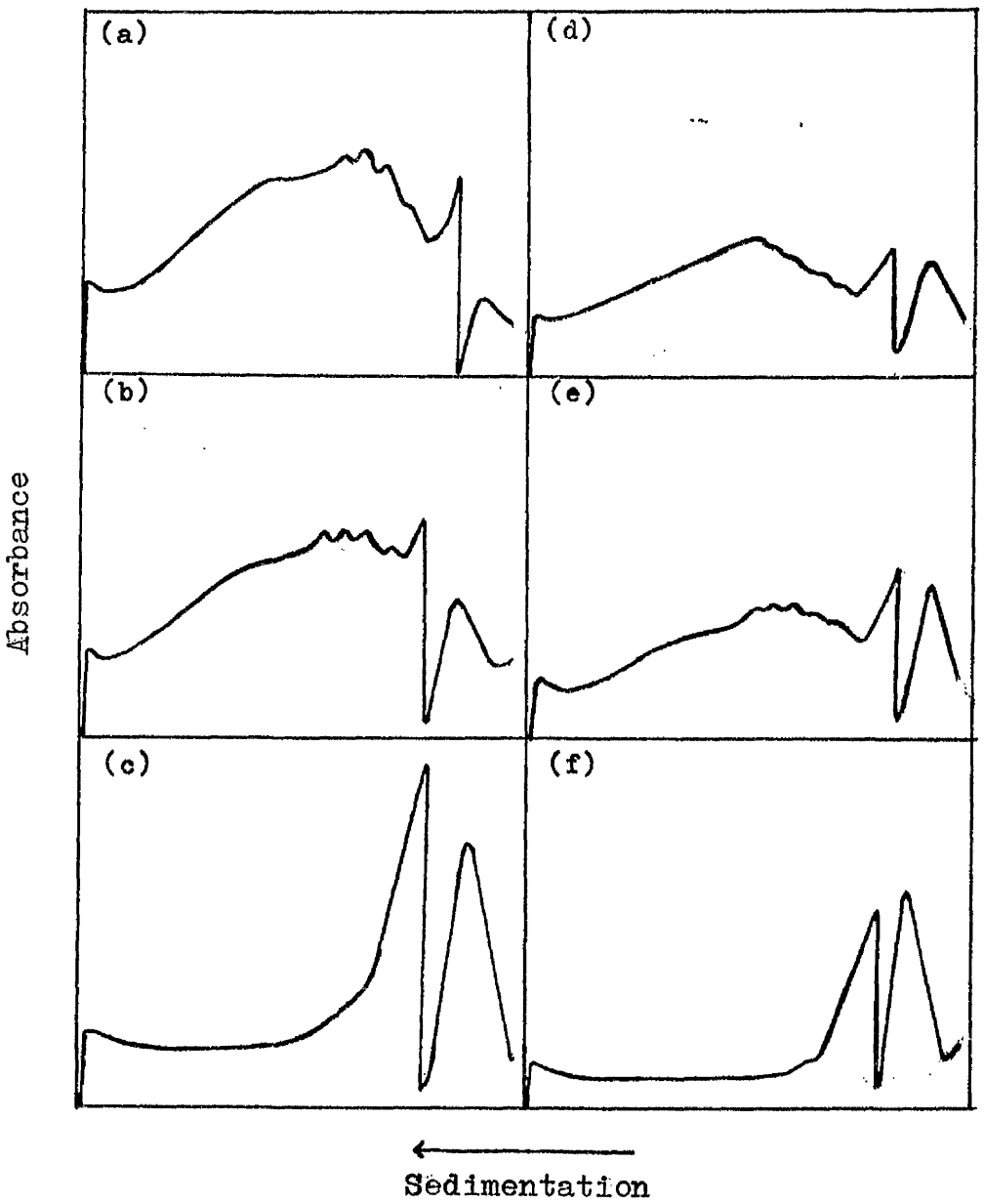
(b) mock-infected cells, hypertonic medium, 160mM NaCl

(c) mock-infected cells, hypertonic medium, 260mM NaCl

(d) PrV-infected cells, isotonic medium, 100mM NaCl

(e) PrV-infected cells, hypertonic medium, 160mM NaCl

(f) PrV-infected cells, hypertonic medium, 260mM NaCl


(a)
(d)
(b)
(e)
(c)
(f)
Absorbance
Sedimentation

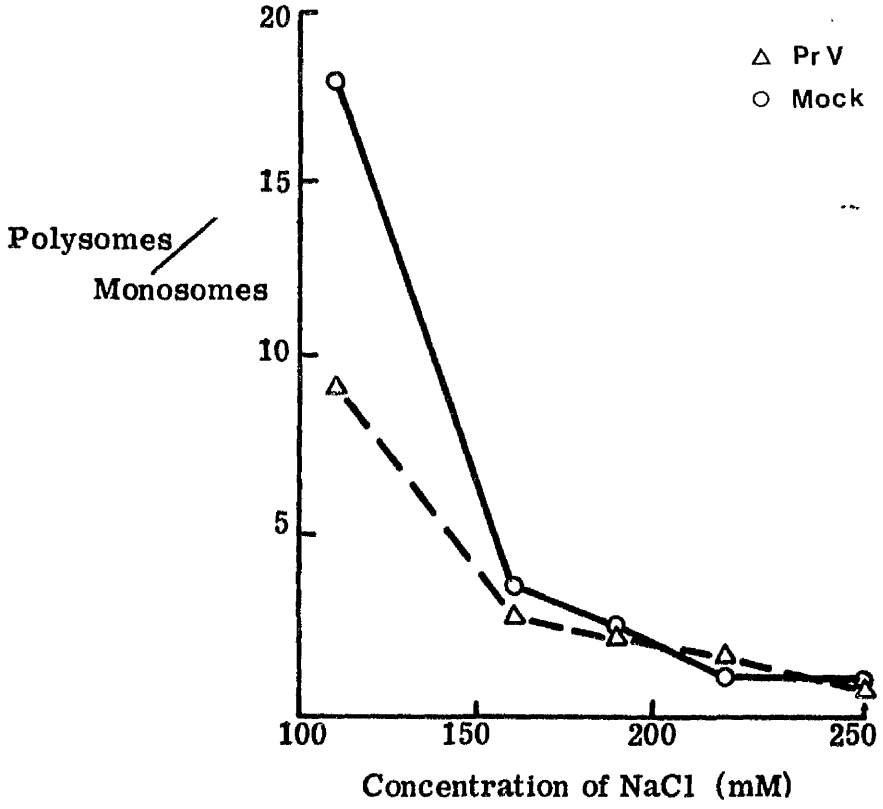


FIG. 6.3.2. DISAGGREGATION OF POLYSOMES IN PrV-INFECTED AND MOCK-INFECTED CELLS IN HYPERTONIC MEDIUM

The ratio of polysomes to monosomes in PrV-infected and mock-infected HeLa cells subjected to 15min treatment with hypertonic medium (see Fig. 6.3.1.) was estimated by cutting out and weighing the appropriate areas of the optical density trace obtained from the recording spectrophotometer (see Fig. 6.3.1.).

CHAPTER 7

Translation of Polysomes

7.1. Introduction.

Polysomes prepared from herpesvirus infected cells show an increase in the number of rapidly sedimenting polysomes when compared with those prepared from mock-infected cells. The shift in polysome size is related to the synthesis of viral polypeptides and the large polysomes are involved in synthesis of viral proteins (see 1.5.4.1.).

A similar increase in polysome size is found in picornavirus infected cells (Penman et al., 1963). In this case the large polysomes can be correlated with the large size of the mRNA, which is a single molecule with molecular weight 2.6×10^6, in contrast to the heterogeneous and generally smaller cellular mRNAs. A correlation between polysome and polypeptide size has been demonstrated in cells infected with the rhabdovirus vesicular stomatitis virus (VSV). VSV G protein, molecular weight 63,000, and the N protein, 50,000, are synthesised on polysomes with a mean size of 8 ribosomes/polysome while the M protein, 24,000 is synthesised on polysomes of considerably smaller size (David, 1978).

Vass (1975) examined the nascent polypeptides on heavy and light polysomes in HeLa cells infected with PrV and mock-infected cells and found no such relationship. Instead at 2½ h post-infection the heavy polysomes seemed to contain a higher proportion of low molecular weight nascent polypeptides than is found in the light polysome fraction.

The availability of a cell-free system makes it possible to study the increased mRNA loading more closely. Using such a system the/

the polypeptide synthesis in different size classes of polysomes can be examined in order to investigate the involvement of large polysomes in viral protein synthesis and the relationship between polysome and polypeptide sizes.

Synthesis of high molecular weight polypeptides in the wheat germ cell free system stimulated by mRNA is very inefficient. Hence, these studies were carried out using polysomes to stimulate polypeptide synthesis.

7.2. Investigation of the Products of Polysome Translation.

7.2.1. Polysome Translation Characteristics.

The wheat germ cell free system has been used mainly for translation of mRNA. However, a few studies involving addition of exogenous polysomes have been carried out.

Polysomes have been added to unfractionated wheat germ extracts which contain endogenous polysomes (Sun et al., 1975; Luthe and Petersen, 1977) and to the S100 fraction prepared by centrifugation of the unfractionated extract at 100,000g to pellet endogenous polysomes (Higgins & Spencer, 1977; Jones et al., 1977). The activities observed are similar.

Translation characteristics differ slightly from those described for mRNA. The optimum $\{Mg^{++}\}$ lies in the range 5-6mM which is twice that observed for mRNA. The optimum $\{K^{+}\}$ has not been carefully examined. Higgins & Spencer (1977) used 130mM K^{+} but other studies have been carried out with 60-70mM K^{+}. H.H. Singer (personal communication) found that maximum stimulation of incorporation was observed at 180mM K^{+}. Generally, the time course is shorter than for mRNA.

7.2.2./

7.2.2. Polysome Translation.

Polysomes were prepared from PrV-infected and mock-infected HeLa cells 5 h post-infection as described in the Materials and Methods. The pellets were rinsed with 2M sucrose and resuspended by vortexing in sterile deionised water. One A_{260} unit of polysomes was added per 50µl assay containing 15µl unfractionated wheat germ extract, 180mM K^+, 0.4mM spermidine and 2.5mM Mg^{++}. Assays were incubated at 25°C for 90 min. Freshly prepared polysomes were used because the activity was found to decrease rapidly on storage at -20°C. Stimulation of the endogenous incorporation by exogenous polysomes is shown in Table 7.2.2.1.

The products of polysome translation were examined by polyacrylamide gel electrophoresis. Figure 7.2.2.1. shows a fluorograph of the products. No endogenous products were detected in a parallel track on the same gel after this exposure time. Comparison of the polysome products (Figure 7.2.2.1.) with the mRNA products (Figure 4.4.1.) shows that the proportion of high molecular weight products synthesised in polysome stimulated assays is considerably greater.

The highest molecular weight detected in products from viral polysomes was 150,000 while the maximum size of mock-infected cell polysome products was 115,000. The proportion of high molecular weight products synthesised on viral polysomes was greater than was synthesised on mock-infected cell polysomes (Fig. 7.2.2.2.).

The fluorograph shows that there are at least 18 polypeptides with molecular weights greater than 20,500 present in infected cell polysome translation products which are not present in the products from mock-infected or PrV infected cycloheximide treated cell polysomes. Eight of these polypeptides co-migrate with polypeptides present/

TABLE 7.2.2.1.

Incorporation of {^{35}S} met into TCA precipitable material in wheat germ extracts stimulated with polysomes.

Polysomes	cpm incorporated /50µl	Stimulation *
Wheat germ endogenous	110,330	
PrV infected HeLa cell	850,490	7.7
HeLa cell	630,610	5.7

* Times endogenous

FIG. 7.2.2.1. PRODUCTS OF IN VITRO TRANSLATION OF POLYSOMES

Polysome pellets were prepared from PrV-infected and mock-infected HeLa cells 5h after infection and from PrV-infected HeLa cells which had been treated with cycloheximide from the time of infection until 15 min before harvesting as described in the Materials and Methods (A5.). Cycloheximide treated polysomes were generously supplied by Mr. M. Chowdhury. The pellets were rinsed with sterile 2M sucrose and suspended in deionised water.

Polysomes were added to wheat germ assay mixes (A3.4.11.) containing 180mM K^+ (added as KCl), 25mM Mg^{++} 0.4mM spermidine and 500µCi/ml {^{35}S}-methionine (1080Ci/mmol) to a concentration of 20 A_{260} units/ml. After incubation, 10µl aliquots of assay mixes were treated for electrophoresis and electrophoresed in parallel with PrV capsid and PrV-infected and mock-infected HeLa cell lysates (A9). The gel was processed for fluorography and exposed to X-ray film.

Track:

A Mock-infected HeLa cell lysate

B PrV-infected HeLa cell lysate

C PrV capsid

D Products of cycloheximide treated PrV-infected cell polysomes

E Products of PrV-infected cell polysomes

F Products of mock-infected cell polysomes

Track E Polypeptides labelled 1-18 are present only in infected cell polysome products

Track F Polypeptides labelled 1-21 are present in both infected and mock-infected cell polysome products

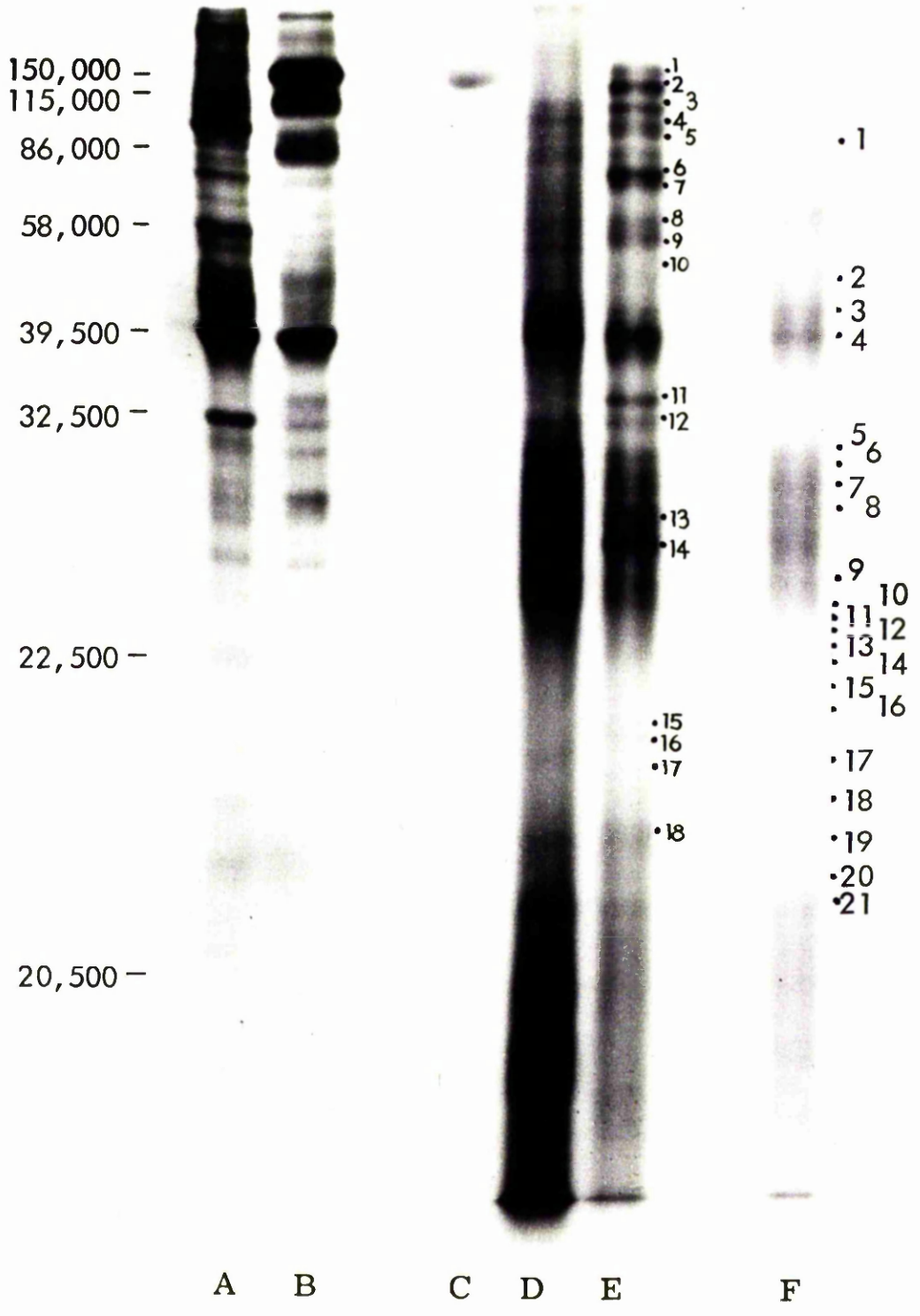
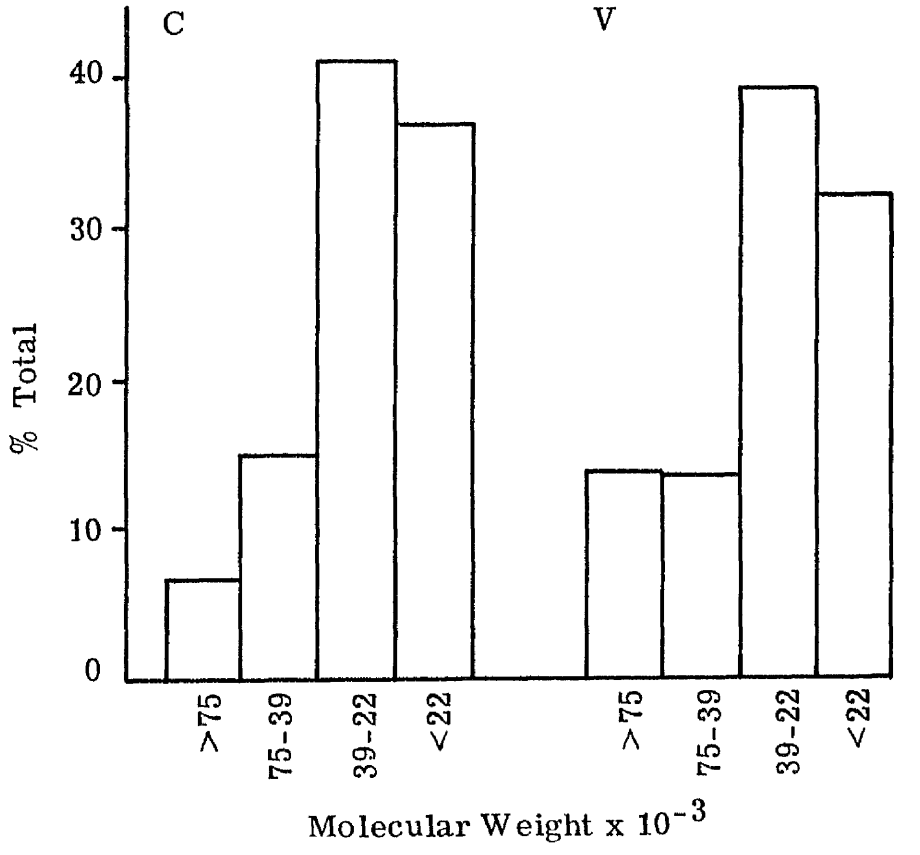

150,000
115,000
86,000
58,000
39,500
32,500
22,500
20,500
A
B
C
D
E
F

FIG. 7.2.2.2. MOLECULAR WEIGHT DISTRIBUTION OF THE IN VITRO PRODUCTS OF PrV-INFECTED AND MOCK-INFECTED CELL POLYSOMES

PrV-infected and mock-infected cell polysomes were harvested in the wheat germ system. The products were separated on a 12% acrylamide gel and detected by fluorography (see Fig. 7.2.2.1.). The film was scanned on a Joyce Loebel Autodensidator. The percentage of total products present in four molecular weight ranges was calculated by cutting out and weighing the area under the curve.

C, mock-infected cell polysome products

V, PrV-infected cell polysome products


C
V
40
30
20
10
0
% Total
>75
75-39
39-22
<22
>75
75-39
39-22
<22
Molecular Weight x 10^{-3}

present only in the infected cell lysate. The other polypeptides may arise either from premature termination of translation or they may be precursor polypeptides which are post-translationally modified in the infected cell.

Co-migrating polypeptides synthesised on both infected and mock-infected cell polysomes are indicated in the Figure. With one exception, these co-migrate with polypeptides in infected and mock-infected cell lysates. A band co-migrating with the cellular polysome product 6 is detectable only in the mock-infected cell lysate. Hence it is possible that the in vitro co-migrating products are not identical. This observation emphasises the importance of a more definitive means of classification.

7.3. Investigation of the polypeptides synthesised on different size classes of PrV-infected and mock-infected HeLa cell polysomes.

Polysomes from PrV-infected and mock-infected HeLa cells were divided into 3 size classes by fractionation of sucrose density gradients of cytoplasmic extracts. Figure 7.3.1. shows the division of polysome gradients into heavy (H), medium (M) and light (L) polysomes. Two different fractionations of PrV-infected cell polysomes were examined. The L fraction defined in 7.3.1(a) contained 2-6 ribosomes/polysome while that defined in 7.3.1(b) contained 2-4 ribosomes/polysome. Hence the L fraction in (a) overlaps with the M fraction in (b). Mock-infected cell polysomes (7.3.1(c)) were fractionated so that the L polysomes contained 2-4 ribosomes/polysome. Polysomes were pelleted from these fractions and translated in the wheat germ system as described previously. The products were examined by electrophoresis and fluorography and are shown in Figure 7.3.2.

It/

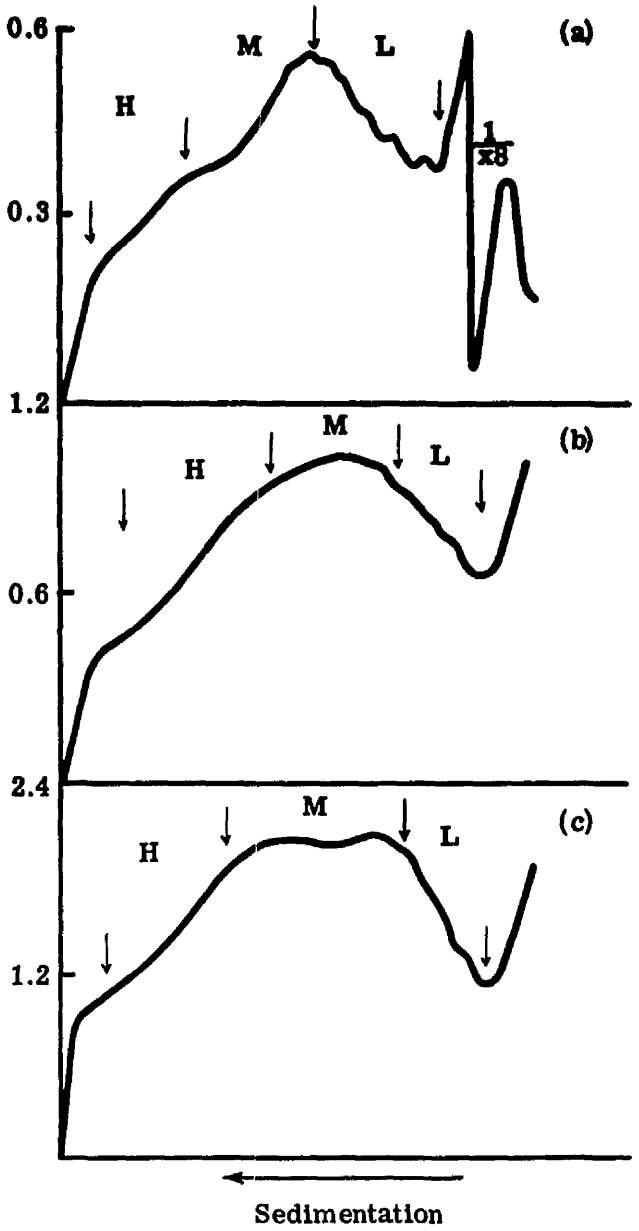

0.6
0.3
1.2
H
M
L
(a)
1/x8
0.6
2.4
H
M
L
(b)
1.2
H
M
L
(c)
Sedimentation

FIG. 7.3.1. FRACTIONATION OF POLYSOMES INTO THREE SIZE CLASSES

Exponentially growing HeLa cells were mock-infected or infected with 20pfu/cell PrV. After 5h the cells were harvested mechanically, lysed, and the cytoplasmic extracts were layered onto 15-30% sucrose gradients. The gradients were centrifuged for 110 min at 27,000 in a SW27 rotor and fractions containing approximately 1ml were collected by pumping from the bottom of the tube through a Gilford 2000 recording spectrophotometer which monitored the absorbance at 260nm. The heavy (H), medium (M) and light (L) polysomes were pooled as indicated by the arrows.

(a) Experiment I, PrV-infected cell polysomes

(b) Experiment II, PrV-infected cell polysomes

(c) Experiment II, Mock-infected cell polysomes

FIG. 7.3.2. IN VITRO TRANSLATION PRODUCTS OF DIFFERENT SIZE CLASSES OF PrV-INFECTED AND MOCK-INFECTED CELL POLYSOMES

The polysome fractions defined in Fig. 7.3.1. were pelleted, rinsed with 2M sucrose and suspended in deionised water. One A_{260} unit of each polysome fraction was added to a wheat germ assay mix (A3.4.11.) containing 180mM K^+ (added as KCl) 2.5mM Mg^{++}, 0.4mM spermidine and 500µCi/ml {^{35}S}-methionine (1080Ci/mmol). After incubation, 10µl aliquots of the assay mixes were treated for electrophoresis and the polypeptides were separated on a 12% polyacrylamide gel together with PrV capsid proteins. The gel was processed for fluorography and exposed to X-ray film.

The major capsid protein of PrV (VP2) is indicated. Polypeptide k is found on light or medium polysomes. Polypeptide l is found mainly on heavier polysomes (CP(X) is a cellular polypeptide with the same migration rate as polypeptide l.

The black "blobs" are due to blackening of the film by DMSO.

Track:

A PrV capsid

B Heavy PrV-infected cell polysomes, experiment I

C Medium " " " " "

D Light " " " " "

E Heavy " " " experiment II

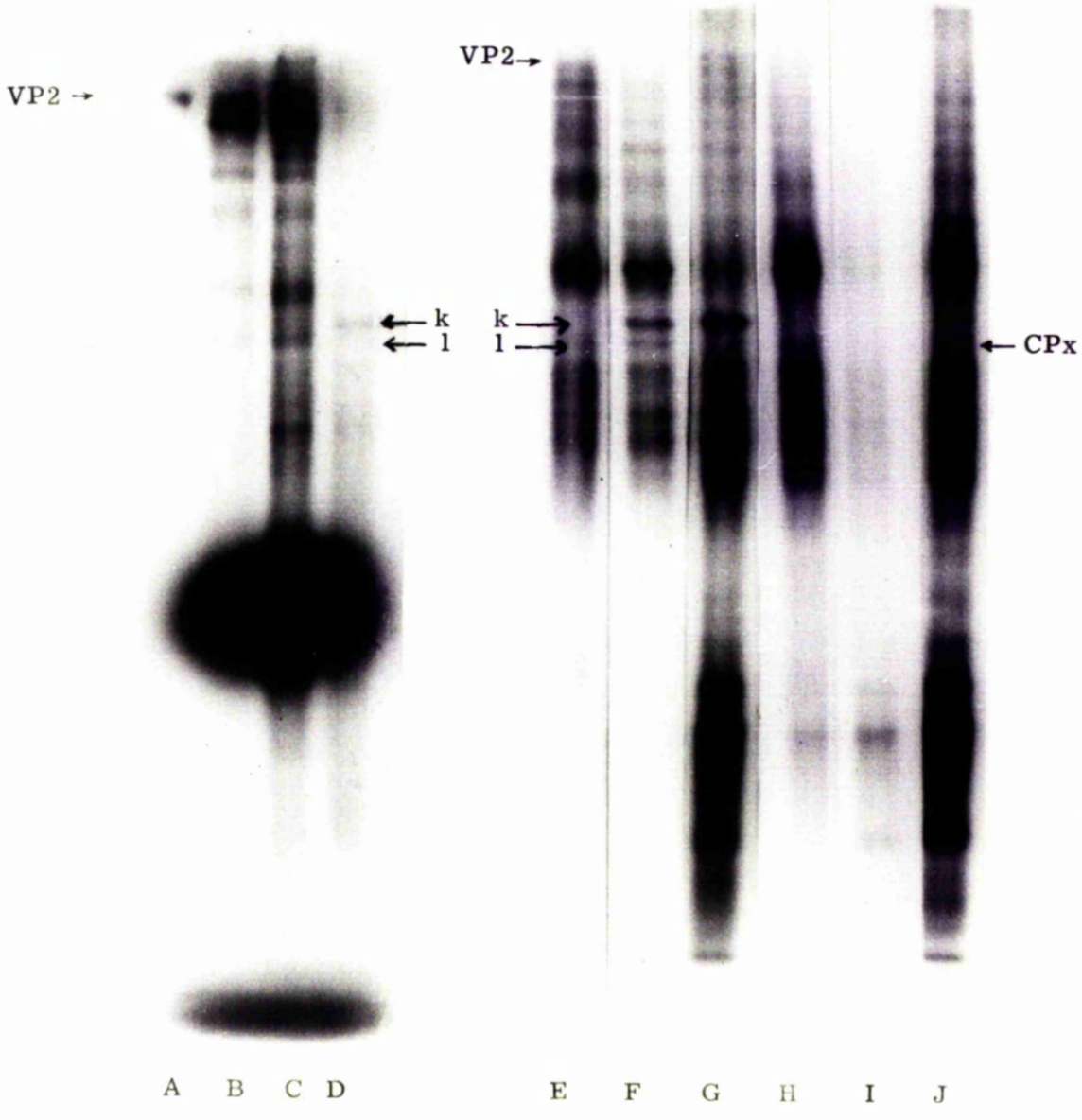
F Medium " " " " "

G Light " " " " "

H Heavy Mock-infected cell polysomes, experiment II

I Medium " " " " "

J Light " " " " "


VP2
VP2
k
l
k
l
CPx
A
B
C
D
E
F
G
H
I
J

It was difficult to obtain the same incorporation for all fractions in polysome translation assays, possibly because of resuspension problems and therefore the exposure times were varied.

High molecular weight polypeptides are synthesised on all size classes of polysomes in both infected and mock-infected cells. There is a decreased proportion of low molecular weight material synthesised on heavy polysomes in both cases, but the decrease is greater in virus infected cells. (Fig. 7.3.3.).

The restriction of synthesis of cellular polypeptides to light polysomes in the infected cells cannot be detected. However, two polypeptides exclusive to infected cell polysomes show restricted synthesis. Polypeptide l is present only on M and H polysomes. The mobility of this protein is similar to that of a cellular protein CP(x) which does not show restricted synthesis. Polypeptide k is present in very much increased proportions on the light polysomes. In experiment I, where the L fraction contains 2-6 ribosomes per polysome, the protein could only be identified in the products of L polysomes. In experiment II where polysomes containing 5-6 ribosomes formed part of the M fraction, the polypeptide was synthesised on both L and M polysomes. Hence its synthesis is confined to polysomes with 2-6 ribosomes per polysome.

The restriction of synthesis of polypeptide k to relatively small polysomes leads to the conclusion that initiation occurs at a low frequency on the mRNA. The low initiation rate, however, seems to be compensated for because relatively large quantities of the protein are synthesised on the light polysomes.

7.4./

FIG. 7.3.3. MOLECULAR WEIGHT DISTRIBUTION OF THE IN VITRO PRODUCTS OF HEAVY AND LIGHT PrV-INFECTED AND MOCK-INFECTED CELL POLYSOMES

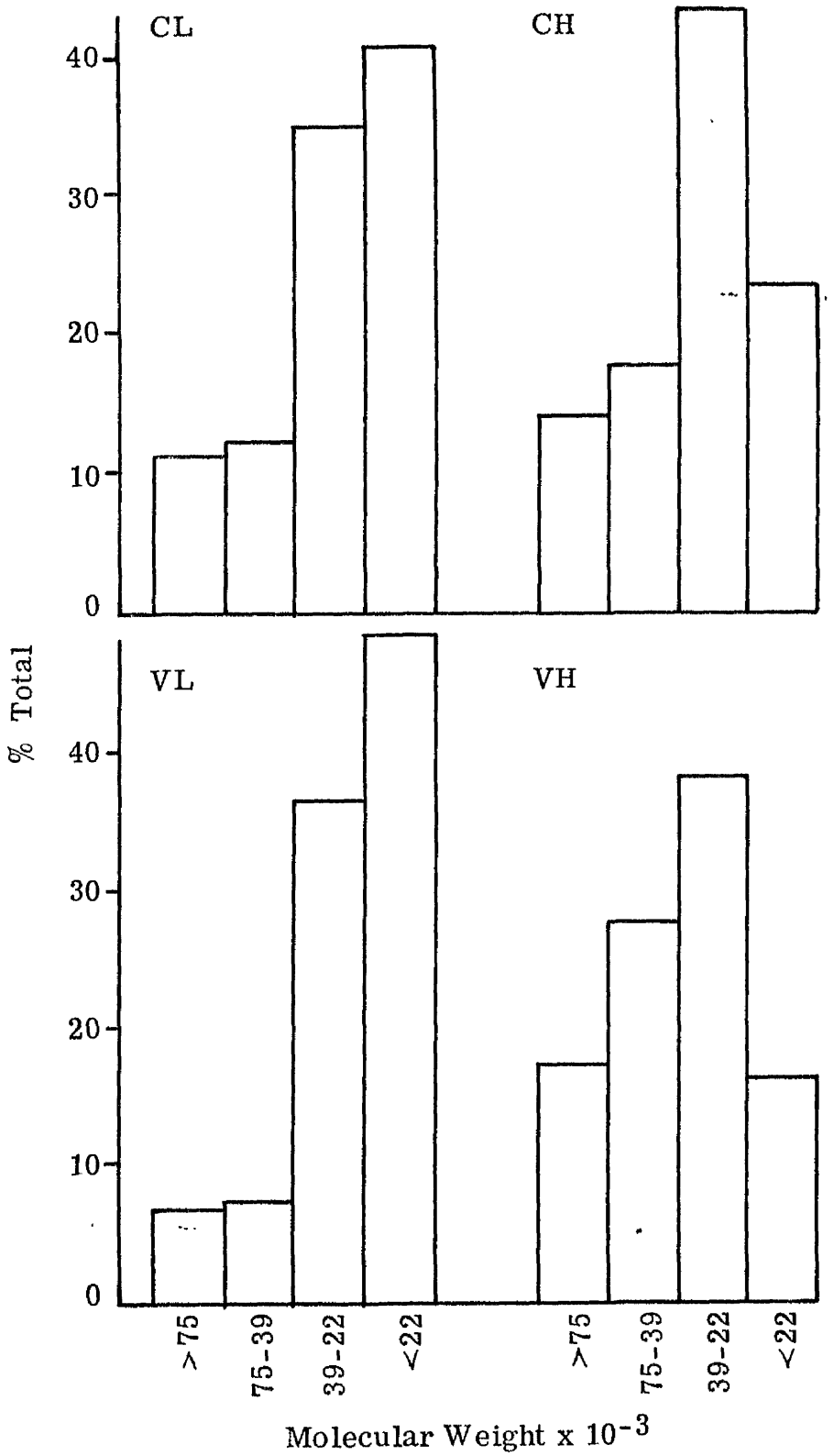
Heavy and light PrV-infected and mock-infected cell polysomes were translated in the wheat germ system. The products were separated on a 12% polyacrylamide gel and detected by fluorography (see Figure 7.3.2.). The film was scanned on a Joyce Loebel Autodensidator. The percentage of total products present in four molecular weight ranges was calculated by cutting out and weighing the area under the curve.

CL, mock-infected cell light polysomes

CH, mock-infected cell heavy polysomes

VL, PrV-infected cell light polysomes (Exp. II, Fig. 7.3.1.)

VH, PrV-infected cell heavy polysomes (Exp. II, Fig. 7.3.1.)


CL
CH
VL
VH
40
30
20
10
0
% Total
>75
75-39
39-22
<22
Molecular Weight x 10^{-3}

7.4. Discussion.

The ionic conditions used for polysome stimulated translation in wheat germ extracts differ from those used for translation of mRNA. These differences may be related to the roles of cations in polypeptide synthesis. Mg^{++} is involved in stabilising RNA-protein complexes and it is possible that the higher Mg^{++} requirement is related to the increase in polysome concentration. K^+ has a role in initiation and elongation. Initiation is inhibited at high $\{K^+\}$. Translation of mRNA is reduced to 19% of that observed at 75mM K^+ when translation is started in 130mM K^+ but if assays are preincubated at 75mM K^+ then K^+ is added to 150mM, this drastic inhibition is not observed (see 4.3.). Higgins and Spencer (1977) found that only 15% of polysome stimulated incorporation was due to initiation in wheat germ assays of 130mM K^+. Hence the polysome stimulated synthesis described here is probably due mainly to elongation of polypeptides initiated *in vivo*.

Elongation rates are increased at high $\{K^+\}$ (Mathews & Osborn, 1974). However, the overall inhibitory effects of high K^+ precludes the use of such conditions for mRNA translation. Polyamines are used to increase the elongation rates *in vitro* (Hunter *et al.*, 1977). The advantage of polysome stimulated translation is that elongation rates can be increased by both methods. This may account in part for the increased proportion of high molecular weight products synthesised in polysome stimulated assays. The faster rate of elongation increases the probability of each ribosome completing translation of a full-length protein before endonucleolytic cleavage.

At least two other factors which reduce mRNA degradation contribute to the decreased synthesis of low molecular weight polypeptides. The/

The use of polysomes reduces the number of operations involved prior to translation and therefore the possibility of mRNA degradation. Further, the large amount of rRNA introduced into the system protects mRNA by providing an alternative substrate for endonucleolytic attack.

These studies were designed to investigate the increase in polysome sedimentation rates in PrV-infected cells. The polysome translation shows that in PrV-infected cells at 5 h post-infection, with two exceptions, there is no difference in the polypeptides synthesised on different size classes of polysomes. It was not possible to identify cellular polypeptides which are synthesised predominantly on light polysomes and hence confirm the involvement of larger polysomes in viral protein synthesis. However, it is not clear how much cellular protein synthesis is taking place at this time, and the polypeptides were only classified by co-migration so it is possible that such effects were not recognised.

Vass (1975) found no relationship between the size of nascent polypeptides and polysome size. At 2½ h post-infection the heavy polysomes seemed to contain a higher proportion of low molecular weight material. These experiments do not show conclusively that there is no relationship between polysome size and polypeptide size because the difference may be caused by different rates of initiation in the mixed viral and cellular mRNA population present at this time.

Elongation in vitro showed that some relationship between the polypeptide and polysome sizes exists. In PrV-infected and mock-infected cells the proportion of low molecular weight polypeptides decreases as polysome size increases. The number of ribosomes is limited by the length of the translated region of a mRNA molecule. If the rates of initiation are the same, a short message will have

less/

less ribosomes than a long message because rounds of translation will be completed earlier. However, although the *in vitro* polysome products are larger, there is no such difference in the maximum size of polypeptides in infected and mock-infected cell lysates so it is unlikely that the increase in polysome size is related to the size of polypeptide in infected cells.

Chapter 8

Discussion and Conclusion

The general aim of this project was to examine some possible mechanisms for translational control in PrV-infected HeLa cells by translation of viral and cellular mRNAs *in vitro*. The initial requirements for such a study were the isolation of mRNAs and the setting up of a cell free protein synthesising system capable of translating these mRNAs.

Selection of mRNAs from other RNA molecules present in cells is made possible by the presence of 3' poly-(A) tracts on mRNA molecules (see 1.2.3.2.). Since herpesvirus infected cells contain both cellular and viral mRNAs, a method of separating these is required. Clearly the definitive way of separating putative viral and cellular mRNAs is by hybridisation to the appropriate DNA. One disadvantage of using such a method is that it requires a large number of manipulations and the probability of damage to the RNA is high. Furthermore, the quantities of mRNA obtained are very low. For these reasons, no attempt to isolate mRNAs by hybridisation was made. Instead, viral mRNAs were preferentially selected by using only polysomes for isolation of mRNAs. The rationale for this is that it has been shown that while a significant amount of cellular mRNA is present in the cytoplasm in PrV-infected cells 5h post-infection most of the polysome associated mRNA is virus specific (Rakusanova *et al.*, 1972). Application of these results to this study is particularly justified because the virus stock used in this study was originally obtained from the above group of workers.

Further to this, in pilot hybridisation experiments 54% of the polysomal poly-(A) containing RNA from PrV-infected cells was shown to contain/

contain sequences complementary to viral DNA. Since saturation analyses were not carried out because of the limited time available and because it was likely that the polysomal poly-(A) containing RNA contained some labelled rRNA (see 2.5.) this may represent a minimum estimate. Thus it seems justified to assume that viral mRNA was preferentially selected by this method.

Isolation of polysomes had the additional advantage that, by inspection of the sucrose density gradient polysome profile, the efficiency of infection could be monitored (see 2.5.).

The mRNA activity of these putative viral and cellular mRNAs was then examined in in vitro protein synthesising systems. The system chosen initially for study was that derived from wheat germ. There were two reasons for this. Firstly, this system was reported to be useful for translating a number of heterologous mRNAs (see 3.1.). Secondly, (and this was important at the early stages of the study), the endogenous activity was low. This made it possible to check the mRNA activity of the putative mRNAs simply by measuring their ability to stimulate incorporation of radioactive aminoacids into TCA precipitable material. Such an assay would not have been possible with the reticulocyte lysate which was the alternative choice. The unfractionated lysate is a very efficient cell free translation system but has high endogenous mRNA activity and identification of exogenous mRNA activity would have required examination of the in vitro products. This was somewhat impractical in the early stages of developing the techniques. Subsequently, however, a messenger dependent reticulocyte lysate became available and this was found to be useful in the later stages of this study.

Incubation of the putative mRNAs in a wheat germ cell free system showed/

showed that both PrV-infected and mock-infected HeLa cell polysomal poly-(A) containing RNAs could stimulate incorporation of amino acids into TCA precipitable material. It was concluded therefore that these RNA species contain significant amounts of mRNA.

Characterisation of the cation concentrations required for maximum stimulatory activity in the wheat germ system showed that the requirements were similar for viral and cellular mRNAs and allowed definition of standard conditions for translation (3.3.4.). The dose response, time course and presence of endogenous aminoacids in the system were also characterised. These features have already been discussed (3.7.).

The mRNA activity was further characterised by an examination of the polypeptides synthesised in vitro. The in vitro products of viral mRNA differed from those of cellular mRNA and preliminary classification of these polypeptides as viral or cellular coded was carried out by comparison of the electrophoretic mobilities of infected cell mRNA products with those of cellular mRNA products. A significant proportion of the polypeptides classified as viral coded were shown to comigrate with polypeptides present in infected but not mock-infected cell lysates. However, classification on the basis of comigration cannot be considered definitive (see 4.5. and 4.8.) and clarification of the origin of the polypeptides by immune-precipitation and hybrid arrested translation was sought.

Immune precipitation with antisera raised against the major capsid protein of PrV verified that this polypeptide was synthesised in assays programmed with viral mRNA. Synthesis of 7 polypeptides which had been classified as viral coded by comigration was shown to be significantly decreased/

decreased when viral mRNA was hybridised to PrV DNA prior to translation thus confirming their origin. Confirmation of the preliminary classification was therefore possible for 8 of the 29 infected cell mRNA products assigned to the "viral coded" class. The usefulness of these methods for classification and the reasons for the limited success obtained have been extensively discussed (4.8.) and will not be dwelt on here.

These results show clearly that PrV mRNA can be translated in vitro in a heterologous cell free system without addition of specific initiation factors. This result is in contrast to that reported for EHV mRNA translation in vitro (Allen & Bryans, 1976) but is in agreement with the observations of Preston (1977) for in vitro translation of HSV mRNA.

The availability of a messenger dependent reticulocyte lysate (MDL) made it possible to explore some of the conclusions that had been drawn from the wheat germ system and to examine critically its usefulness. The low proportion of high molecular weight polypeptides synthesised in wheat germ extracts relative to the proportions found in cell lysates has already been noted (see 4.8.). One possible explanation for this was that the mRNA was degraded. This could also account for the presence of some of the polypeptides synthesised in vitro which did not comigrate with any of those detectable in cell lysates. When the same mRNA preparations were translated in the MDL, and the wheat germ system, the major polypeptides synthesised were the same but the proportion of high molecular weight products in the MDL differed considerably from that in the wheat germ system and was more representative of that found in cell lysates. This showed that the low proportion of high molecular weight products synthesised in wheat germ extracts could not be attributed to degradation/

degradation of mRNA prior to addition to assays. Further, it leads to the conclusion that although the wheat germ system is capable of recognising initiation and termination signals and translating heterologous mRNAs to give apparently the same products as are synthesised in the more homologous reticulocyte system, the proportions of polypeptides synthesised are not representative of the proportions of mRNAs present. Thus comparison of the two systems justifies the use of the wheat germ system in the experiments described in this thesis but shows that its overall usefulness is limited to studies where it is not essential that the amount of product should reflect the concentration of its mRNA. The products synthesised in the MDL appear to be more representative of the mRNA species but they may not be quantitively so and a similar limitation may apply to this system.

The results described in this thesis require comment with regard to the role of K^+ in protein synthesis. As was previously noted (4.3.) synthesis of proα collagen (molecular weight 155,000) in the wheat germ system is only detectable at a $\{K^+\}$ of 150mM (Benveniste et al., 1976; Harwood et al., 1975). The elongation rate in the ascites system is elevated at high $\{K^+\}$ (Mathews and Osborn, 1974) and hence the requirement for high $\{K^+\}$ for synthesis of high molecular weight proteins in wheat germ extracts may be related in part to an increase in the likelihood that translation of large mRNAs will be completed before they are degraded by nucleases.

The experiments described in this thesis showed that increasing the $\{K^+\}$ in wheat germ assays stimulated by PrV or cellular mRNA to more than 100mM almost completely inhibited synthesis of all polypeptides. A similar result has been reported for wheat germ assays programmed with tobacco/

tobacco mosaic virus RNA (Marcu and Dudock, 1974). Recent evidence suggests that this apparent discrepancy may be due to differences in the anions. The optimum $\{K^+\}$ for translation has been shown to be higher when CH_3COOK is used as the source of K^+ than when KCl is used and hence it has been concluded that in vitro translation systems are inhibited by Cl^- (Weber et al., 1977; Kemper & Stolarsky, 1977).

In the experiments described above Benveniste and coworkers used CH_3COOK as a source of K^+. They reported that at 150mM K^+ protein synthesis was 70% of that at optimal $\{K^+\}$. Harwood et al., (1976) used high concentrations of KCl and examination of their data suggest that at 150mM K^+ the total protein synthesis was less than 10% of the maximum possible. It seems likely that in this case proα collagen was only detected because the mRNA used was enriched for its mRNA. It is probable also that the failure to increase synthesis of high molecular weight products in the experiments described herein and in those carried out by Marcu and Dudock (1974) was due to the use of KCl and a mRNA population which was not enriched for species coding for large polypeptides.

Further to this, when the optimum $\{K^+\}$ for translation of PrV and cellular mRNAs in the wheat germ system was examined using CH_3COOK, significant stimulation of protein synthesis was observed at higher $\{K^+\}$ than when KCl was used. In retrospect, it seems likely that supraoptimal K^+ with CH_3COOK as the anion would be useful in increasing the synthesis of large polypeptides in the wheat germ system.

Having established that viral and cellular mRNA have been isolated and can be translated in vitro, features of this translation in relation to the herpesvirus-induced suppression of host cell protein synthesis can be discussed.

Virus/

Virus-induced inhibition of cellular protein synthesis at the level of translation can be brought about in three ways:

(a) removal of host mRNA by degradation
or sequestration

(b) inhibition of initiation of host mRNA

(c) suppression of elongation of host mRNA.

The information available on herpesvirus-induced shut-off is limited and definitive support for any one mechanism is lacking. Ben-Porat et al., (1971) have some evidence that RNA and protein synthesis are required for the shut-off and have suggested that synthesis of a virus specific protein which is rapidly turned over is necessary for inhibition. Such a protein could act by any one of the above mechanisms. Some degradation of host mRNA does take place in herpesvirus-infected cells but cellular mRNA is still present late after infection (see 1.6.2.). Thus some direct effect on initiation or elongation is likely (see also 1.7.4.).

Hackett et al., (1978) have some evidence that changes in the elongation rate may occur in mengovirus-infected Erlich ascites tumour cells. They compared the translation of viral RNA and uninfected cell mRNA in fractionated cell free translation systems derived from virus-infected and uninfected cells and found that preferential translation of viral message occurred in the infected cell system. 70% of this partiality could be attributed to initiation factors and 30% was due to the pH5 enzyme fraction which contains elongation factors, tRNA and tRNA synthetases. Their evidence supports the hypothesis that the influence of the enzyme fraction could be the result of a slowdown in the elongation of host-specific polypeptides. It is generally agreed, however, (and the above study corroborates this) that most translational controls/

controls operate at initiation (Lodish, 1976).

At present there is little evidence to support the view that message specific initiation factors have a major role in translational control. It is likely that the initiation rate of individual mRNAs depends on interactions among a set of initiation factors which are common to all eucaryotic cells. Changes in this population alter the initiation rates (see 1.7.3.). Thus a virus might control protein synthesis by altering the availability of initiation factors or changing their interactions so that the resulting environment favoured viral mRNA translation.

A specific mechanism for such a change in the "initiation state" of a cell has been proposed by Carrasco (1977). His hypothesis concerns the shut-off of host cell protein synthesis in virus-infected cells which is attributed to a virus-induced increase in the intracellular monovalent cation concentration. The model is based on the fact that optimal ionic conditions for cellular and EMC virus RNA directed protein synthesis in vitro are different (Carrasco and Smith, 1976). It suggests that viral proteins alter the membrane permeability so that Na^+ leaks in and K^+ leaks out. This change in ionic conditions specifically inhibits cellular mRNA translation.

The idea that ion concentrations can play a part in cellular metabolism is not novel and central roles for Mg^{++} (Rubin, 1977) and Ca^{++} (Durham, 1977) have been proposed. However, such a role for monovalent cations has not been previously explored.

The model is supported by observations made in a number of systems which show that real differences in $\{K^+\}$ optima for translation do exist. Furthermore, where differences in optima are not detectable, some mRNAs show less stringent dependence on optimal conditions than others and are more/

more readily translated in suboptimal conditions. Changes in membrane permeability are seen in EMC, mengo and Semliki Forest virus infected cells concomitant with the onset of viral protein synthesis (Carrasco, 1978) and virus induced changes in cell permeability have been documented by others (Durham, 1978; and see Carrasco, 1978). Moreover, alterations in the internal $\{Na^+\}/\{K^+\}$ ratio by inhibition of the sodium pump have been shown to inhibit protein synthesis in HeLa cells (Ledbetter and Lubin, 1977).

The critical point in this theory, however, is that an increase in the internal $\{Na^+\}/\{K^+\}$ ratio takes place concomitantly with inhibition of host protein synthesis. Carrasco and Smith (1976) have shown that uptake of $^{86}Rb^+$ (which is an authentic analogue of K^+) decreases in EMC virus infected L cells at the time when viral protein synthesis becomes detectable. This change is attributed to the synthesis of a viral structural protein. Such a scheme accounts for the fact that inhibition of host protein synthesis takes place in picornavirus-infected cells without viral protein synthesis if high multiplicities of infection are used (Baltimore, 1969). However, other evidence suggests that if such changes are resppnsible for the virus-induced shut-off, the mechanism is not generally applicable. Francoeur and Stanners (1978) could not detect any change in the uptake of $^{86}Rb^+$ in vesicular stomatitis virus infected cells until some time after inhibition of cellular protein synthesis had commenced (Stanners et al., 1977). Moreover, Egberts et al., (1977) have shown that in mengovirus infected cells, while a decline in the internal $\{K^+\}$ and a rise in the $\{Na^+\}/\{K^+\}$ ratio begins when viral protein is maximal, during the early shut-off of host protein synthesis, the intracellular $\{Na^+\}/\{K^+\}$ ratio falls.

Results described in this thesis suggest that the model is not applicable/

applicable to herpesviruses. Experiments have shown that the basic premise - that viral mRNA is translated at a higher monovalent cation concentration than cellular mRNA - is not true for late PrV mRNA and HeLa cell mRNA. This conclusion was reached from studies on translation of exogenous RNAs in the wheat germ and Krebs II ascites cell free translation systems. In the wheat germ system the optimum $\{K^+\}$ for the maximum stimulatory activity of Pr viral and cellular mRNAs were the same but maximum activity with reticulocyte mRNA required a slightly higher $\{K^+\}$. The stimulatory activity of EMC virus RNA in wheat germ extracts showed less stringent dependence on $\{K^+\}$ further verifying that differences in optima are detectable in this system. An examination of the stimulatory activity of these RNAs in Krebs II ascites extracts also supported the conclusion. EMC virus RNA showed maximum mRNA activity at $\{K^+\}$ similar to those described by others while reticulocyte mRNA had a significantly lower optimum $\{K^+\}$ for translation. Although PrV mRNA did not translate well in this system, the only mRNA activity detectable was found at similar $\{K^+\}$ to that found to be optimum for reticulocyte mRNA.

These results do not, however, rule out the possibility that although the optimum $\{K^+\}$ for maximum stimulatory activity of viral and cellular mRNAs are similar, the translation of some viral mRNAs might be more resistant to supra-optimal $\{K^+\}$. Examination of the polypeptides synthesised in the wheat germ system in response to viral mRNA at elevated $\{K^+\}$ showed that the synthesis of some polypeptides was resistant to the high $\{K^+\}$. However, when these polypeptides were classified by comigration and HART they were found to contain both viral and cellular coded species. Thus there was no correlation between the genome coding for the protein (viral or cellular) and the resistance to elevated $\{K^+\}$. It is unlikely, therefore, that an increase in the intracellular monovalent cation concentration has a role

in/

in the switching off of cellular protein synthesis in HeLa cells late after infection with PrV.

The possibility that monovalent cations have a role in the early switch from cellular to viral protein synthesis was not examined. The population of mRNA molecules on polysomes changes throughout infection and a scheme whereby the initial switch from viral to cellular protein synthesis is controlled by ion concentrations but later becomes independent can be envisaged. However, if Carrasco's hypothesis does account for the early changes after PrV infection, some modification is necessary: synthesis of structural polypeptides which have a role in altering membrane permeability in this model is a late event in replication in PrV-infected cells and the shut off of host cell protein synthesis is achieved early in infection even when low multiplicities of infection are used.

At this juncture it seems pertinent to consider the applicability of the model to picornaviruses in general. Recent data (Rose et al., 1978; Helentjaris and Erhenfeld, 1978), suggests that changes in the initiation factor population in poliovirus infected cells may be responsible for the shut off of host cell protein synthesis. This information is by no means definitive but implies that the mechanism of shut off is much more complex than Carrasco's model predicts.

One further point requires comment. The optimum $\{K^+\}$ for translation of EMC virus RNA was determined in all cases by addition of KCl to in vitro translation systems. In view of the fact that Cl^- seems to inhibit protein synthesis, it may be necessary to interpret these results to mean that the stimulatory activity of EMC virus RNA is less inhibited by Cl^- than that of cellular mRNAs.

A more general model for virus-induced inhibition of host protein synthesis/

synthesis has been proposed by Nuss, Oppermann and Koch (1975). According to this model the preferential synthesis of viral proteins is brought about by a non-specific lowering of the peptide chain initiation rate with the result that only viral mRNAs, which have a higher rate of initiation than most cellular mRNAs, are translated. The hypothesis is based on the observation that protein synthesis in HeLa cell infected with poliovirus, vesicular stomatitis virus and vaccinnia virus is less susceptible to hypertonic initiation block (see 6.1.) than protein synthesis in mock-infected cells. Furthermore, when cells are grown in hypertonic media the polypeptides synthesised are predominantly viral (Nuss et al.,1975; Oppermann and Koch, 1976a).

This model is an application of the Lodish model for translational control which predicts that if the overall initiation rate in a system is lowered then only those mRNAs with high initiation rate constants will be translated. The Lodish model is based on a static initiation rate constant in contrast to the more recent proposal (Revel and Groner, 1978) that the initiation rate constant for a mRNA is determined by the interactions between initiation factors and is altered by changes in the initiation factor availability (see 1.7.2.3.). Despite the possibility that it is an oversimplification, the Lodish model is useful in examining a complex situation about which very few details are known. Likewise the proposed mechanism for viral control of translation provides a framework within which to examine the differences in mRNA translatability.

The experiments described in this thesis showed that both early and late after infection of HeLa cells with PrV total protein synthesis was no less susceptible to media containing elevated concentrations of NaCl than it is in mock-infected cells. At 5h post-infection the total amount of protein synthesis is decreased and therefore it is possible that the initiation/

initiation rate at this time is lowered to such an extent that viral mRNA is already initiating under limiting conditions and cannot maintain initiation when a further block is applied. However, at 2h post-infection conditions are not limiting since total protein synthesis is unaltered and therefore such an explanation cannot account for the difference between the observations made with PrV and those described by Koch's group for other viruses. It must be concluded then that PrV mRNA is not more resistant to inhibition of initiation than cellular mRNA. Hence it is unlikely that this mechanism for virus-induced suppression of host cell protein synthesis is applicable to PrV.

This conclusion cannot, however, be extended to the herpesvirus family in general. Gupta and Rapp (1978) have reported that protein synthesis in human embryonic lung cells infected with cytomegalovirus is resistant to hypertonic initiation block and have identified viral structural proteins in the resistant polypeptides. Although PrV and cytomegalovirus belong to the same family, they exhibit quite different effects on cellular metabolism under normal growth conditions. After PrV infection host protein synthesis is inhibited and there is an overall decrease in total protein synthesis. In cytomegalovirus-infected cells, however, the total protein synthesis increases after infection and the virus polypeptides form only a minor portion of the total population of polypeptides synthesised (Gupta and Rapp, 1978). Hence there may be no conflict between these two results.

A further part of this study was designed to investigate the reason for the increased number of rapidly sedimenting polysomes in herpesvirus-infected cells, Increased loading of polysomes may be brought about in three ways:

(a) an increase in the size of mRNA species

(b)/

(b) a lower rate of elongation in infected cells

(c) a higher rate of initiation in infected cells.

If the average length of the translated region of infected cell mRNA is longer than that of cellular mRNAs, then, provided the initiation and elongation rates are the same for both species, the longer mRNA will load more ribosomes and thus contain more rapidly sedimenting polysomes. Such correlations between the size of polypeptide and polysomes are known (see 7.1.). Evidence discussed here, however, leads to the conclusion that this is not the reason for the increase in polysome size found in PrV-infected cells.

Harris and Wildy (1975) have shown that the size of total poly-(A)-containing RNA isolated from HSV-1 infected cells does not differ from that isolated from mock-infected cells. This was extended by Vass (1975) who showed that there is no detectable difference in the size of poly-(A) containing RNA found on heavy and light polysomes in PrV-infected and mock-infected HeLa cells. These results suggest that there is no correlation between polysome size and mRNA size but the possibility remains that the length of the translated region is independent of the size of the mRNA molecule. That this is not so is suggested by experiments which show that when PrV-infected and mock-infected HeLa cells are grown in low concentrations of cycloheximide so that elongation is the rate limiting step in protein synthesis, small mRNAs are found on light polysomes and larger mRNAs on heavy polysomes (Vass, 1975).

Furthermore, there is no evidence that the polypeptides synthesised in HSV-1 infected cells are larger than those found in uninfected cells or are processed from larger precursors (Honess and Roizman, 1973) as is the case in picornavirus-infected cells where a similar increase in polysome size is observed.

The/

The experiments described in this thesis extend these results. Fractionation of polysomes into three size classes and the run-off of nascent polypeptide chains in an *in vitro* translation system showed that the polypeptides synthesised by all three classes were similar (7.3.). The proportion of large polypeptides synthesised on heavy polysomes was higher than that on light polysomes but some correlation between the size of the translated region and the polysome size is expected and this change in proportion was similar for both virus-infected and mock-infected cell products.

Although the maximum size of total infected cell polysomal products was slightly greater than that of the largest cellular products detected (7.2.2.), this is not thought to be significant. There was no difference in the maximum size of mock-infected and infected cell polypeptides labelled *in vivo* and it is unlikely that the high molecular weight polysome products found *in vitro* are unprocessed precursors because they comigrated with *in vivo* polypeptides. Hence, the large polysomes could not be correlated with synthesis of large polypeptides.

A decrease in the elongation rate would lead to an increased density of ribosomes on mRNA and therefore a shift in the sedimentation profile. The importance of such a mechanism is difficult to assess because the effect on polysome size will depend on the degree of decrease in the elongation rate. If the elongation rate is lowered to such an extent that elongation becomes rate limiting for translation, then the length of the translated region of the mRNA will be proportional to the polysome size and this clearly is not the case. However, the possibility that some decrease in the elongation rate does occur cannot be excluded.

An increase in the average rate of initiation of polypeptide chains coupled with no change in the elongation rate would lead to a higher density/

density of ribosomes on mRNA. The observation that in PrV infected cells at 2½h post-infection heavy polysomes contained a higher proportion of low molecular weight nascent polypeptides suggests that the increased loading of ribosomes on mRNA may be related to an increased rate of initiation. Evidence presented here (see 7.3.) that synthesis of two polypeptides was restricted to particular size classes of polysomes verifies that differences in initiation rates do occur. Although no conclusions on possible changes in initiation rates can be drawn from this data, the results do not disagree with such a model.

Final conclusions on the mechanisms involved in switching off host cell protein synthesis in herpesvirus infected cells and in the increased loading of ribosomes on mRNA are not possible from the experiments carried out in the course of this study. The results merely serve to eliminate some possibilities. The hypothesis that an elevated internal monovalent(Na^+) cation concentration allows preferential synthesis of viral proteins late after infection of HeLa cells with PrV is unsubstantiated. The possibility remains that such a change controls early events in viral replication, but in view of the still highly hypothetical nature of the model this idea must be regarded with caution. The proposal of Ben-Porat et al., (1971) that the switch-off is due to synthesis of a viral protein remains viable, but, beyond the conclusion that such a factor does not operate by a non-specific lowering of the initiation rate in infected cells, no conclusions as to its possible mode of action can be made. The increased loading of polysomes may be caused by changes in initiation or elongation.

It seems likely that viral gene products will be involved in changing initiation factor interactions and the "initiation state". The coordinate control and sequential ordering of polypeptide synthesis in herpesvirus-infected/

infected cells suggests that the regulatory mechanisms will be complex. Immediate-early proteins may be responsible for an initiation state unfavourable for cellular mRNA translation; the early proteins, which decrease translation of immediate-early proteins, are likely to alter this initiation state, and the late proteins which inhibit synthesis of early proteins again are likely to change initiation conditions from those established by the early proteins. Thus controls on host mRNA translation may change throughout the course of infection. Overall changes in the energy status of the cell and a decrease in the efficiency of the protein synthetic apparatus related to inhibition of host cell macromolecular synthesis may also contribute to alterations in protein synthesis in herpesvirus-infected cells.

This project represents a very preliminary study of these control mechanisms. However, the experiments described suggest that it is likely that future work based on the approach adopted here, (namely the in vitro translation of isolated mRNA) will yield much interesting information on the nature of the intricate controls operative in herpesvirus-infected cells.

Appendices. I. Materials

A1. Biological 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). The line has since been adopted to grow in the absence of tryptose phosphate (Cl3A).

The monolayer adapted human epithelium cell line derived from a cervical carcinoma (Gey et al., 1952) and known as HeLa cells was also used in this study.

3. Wheat germ.

Jordan's Natural Wheat Germ, Holme Mills, Biggleswade.

A2. Chemicals

1. Radiochemicals

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

{5,6-^{3}H}uridine	40 - 60 Ci/mmol
L-{4,5-^{3}H}leucine	50 - 60 Ci/mmol
L-{^{35}S}methionine	800 Ci/mmol
L-{methyl-^{3}H}methionine	5 - 15 Ci/mmol

2. Chemicals for Liquid Scintillation Spectrophotometry.

The chemicals were obtained as follows:-

2,5 diphenyloxazole (PPO)	Koch Light Laboratories Ltd., Colnbrook, Bucks.
Toluene, A.R. Grade	"
p-Bis(o-methylstyryl) benzene (Bis MSB)	Eastman Biochemicals, Kodak Co.
Triton X-100	Rohn & Haas (U.K.) Ltd., Croydon.
NCS Tissue Solubilizer	The Radiochemical Centre, Amersham, Bucks.

3. Materials for Polyacrylamide Gel Electrophoresis.

Acrylamide	BDH
NN' Methylenebisacrylamide	"
Ammonium persulphate	"
NN' Diallyltartardiamide (DATD)	Aldrich Chemical Co. Inc., Milwaukee, Wis.
NNN'N' tetramethylethylenediamine (TEMED)	Kock-Light Laboratories Ltd., Colnbrook, Bucks.

4./

4. Materials for in vitro Translations.

N'-2-ethanesulphonic Acid (HEPES)	Sigma Chemical Company.
Dithiothreitol (DTT)	"
Spermidine trihydrochloride	"
Spermine tetrahydrochloride	"
Glutathione Reduced Form	"
Phenylhydrazine hydrochloride	"
Haemin Bovine crystalline	"
Ethylene-glycol-bis (2-aminoethylether) -N,N'tetraacetic acid (EGTA)	"
Sodium pentobarbitone (Nembutal)	May & Baker Ltd., Dagenham.
Creatine kinase	Boehringer Mannheim, GmbH, Mannheim, West Germany.
Creatine phosphate	"
Micrococcal nuclease (8000 units/mg)	"
L-amino acids	Calbiochem Ltd., La Jolla, California.
Adenosine 5'-triphosphate (ATP)	P-L Biochemicals Inc., Milwaukee, Wis.
Guanosine 5'-triphosphate (GTP)	"

5. Chromatographic Materials.

Sephadex G25 (coarse grade)	Pharmacia, Ltd., Uppsala, Sweden
Sephadex G25 (medium grade)	"
CN-Bromide activated Sepharose 4B	"
Oligo-(dT) Cellulose	Collaborative Research Inc., Waltham, Mass.

6./

6. Photographic Materials

Kodak X-Omat H X-ray film, Kodak DX-80 developer and Kodak FX-40 X-ray liquid fixer were supplied by Kodak Ltd., London.

7. Other Materials.

Heparin, freeze dried	Evans Medical Ltd., Speke, Liverpool
Absolute Alcohol, A.R. Grade	James Burroughs Ltd., London.
Whatman No. 1 2.5cm filter paper discs	Whatman Ltd., Maidstone, Kent.
β-mercaptoethanol	Koch-Light Laboratories Ltd., Colnbrook, Bucks.
3-Methyl-1-butanol (isoamyl alcohol)	"
Trichloroacetic acid (TCA)	"
Bovine serum albumin trypsin inhibitor, RNA polymerase molecular weight standards	Boehringer Mannhein, GmbH, Mannheim, West Germany.
Ovalbumin, chymotrypsinogen A molecular weight standards	Mann Research Laboratories Ltd., Inc., New York.

Other materials were purchased from BDH or Sigma and were "Analar" or its equivalent.

A3. Composition of Standard Solutions.

A3.1. Cell culture media.

1. Eagles Minimal Essential Medium (MEM) (Modified).

(Busby, House & Macdonald, 1964).

Amino Acids	mg/l
L-arginine	126.4
L-cystine	24.0
L-glutamine	292.0
L-histidine HCl	41.9
L-isoleucine	52.5
L-leucine	52.5
L-lysine HCl	73.1
L-methionine	14.9
L-phenylalanine	33.0
L-threonine	47.6
L-tryptophan	10.2
L-tyrosine	36.2
L-valine	46.8

Vitamins	mg/l
D-calcium pantothenate	2.0
Choline chloride	2.0
Folic acid	2.0
Inositol	4.0
Nicotinamide	2.0
Pyridoxal-HCl	2.0
Riboflavin	0.2
Thiamine HCl	2.0

Inorganic Salts and Other Components	mg/l
$CaCl_2.2H_2O$	393
KCl	400
$MgSO_4.7H_2O$	200
NaCl	6800
$NaH_2PO_4.2H_2O$	140
$NaHCO_3$	2200
Phenol Red	17
D-glucose	4500
Penicillin	10^5 units

Inorganic Salts and Other Components (Contd) mg/l

Streptomycin 10^5 µg/ml

MEM was prepared from a powder supplied by Flow Laboratories which was dissolved to give a 10 x concentrate of the medium. Bicarbonate, penicillin, streptomycin and phenol red were added separately.

2. EC10

90% Eagles MEM supplemented with 10% calf serum (v/v).

3. Earle's Balanced Salt Solution (BSS).

0.13M NaCl, 6mM KCl, 1mM $MgSO_4$, 1mM NaH_2PO_4, 2mM CaCl,0.015% (w/v) phenol red, chloroform to a final concentration of 0.1%. Sterilised by autoclaving at 15 lbs/in^2 for 20 min.

4. Sodium bicarbonate.

5.6% w/v $NaHCO_3$, 0.0015% (w/v) phenol red. Sterilised by millipore filtration using a 0.22 micron membrane.

5. BSS + bicarbonate.

450ml BSS + 20ml sodium bicarbonate.

6. Phosphate Buffered Saline (PBS).

0.17M NaCl, 3.4mM KCl, 10.0mM Na_2HPO_4, 1.8mM KH_2PO_4 0.7mM $CaCl_2$, 0.5mM $MgCl_2$ pH 7.2.

7./

7. Formol-saline.

0.077M NaCl, 0.1M Na_2SO_4 3.6% (v/v) formaldehyde.

8. Giemsa Stain.

0.75% (w/v) Giemsa in glycerol - methanol 1:1 (v/v).

A3.2. Solutions for mRNA preparation.

Sucrose solutions were sterilised by autoclaving at 5lbs/in^2 for 50 min. All other solutions were autoclaved at 15 lbs/in^2 for 50 min.

1. Reticulocyte Standard Buffer (RSB).

0.01M NaCl, 0.003M $NgCl_2$, 0.01M Tris-HCl, pH 7.4.

Heparin was added just prior to use.

2. pH 9.0 Extraction Buffer.

0.1M NaCl, 0.001M EDTA, 2.0% (w/v) SDS, 0.1M Tris-HCl, pH 9.0.

3. NETS.

0.1M NaCl, 0.01M EDTA, 0.2% (w/v) SDS, 0.01M Tris-HCl, pH 7.4.

4. Lysing NETS.

As above but with 1.0% (w/v) SDS.

5. Elution Buffer I.

0.01M K_2HPO_4, 0.01M EDTA, 0.2% (w/v) SDS in 90% (v/v) formamide, pH 7.5.

The formamide was deionised by stirring with Bio-Rad Mixed Bed/

Bed Ion Exchange Resin AG 501-X8, 20-50 mesh.

6. Concentrated Salt Buffer.

0.7M NaCl, 0.05M Tris HCl, 0.01M EDTA in 25% (v/v) formamide, pH 7.5.

7. Elution Buffer II.

0.01M Tris HCl, 0.2% (w/v) lauryl sarcosine, in 90% (v/v) formamide, pH 7.4.

8. Oligo-(dT) Cellulose Loading Buffer.

0.5M LiCl, 0.001M EDTA, 0.1% (w/v) SDS, 0.01M Tris-HCl, pH 7.5.

9. Oligo-(dT) Cellulose Intermediate Buffer.

0.1M LiCl, 0.001M EDTA, 0.1% (w/v) SDS, 0.01M Tris-HCl, pH 7.5.

10. Oligo-(dT) Cellulose Elution Buffer.

0.001M EDTA, 0.01M Tris-HCl, pH 7.5.

A3.3. Hybridisation solutions.

1. Standard Saline Citrate (SSC).

0.15M NaCl, 0.015M Na citrate.

2. Hybridisation Buffer.

0.75M NaCl, 0.005M EDTA, 0.25% (w/v) SDS, 0.01M Tris-HCl, pH 7.5.

3. Formamide-NaCl-PIPES (FNP).

80% formamide, 0.4M NaCl, 10mM PIPES, pH 6.4 at room temperature.

A3.4. Solutions for Wheat Germ Preparation.

1. Extract Buffer.

0.1M KCl, 0.001M $Mg(CH_3COO)_2$, 0.002M $CaCl_2$, 0.02M HEPES 0.006M β-mercaptoethanol. Solution made in deionised water, to pH 7.6 with 5N KOH and autoclaved prior to addition of β-mercaptoethanol.

2. Column Buffer.

0.12M KCl, 0.005M $Mg(CH_3COO)_2$ 0.02M HEPES, 0.006M β-mercaptoethanol, pH 7.6. As above.

3. Energy Mix.

1mM ATP, 20uM GTP, 5.5mM creatine phosphate, pH 7.6 with KOH.

4. 0.12M Dithiothreitol (DTT).

Dissolved in sterile deionised water and bubbled with N_2 for 10 min. Stored in small aliquots at $-70^{\circ}C$.

5. Creatine Kinase.

1mg/ml in 50% (v/v) sterile glycerol.

6. Salt mix.

1M HEPES, pH 7.6, sterile.

7. Amino acids.

Approximately 400μM glycine, alanine, arginine, aspartate, histidine, isoleucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, cysteine, glutamine, glutamate, asparagine, (methionine or leucine). Heated gently in sterile deionised water to dissolve and filtered through a 0.45 micron HA millipore filter. Stored in small aliquots at -20°C.

8. Spermidine.

10mM Spermidine trihydrochloride pH 7.6 with 1M KOH in sterile deionised water. Stored at -20°C.

9. Spermine.

0.86mM Spermine tetrahydrochloride pH 7.6 with 1M KOH in sterile deionised water. Stored at -20°C.

10. Salt solutions.

Solutions of KCl, KCH_3COO, $Mg(CH_3COO)_2$ were prepared to vary the cation concentrations.

11. ATP mix (100μl).

Standard, to give conditions defined in 3.3.4.

25μl energy mix

5μl creatine kinase

10μl DTT

10μl spermidine

10μl 0.9M KCl

10μl Salt mix

30/

11. ATP mix (100µl) (Contd):

30µl H_2O, isotope or other

12. Assay mix (50µl).

15µl wheat germ extract

10µl ATP mix

25µl H_2O, isotope or other

A3.5. Solutions for Reticulocyte Lysate Translation.

1. Glutathione.

0.5M glutathione in H_2O, pH 6.0 with NaOH.

2. Phenylhydrazine.

2.5% (v/w) phenylhydrazine hydrochloride in H_2O containing 0.5% (v/v) 0.5M glutathione (2.5mM), pH 7.0 with NaOH. Stored in 10ml aliquots in light free tubes at -20°C.

3. Haemin.

10mM haemin, 0.5M KCl, 0.2M Tris-HCl pH 8.2 in 95% (v/v) ethylene glycol.

4. Creatine Kinase.

5mg/ml in 50% (v/v) glycerol, sterile.

5. K^+/Mg^{++} Solution.

2M KCl, 0.01M $MgCl_2$, sterile.

6./

6. Creatine Phosphate.

0.2M creatine phosphate in sterile H_2O.

7. Amino acids.

Approximately 1.4mM glycine, alanine, arginine, aspartate, histidine, isoleucine, lysine, phënylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, cysteine, glutamine, glutamate, asparagine (methionine or leucine). Heated gently to dissolve in sterile deionised water and filtered through a 0.45 micron HA millipore filter. Stored in small aliquots at -20°C.

8. Master Mix.

K^+/Mg^{++} solution, creatine, phosphate, amino acids in ratio 1:1:1 (v/v/v).

9. $CaCl_2$.

0.1M $CaCl_2$, sterile.

10. Micrococcal nuclease.

1mg/ml in sterile water stored frozen at -20°C.

11. EGTA.

0.1M EGTA to pH 7.4 with KOH (tetrapotassium salt). Stored at 4°C. Sterile.

12. Assay Mix.

50µl MDL

7.5µl H_2O, RNA or isotope

A3.6. Solutions for Krebs II Ascites Cell Free Translation.

1. Tris Buffered Saline (TBS).

0.146M NaCl, 0.035M, Tris-HCl, pH 7.6.

2. Reticulocyte Standard Buffer (RSB).

0.01M KCl, 0.0015M $Mg(CH_3COO)_2$, 0.01M Tris-HCl, pH 7.5, sterile.

3. Medium 125K.

0.125M KCl, 0.005M $Mg(CH_3COO)_2$, 0.005M β-mercaptoethanol, 0.025M Tris-HCl, pH 7.5. β-mercaptoethanol added after autoclaving at 15lbs/in^2 for 50 mins.

4. Medium 50K.

As above but 0.05M KCl.

5. Dithiothreitol (DTT).

0.5M dissolved in sterile deionised water and bubbled with N_2 for 10 min. Stored in small aliquots at -70°C.

6. Creatine Kinase.

20mg/ml in 50% (v/v) glycerol, sterile. Stored at -70°C.

7. Energy Mix.

50mM ATP, 5mM GTP, 250mM creatine phosphate pH 7.5 with KOH. Stored in small aliquots at -20°C.

8./

8. Amino Acids.

As for Wheat Germ (9.3.4.7.)

9. Spermidine.

As for Wheat Germ (9.3.4.8.)

10. tRNA.

Wheat germ tRNA 3.75 mg/ml in sterile deionised water. Stored at -20°C.

11. Tris-buffer solution.

0.25M Tris-HCl, pH 7.5.

12. Potassium solutions.

The total K^+ concentration contributed by the S30 and energy mix is 32mM. K^+ was added to give the final concentration indicated in the Results by addition of concentrated KCl to the assay or ATP mixes.

13. ATP Mix (100µl).

20µl tris buffer solution

10µl amino acids

10µl energy mix

10µl tRNA

10µl spermidine

5µl DTT

5µl creatine kinase

30µl KCl, H_2O or radioactive amino acids.

14. Assay Mix (50µl).

30µl S30

10µl ATP mix

10µl RNA, H_2O, KCl or radioactive amino acid.

A3.7. Solutions for Electrophoresis of Proteins.

1. Electrode Buffer.

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

2. Main Gel Solution.

0.1% (w/v) SDS, 0.1% (v/v) TEMED, 0.14% (w/v) ammonium persulphate, 0.375M Tris-HCl, pH 8.8 containing acrylamide and bis-acrylamide in the ratio 1:0.033 or acrylamide and DATD in the ratio 1:0.075.

3. Stacking Gel Solution.

0.1% (w/v) SDS, 0.1% (v/v) TEMED, 0.09% (w/v) ammonium persulphate, 1.0% (w/v) agarose, 0.125M Tris-HCl, pH 7.0, containing 3% (w/v) acrylamide, 0.1% (w/v) bis-acrylamide or 3% (w/v) acrylamide, 0.15% (w/v) DATD.

4. Coomassie Blue Stain.

0.25% (w/v) Coomassie Brilliant Blue in 45% (v/v) methanol, 5% (v/v) glacial acetic acid.

5./

5. Destaining solution.

45% (v/v) methanol, 5% (v/v) glacial acetic acid.

A3.8. Solutions for Immunological Techniques.

1. Phosphate Buffered Saline A (PBS A).

0.17M, NaCl, 3.4mM KCl, 10.0mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.2.

2. Polyethylene Glycol (PEG).

12% (w/v) PEG, in PBS A. Dissolve 4g PEG in 4ml water by heating gently, then adjust the volume to 33ml with PBS A.

3. Phosphate Buffered Saline with Detergent (PBS-det).

1% (w/v) SDS, 1% (w/v) sodium deoxycholate, 0.5% (v/v) Triton X-100.

4. Tris-Potassium-Magnesium with detergent (TKM-det).

0.1M KCl, 0.005M $MgCl_2$ 0.1M Tris-HCl, pH 8.0, 1% (w/v) Sodium deoxycholate, 1% (v/v) Triton X-100, 0.5% (w/v) SDS.

A3.9. Scintillation Solution.

1. Toluene-PPO.

0.5% (w/v) PPO in toluene.

2./

2. Triton-toluene.

0.5% (w/v) PPO, 0.05% (w/v) Bis MSB, 35% (v/v) Triton X-100, in toluene.

II. Methods

A4. Cell Culture Techniques.

A4.1. Propagation of cells.

HeLa and BHK/21 (C13A) cells were routinely cultured as monolayers in 8Ooz Winchester bottles (burlers) according to the method of House and Wildy (1965). The burlers were gassed to give an atmosphere of 5% CO_2/95% 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 EClO (A.3.1.2.), counted and dispensed into sterile burlers containing 180ml of EClO, in aliquots of 18 - 22 x 10^6 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.

A4.2. 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 after 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.

PPLO 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_2 in N_2 at 37°C.

Infected/

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

A4.3. Propagation of Virus.

Monolayer cultures of BHK/21 (C13A) cells (approximately 10^8 cells per burler) were infected at a multiplicity of 1 plaque forming unit (pfu) per 300 cells in 20ml medium. Virus was allowed to adsorb for 1h, then the innoculum was removed and replaced with 100ml of medium. The cultures were rolled at 37°C for 36h, then harvested aseptically by shaking the bottle to dislodge cells into the medium, transferring to centrifuge bottles and spinning at 600g for 10 min. This pellets the cells; the supernatant was then spun at 15,000g for 2h to pellet the virus. The pelleted supernatant virus was resuspended in EC10 (1ml for every burler used), and gently sonicated to make the suspension uniform. Aliquots were stored at -70°C.

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 10^9 - 10^{11} pfu/ml.

A4.4. Plaque assay for PrV.

Confluent monolayers of BHK/21 cells in 50mm Petri dishes, which had been seeded at 4 x 10^6 cells/dish 18 hours previously, were infected with serial dilutions of PrV in 0.2ml EC10. After 1h adsorption/

adsorption at 37°C, the excess was poured off, 4ml medium was added and incubation continued. After a further h, 50µg/ml heparin was added to the medium to prevent vertical transmission of the virus. 28h after infection, incubation was arrested, the cell sheet was washed with PBS and fixed with 3ml formol-saline per dish (30 min at room temperature). Plates were then stained with 0.5ml Giemsa stain/ dish for 1 h at room temperature, excess stain was washed off very gently with H_2O and plaques were counted under a low power microscope.

A5. Preparation of HeLa Cell Polysomes.

HeLa cells were seeded at 20 x 10^6 per burler and incubated at 37°C in 5% CO_2 for 2-3 days. When the cell density had reached 80-100 x 10^6 the cells were infected at 20pfu/cell in 20ml of the medium in which they had been growing. This slightly acid medium was used because adsorption is more efficient at lower pH's. After 1h, the excess was decanted and 50ml of fresh medium prewarmed to 37°C was added. Radioactive uridine was added at the appropriate time after infection if the RNA was to be labelled. If poly-(A) containing RNA was to be prepared from the polysomes, a minimum of 16 burlers was used.

At the appropriate time after infection the cells were harvested mechanically. The growth medium was poured off and the bottle rotated in an ice-bath. 10ml ice-cold BSS + bicarbonate (A3.1.3.) was pipetted into the burler and the cells were removed from the glass by means of a rubber scraper, and pelleted by centrifugation at 1000rpm for 5 min at 4°C in a MSE Major Centrifuge. Pellets containing up to 300 x 10^6 cells were suspended in 2.5ml RSB (A3.2.1.) containing 0.1mg/ml heparin and the cells allowed to swell for 5 min before being disrupted/

disrupted by gentle homogenisation (4 strokes in a teflon/glass homogeniser at low speed). The nuclei and large cell debris were pelleted at 2,500 rpm for 10min. 2.5ml aliquots of cytoplasmic extract were immediately layered onto 34ml 15 - 30% (w/v) sucrose gradients in RSB containing 1mg/ml heparin. Gradients were centrifuged in a SW27 rotor at 27,000 rpm for 110 min at 4°C, then harvested by placing a needle down the side of the tube to the bottom and pumping through a Gilford 2000 recording spectrophotometer. The absorbance was monitored at 260nm and the gradient divided into polysomal and monosomal fractions. The polysomal fractions were pelleted by centrifugation in a SW27 rotor at 20,000 rpm for 17h or in a 60Ti rotor at 45,000 rpm for 3h at 4°C.

To examine the effect of hypertonic conditions on polysomes the growth medium was replaced with fresh medium (prepared from calf serum which had been dialysed overnight against distilled water) containing the appropriate NaCl concentration 15 min prior to harvesting. Polysome to monosome ratios were computed by cutting out and weighing the A_{260} scan of the gradient.

A6. Extraction of RNA.

RNA was extracted from polysomes essentially as described by Mendecki et al., (1972). Polysome pellets were rinsed with sterile 2M sucrose, drained and resuspended in pH 9.0 RNA extraction buffer (A3.2.2.) by repeated pipetting and vigorous mixing at a concentration of approximately 10 A_{260} units/ml. The polysome suspension was diluted with an equal volume of phenol:chloroform:isomyl alcohol (50:50:1) (v/v/v) mixed vigorously for 10 min and centrifuged at 3000 rpm in a MSE Major Centrifuge for 10 min at 8°C. The aqueous layer

was/

was removed and the organic layer re-extracted with an equal volume of extraction buffer. The two aqueous phases were pooled, and re-extracted with the phenol-chloroform solution. The final aqueous phase was diluted with 2 volumes of ethanol and the RNA allowed to precipitate at -20°C for at least 2h.

RNA was extracted from rabbit reticulocytes essentially by the method of Perry et al., (1972). Washed red blood cells were lysed by addition of an equal volume of lysing NETS (A3.2.4.) and immediately diluted with one half volume of phenol:chloroform:isoamyl alcohol (100:100:1) (v/v/v) saturated with NETS (A3.2.3.). The mixture was shaken at room temperature for 30 min, then centrifuged for 10 min at 12,000g. The aqueous phase was removed and the RNA precipitated by addition of 2 volumes of ethanol and standing at -20°C for at least 4h.

A7. Isolation of Poly-(A) Containing RNA.

A7.1. Poly-(U) Sepharose Affinity Chromatography.

Poly-(U) Sepharose was prepared by a modification of the method of Wagner et al., (1971) for preparation of Poly-(rI:rC) Sepharose (L. Fitzmaurice, personal communication). 15g of activated Sepharose 4B was washed with 600ml of 0.1M KPO_4, pH 8.0, which had been stirred overnight with 0.01% diethylpyrocarbonate (DEP) to destroy RNAse and then autoclaved to remove all traces of DEP, on a baked Buchner funnel. The Sepharose was re-suspended in 15ml of the same buffer containing 9mg poly-(U) and stirred gently for 17h at 4°C. The poly-(U) Sepharose was then washed with elution buffer I (A3.2.5.) and concentrated salt buffer (A3.2.6.) and stored in 50% glycerol, 50% NETS.

Poly/

Poly-(U) Sepharose columns with a column volume of approximately 1ml were poured in mini columns made from pasteur pipettes plugged with glass wool and baked at 200°C. The columns were washed with 10 column volumes of elution buffer II (A3.2.7.) and then equilibrated with NETS. Polysomal and reticulocyte RNAs were pelleted by centrifugation at 19,000g for 20 min at 0°C. The RNA was dissolved in 1-2ml NETS and the solution was cycled three times through the poly-(U) Sepharose column. The bound fraction was eluted from the column with 3ml of elution buffer II and the RNA precipitated with two volumes of ethanol at -20°C for 17h. The RNA was pelleted as above, dissolved in 0.4M NaCl and re-precipitated twice more. It was then dissolved in 50-200µl sterile deionised water and stored at -20°C until required for translation.

A7.2. Oligo-(dT) Cellulose Chromatography.

Oligo-(dT) cellulose was suspended in deionised water and poured into minicolumns (see A7.1.). Approximately 0.1g dry weight oligo-(dT) cellulose was used for affinity chromatography of up to 120 A_{260} units of polysomal RNA. The columns were washed with 10ml 2% (w/v) SDS and then equilibrated with loading buffer (A3.2.8.). The RNA solution was warmed for about 10 min in a water bath at 50°C and cycled three times through the column. Columns were washed with intermediate buffer (A3.2.9.) until the A_{260} was less than 0.01 and then the bound RNA was eluted with 1.5ml elution buffer (A3.2.10.). The bound fraction was made 0.4M NaCl by addition of a tenth volume of 4M NaCl and precipitated and dissolved for translation as previously described.

A8. DNA-RNA Hybridisations.

A8.1. Hybridisation on Nitrocellulose Filters.

PrV DNA was prepared as described by Stevely (1977). CsCl was removed by dialysis against distilled water. The DNA solution was adjusted to 0.1 x SSC (A3.3.1.) and sheared by passing 12 times through a 25g needle. The DNA was then denatured by addition of an equal volume of 1M NaOH which was neutralised 15 - 20 min later by addition of 3 volumes of a solution containing 3M NaCl, 1M Tris-HCl, pH 8.0, 1M HCl in a ratio of 2:1:1 (Birnstiel et al., 1968). The solution was then passed by gravity through 13mm HAWP Millipore filters presoaked for 2h in 2 x SSC and held in Swinnex-13 filtration units which were fitted with 10ml perspex tubes. Filters were then washed with 2ml 2 x SSC, dried at 80°C for 2h, numbered with a soft lead pencil and stored dry at -20°C (Gillespie and Spiegelman, 1968).

When required, the filters were soaked for 2 h in 2 x SSC. RNA dissolved in 0.1 x SSC was denatured by heating at 115°C for 5 min. Aliquots of RNA were added to filters placed in sterile vials and enough hybridisation buffer (A3.3.2.) was added to allow the disc to float freely (approximately 400µl). (Jacquemont and Roizman, 1975). The vials were incubated at 66°C for 20h. The filters were removed from the vials and washed by a batch method (Birnstiel et al., 1968); filters were placed in a 2l beaker with 1l 2 x SSC at room temperature and kept under continuous agitation for 30 min. This washing procedure was repeated twice. The filters were then incubated in 5ml 2 x SSC containing pancreatic RNAase (50µg/ml) for 1h at room temperature, rinsed twice with 2 x SSC and dried under a heat lamp. Radioactivity incorporated was determined by liquid scintillation counting in toluene-PPO.

A8.2/

A8.2. Hybridisation in Solution.

PrV poly-(A) containing RNA was hybridised to excess DNA in solution as described by Paterson et al., (1977). Appropriate amounts of DNA and mRNA were mixed in a very small volume of water in a 1.5ml Eppendorf centrifuge tube. Samples were heated at 100°C for 60s, quick chilled in a dry ice methanol bath and spun for 10s in a microfuge. 25µl of FNP (A3.3.3.) was added per sample and hybridisation mixtures were incubated at 66°C for 2.5h. Reaction was terminated by addition of 200µl cold distilled water. 20µg E. coli tRNA was added as carrier. Samples were divided in two. One portion was retained in hybrid form while the other was heated for 60s at 100°C and then quick chilled in dry ice methanol. Samples were then adjusted to 0.2M sodium acetate, pH 5.5 and the nucleic acids precipitated with 2.5 volumes of ethanol at -20°C. The pellets were collected by centrifugation at 12,000g for 10 min at 4°C, washed and recentrifuged twice with 0.75ml 70% (v/v) chilled ethanol. Residual ethanol was removed by lyophilisation just prior to translation. Pellets were suspended in deionised water for in vitro translation.

A9. Preparation of PrV Capsid and Cell Lysates.

PrV capsid was prepared labelled with {^{35}S} methionine as described by Stevely, (1975). {S^{35}} methionine labelled infected and mock-infected cell lysates were prepared as follows. Two burlers of exponentially growing HeLa cells were mock-infected or infected with PrV at 20pfu/cell. 4½h post-infection 200µCi {^{35}S} methionine for a further hour. At 5½h post-infection, the cells were harvested and lysed in 1ml RSB (A3.1.), then adjusted to 2% (w/v) SDS, 5% (v/v)

β/

β-mercaptoethanol and heated in a boiling water bath for 5 min.

A10. Wheat Germ Cell-free Translation of mRNA.

The method used was a combination of the methods described by Roberts and Paterson (1973) and Marcu and Dudock (1974).

A10.1. Preparation of the Wheat Germ Extract.

2g wheat germ (A1.3.) and 2g acid washed (2M HCl) baked sand were ground in a baked mortar and pestle (precooled to $4^{o}C$) for 1 min. 4ml extract buffer (A3.4.1.) was added and the mixture transferred to a centrifuge tube and spun at 16,000 rpm in a Sorvall SS34 rotor for 12 min. The supernatant was carefully removed avoiding the yellow fat layer and 1.5ml was applied to a 1.7 x 21cm Sephadex G25 (coarse grade) column pre-equilibrated with column buffer (A3.4.2.). The eluate was collected into sterile tubes and the optical density at 260nm was measured. Fractions with optical densities greater than 90 A_{260} units/ml were pooled and spun at 16,000rpm as above for 20 min. The supernatant was divided into 200 - 400µl aliquots, quick-frozen and stored in liquid N_2. Extracts were thawed immediately before use and repeated thawing and refreezing did not signficantly alter the translational activity if the extract was thawed for a short period and quickly refrozen in liquid N_2. The whole procedure was carried out at $4^{o}C$. Sephadex was autoclaved prior to use and all tubing was filled with 15% (v/v) H_2O_2 for 20 min and then rinsed with sterile deionised to inactivate RNAase before packing.

A10.2/

A10.2. Translation Assays.

Assays were started by adding wheat germ extract to the mixture of ATP mix (A3.4.11.) mRNA and isotope. The assay mixes (A3.4.12.) were incubated at 25°C for 90 min (unless otherwise stated in the Results). Assays were stopped by addition of 10µl 10% (w/v) SDS and 3µl β-mercaptoethanol and heating for 2 min in a boiling water bath, or by cooling on ice.

The TCA precipitable radioactivity incorporated was determined in one of two ways:

(i) Aliquots were spotted on 2.5cm Whatman No. 1 filter discs supported on a pin and placed, while still wet, gently into ice-cold 10% (w/v) 'TCA' containing the amino acid used for labelling at a concentration of 10^{-5}M. The filters were left at least 10min in this solution and then washed successively in 5% (w/v) TCA at room temperature, 5% (w/v) TCA at 90°C, ethanol-diethyl ether (1:1) and ether for 10min. Filters were air dried for 5 min, then placed under a heat lamp for 5 min prior to counting in toluene-PPO (Bollum, 1968). 3µl aliquots were counted from assays labelled with {^{35}S} methionine and 5µl aliquots from assays labelled with {^{3}H} leucine.

(ii) Assays were diluted 10fold with TKM-det (A3.8.4.). 40µl aliquots were removed; an equal quantity of 0.2M NaOH was added and the samples were incubated at 37°C for 15min. Samples were neutralised with acetic acid, approximately 20µg bovine serum albumin was added as carrier and protein was precipitated by adding 50µl 25% (w/v) TCA, then 1ml 10% (w/v) TCA. Samples were left for several hours at

4/

4°C, then precipitates were pelleted by centrifugation in an Eppendorf 3200 microfuge at 800g for 30 secs. The pellets were washed three times with 10% (w/v) TCA and dissolved in 40µl NCS tissue solubiliser. Radioactivity incorporated was determined by counting 20µl samples in 10ml triton-toluene scintillant.

A11. Reticulocyte Lysate Translation of mRNA.

A11.1. Reticulocytosis.

New Zealand White Rabbits were made anaemic by a modification of the procedure of Waxman and Rabinowitz (1966). The rabbits were injected subcutaneously on four successive days with approximately 0.4ml phenylhydrazine solution (A3.5.2.) per kg body weight. The rabbits were rested for two days and on the seventh day they were anaesthetised by injection of a solution of equal volumes of 1% (w/v) heparin and sodium pentabarbitone solution (Nembutal) into the ear vein (1.5 - 2.0ml per 2.5kg rabbit). Rabbits were bled out by heart puncture into heparinised syringes and the blood was transferred to sterile tubes. To ensure that the animal was dead the chest cavity was opened and drained of blood. This procedure yielded 80 - 150ml blood per 2.5kg rabbit.

A11.2. Lysis of Reticulocytes.

The red blood cells were pelleted by centrifugation at 2000rpm for 10 min at 4°C in a MSE Major centrifuge. The serum was removed and discarded. The cell pellet was resuspended in 3-4 volumes of BSS and centrifuged again at 2000rpm. The supernatant and buffy coat (white cells) were removed by suction. This was repeated twice more/

more to remove all white cells. If the reticulocytes were to be used for preparation of mRNA they were then lysed by addition of lysing NETS and RNA was extracted as described in A6. If a translation system was to be prepared they were lysed by addition of an equal volume of sterile deionised water, mixed vigorously and left on ice for 3-4 min. The lysate was then centrifuged at 16,000g for 10 min to pellet the cell debris. The ruby red supernatant was stored in 800µl aliquots at -70°C.

A11.3. Preparation of Messenger Dependent Lysate (MDL).

MDL was prepared by the batch method described by Pelham and Jackson (1976). Lysate was thawed and 800µl aliquots were rapidly made 50µg/ml in creatine kinase and 25uM in haemin by addition of 25µl haemin (A3.5.3.) and 10µl creatine kinase (A3.5.4.). 150µl master mix (A3.5.8.) was added to each tube; then the lysate was made 1mM in $CaCl_2$ and 10µg/ml in micrococcal nuclease by addition of 10µl $CaCl_2$ (A3.5.9.) and 10µl nuclease (A3.5.10.). After thorough mixing it was transferred from ice to a water bath and incubated at 20°C for 15 min. The digestion was stopped by addition of 20µl EGTA (A3.5.11.) to chelate Ca^{++}. The MDL was stored in 200µl aliquots at -70°C.

A11.4. Translation Assays.

Assays were started by adding 50µl MDL to mRNA and isotope to a total volume of 57.5µl. Assay mixes (A3.5.12.) were incubated at 30°C for 90 min, then cooled on ice and the TCA precipitable radioactivity was determined by the filter method.

A12/

A12. Krebs II Ascites Cell Free Translation of mRNA.

Cell free translation in Ascites S30 extracts was carried out essentially as described by Mathews and Korner (1970). Wheat germ tRNA was added (Aviv et al., 1971).

A12.1. Growth of Krebs II Ascites Cells.

Ascites cells were maintained by the intraperitoneal injection of 0.2ml ascitic fluid (approximately 25×10^6 cells) into mice at 7 day intervals.

A12.2. Harvesting of Ascites Cells.

Seven days after inoculation, mice were killed by cervical dislocation. The abdominal skin was swabbed with 70% (v/v) alcohol and drawn back. The peritoneum was cut open over a filter funnel covered by muslin and the cells were collected into a 250ml glass centrifuge bottle (on ice) containing a few mls of ice-cold TBS (A3.6.1.). The mouse peritoneum was rinsed with ice-cold TBS. Very bloody tumours were rejected. All subsequent operations were carried out at 0-4°C. The cells were washed about three times in TBS by repeatedly pelleting by centrifugation at 1500 rpm for 3 min in a MSE Major centrifuge. When most of the red blood cells had been removed, the volume of cells was measured by resuspending in sterile TBS and spinning at 2000 rpm for 10 min in a graduated centrifuge tube. All subsequent operations were carried out in sterile solutions with RNAase free apparatus. The cells were resuspended in 1.5 volumes of RSB (A3.6.2.) and allowed to swell for 15 min. The cells were then burst open by homogenisation in a tight teflon/glass homogeniser (20 strokes at maximum speed) and 0.11 volumes of ten times concentrated medium/

medium 125 K (A3.6.3.) was added. The extract was centrifuged for 10 min at 15,000 rpm (30,000g) in a Sorvall SS34 rotor. The supernatant (S30) was removed, and recentrifuged. The second S30 extract was used immediately to prepare translation extract.

A12.3. Preparation of Translation Extract.

15ml of S30 was applied to a 45 x 2.7cm Sephadex G25 (medium grade) column pre-equilibrated with medium 125K. 15ml concentrated eluate was collected and the concentration of KCl and $Mg(CH_3COO)_2$ adjusted to 125mM and 5mM respectively. Other reagents necessary for translation were added to give the following concentrations: 5mM DTT, 50mM ATP, 5mM GTP, 250mM creatine phosphate, 0.2mg/ml creatine kinase and 50mM each of the 20 essential amino acids (A3.6.8.). The mixture was incubated for 40 min at 37°C, cooled on ice for 15 min., then centrifuged at 2000 rpm for 15 min. The preincubated S30 was reapplied to a 45 x 2.5cm Sephadex G25 (medium grade) column equilibrated with medium 50K (A3.6.4.). The most concentrated fractions were collected and stored in 400µl aliquots in liquid N_2.

S30 was thawed immediately before use and repeated thawing and refreezing did not significantly reduce the translational activity if the extract was thawed for a short period and quickly refrozen in liquid N_2. RNAase free conditions were obtained as detailed for wheat germ preparations.

A12.4. Translation Assays.

Assays were started by adding S30 to the mixture of ATP mix (A3.6.13.) mRNA and isotope. Assay mixes (A3.6.14.) were incubated at/

at 37°C for 60 min. then cooled on ice and the TCA precipitable radioactivity was determined by the filter method detailed for wheat germ assays.

A13. Separation and Detection of Polypeptides in Polyacrylamide Gels.

A13.1. Polyacrylamide Gel Electrophoresis.

Electrophoresis was carried out using a discontinuous system as described by Dimmock and Watson (1969) and Laemmli (1970).

The gel apparatus was constructed from two 20 x 20cm glass plates separated by perspex spacers 0.15cm thick. The plates were held together by fold-back bulldog clips and vacuum grease was applied to the outside edges of the specers to prevent leakage. The bottom of the gel was sealed by supporting the plates vertically in a trough and pouring a plug of 15% acrylamide main gel solution (A3.7.2.) which filled the trough and the bottom of the plates. The solution was allowed to run down the inside edge of the spacers to form a thin seal. When the plug had polymerised the main gel (18 x 18cm) was poured and overlaid with water. After at least 30 min the water was poured off the main gel and a stacking gel (A3.7.3.) was applied. Sample wells were made by pushing a plastic silicone-coated comb into the stacking gel before it set. This was withdrawn after at least 15 min. leaving a stacking gel with a height of 0.7 - 1.0 cm at the bottom of the wells. For electrophoresis the gel was supported vertically in a perspex electrophoresis tank. Contact with an upper tank was made by way of a wick of Whatman 3MM Chromatography Paper and a 0.5cm sponge.

Proteins were denatured prior to electrophoresis by addition of concentrated reagents to yield final concentrations of 2% (w/v) SDS and 5% (v/v) β-mercaptoethanol followed by heating in a boiling water bath/

bath for 2 min. Bromophenol blue and glycerol were added to final concentrations of 0.002% (w/v) and 20% (v/v). Samples with a total volume of up to 100µl were applied to the gel and electrophoresed at 100V until the tracking dye was approximately 1cm from the end of the gel.

A13.2. Detection of {^{35}S} Methionine Labelled Polypeptides Separated on Gels.

Radioactive polypeptides were detected by fluorography (Bonner & Laskey, 1974). After electrophoresis, gels were soaked in three changes of 8-10 volumes of dimethyl sulphoxide (DMSO) for 20 min. each change. DMSO baths were used several times in the same sequence. The gel was then shaken for 3 h in 4 volumes of 22.2% (w/v) PPO in DMSO and then washed under running water for a further 3 h before drying under vacuum. The dried gel was placed in contact with Kodak X-Omat H X-ray film between heavy glass plates and exposed at -70°C for the appropriate time. The film was developed in Kodak DX-80 developer and fixed with Kodak FX-40 X-ray liquid fixer.

A13.3. Estimation of Molecular Weights.

Gels were calibrated by coelectrophoresis of albumin (68,000), trypsin inhibitor (21,000), chymotrypsinogen A (25,000), ovalbumin (45,000), β-galactosidase (130,000) and RNA polymerase (39,000, 155,000, 165,000). After electrophoresis gels were stained by immersion for 1 h in Coomassie Blue Stain (A3.7.4.) and destained by several changes of destainer (A3.7.5.). Gels were impregnated with PPO as described in A.13.2. and dried. The molecular weight of the standards was plotted against their migration distances on semi-logarithmic/

logarithmic paper (Fig. A13.1.). The relationship is non-linear. The molecular weights of polypeptides synthesised in vitro were estimated from the calibration curve and used to calibrate other unstained gels because staining reduced the efficiency of fluorography.

A14. Immunological Techniques.

A14.1. Preparation of Antisera.

Antisera were raised in rabbits against the major capsid protein of PrV and a PrV-infected BHK 21 cell lysate harvested 5 hr post-infection. Purified capsid proteins (A15) were made 2% (w/v) SDS, 5% (w/v) β-mercaptoethanol and electrophoresed into a 10% acrylamide slab gel (A13). A section of the gel was stained to locate the major capsid protein and the band containing this protein excised from the unstained area, crushed, mixed with Freunds adjuvant and injected into rabbits.* The BHK 21 infected cell lysate was similarly electrophoresed about 1 cm into a 15% acrylamide slab gel and the total proteins cut out and injected into rabbits. Antisera were collected at intervals and assayed for the presence of antibodies. Goat anti-rabbit IgG antiserum was prepared by immunisation of goats with rabbit IgG and was a generous gift from Mrs. H.H. Singer.

*Three doses of 200µg protein were injected at two week intervals.

A14.2. Double Diffusion.

Antisera were assayed for antibody activity by a modification of the technique described by Ouchterlony (Ouchterlony, 1964). Hot (60°C) 1.5% (w/v) agarose in PBS A (A3.8.1.) PEG (A3.8.2.) and PBSA were mixed in a ratio of 4:1:1 and 2.5 ml aliquots were immediately applied to 7.5 x 2.5 cm glass slides by moving a pipette along the centre of the slide. The solution was left 15 min to set, holes

were/

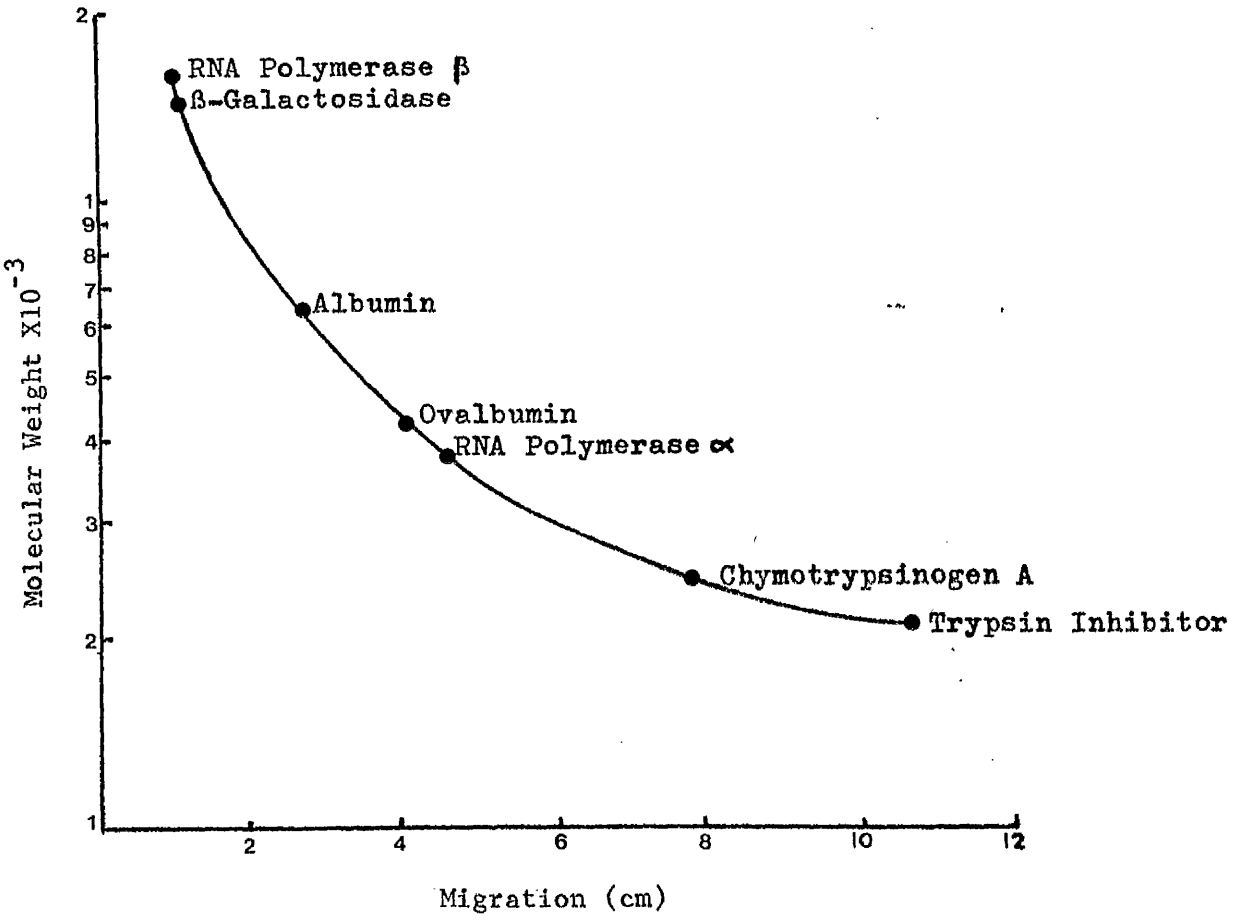


FIG. A13.1. ELECTROPHORETIC MOBILITY AS A FUNCTION OF POLYPEPTIDE MOLECULAR WEIGHT IN A 12%BIS-CROSSLINKED POLYACRYLAMIDE GEL

Commercially supplied molecular weight standards were denatured according to the manufacturers' instructions and electrophoresed into a 12% bis-crosslinked polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue, and processed for fluorography. The migration distances from the top of the main gel were measured and are plotted against the molecular weight on a logarithmic scale.

were cut with a punch and the agarose removed with a pasteur pipette. The holes were filled with antiserum or antigen and the slides placed in an airtight box with a damp filter paper and left overnight at room temperature.

A14.3. Absorption of Antibodies.

Uninfected HeLa cells were harvested and lysed in the same way as for polysome preparation (A5.). If the lysate was to be treated with SDS, the volume was kept as small as possible; SDS was added to a concentration of 1% (w/v), the lysate was boiled for 2 min. and then diluted with distilled water to 10^8 cells/ml. Antisera were absorbed by adding the lysates from 10^9 SDS-treated or untreated cells to 1 ml antiserum and incubated overnight at $4^{\circ}C$ (Sim and Watson, 1973). The absorbed antisera were centrifuged at 40,000 rpm for 1 h in a Beckman SW40 rotor and the supernatant fluid concentrated to the original volume of serum in an Amicon microfiltration cell Model 8MC.

A14.4. Preparation of IgG.

Two volumes of 27% (w/v) Na_2SO_4 (at $37^{\circ}C$) were added dropwise to one volume of serum stirred gently at room temperature. The precipitate was pelleted immediately by centrifugation for 10 min. at 3,000 rpm in a Mistral 4L centrifuge. The precipitate was dissolved in approximately one-fifth of the original serum volume of water and dialysed overnight against 50 - 100 volumes 15% (w/v) Na_2SO_4 at room temperature. The precipitate was pelleted as above, dissolved in one tenth of the original serum volume of PBS and dialysed overnight at $4^{\circ}C$. The IgG solution was spun at 12,000g to pellet denatured protein and debris and stored at $-20^{\circ}C$.

A14.5. Indirect Precipitation.

Cell lysates and wheat germ assays were diluted with buffers containing detergent (A3.8.3.; A3.8.4.) in 1.5ml Eppendorf microtubes and incubated for 15 min. at 37°C with 5µl antiserum or IgG. The appropriate volume of goat anti-rabbit IgG antiserum was added to precipitate all the rabbit IgG and incubation was continued at 37°C for a further 45 min. The precipitates were left overnight at 4°C, then pelleted by centrifugation at 800g in an Eppendorf 3200 centrifuge for 4 min. The precipitates were washed three times with buffer then dissolved in 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS by treating at 100°C for 2 min.

The volume of goat anti-rabbit IgG antiserum required to precipitate the rabbit IgG was determined by titration. Increasing volumes of goat anti-rabbit IgG antiserum were incubated with a constant volume of antiserum or IgG for 1 h at 37°C then overnight at 4°C. The precipitates were pelleted as above, washed, dissolved in 0.1 ml 0.2M NaOH and the concentration of protein was determined spectrophotometrically using the following formula:

$$\{\text{Protein}\} = \frac{E_{280} - E_{320}}{1.35} \quad \mu g/ml$$

The volume of goat anti-rabbit IgG antiserum which gave the largest precipitate was thereafter used in indirect precipitation.

A15. In vivo Labelling at Elevated {NaCl}.

HeLa cells were seeded at 10^6 cells in 50mm Petri dishes containing glass coverslips. When confluent (2-3 days) they were mock-infected or infected with 20pfu/cell PrV. After 1 h at 37°C the medium was replaced with EC10 (prepared with dialysed serum,

see/

(see A5). NaCl was added to the appropriate concentration 30 min. before harvesting and 15μCi {^{3}H} methionine was added to each dish before harvesting. Coverslips were removed at the appropriate time after infection, washed three times with ice-cold 5% (w/v) TCA, once with ethanol and then counted in toluene-PPO.

A16. Liquid Scintillation Spectrophotometry.

The amount of radioactivity present in proteins was determined either by TCA precipitation on filter paper discs which were subsequently dried and counted in toluene-PPO (A3.9.1.) or by dissolving the protein solution directly in triton-toluene scintillant (A3.9.2.). {^{3}H}-labelled RNA was either dried on filters which were counted in toluene-PPO or dissolved in triton-toluene. The efficiency of counting for {^{3}H} on filters was 7.5% and for {^{35}S} was 65%. The efficiency of counting for {^{3}H} in solution was 57% and for {^{35}S} 85%.

REFERENCES

Aboodeely,R.A., Palmer,E., Lawson,L.A. and Randall,C.C. (1970). Virology 44, 146-152.

Allen,G.P. and Bryans,J.T. (1976). Virology 69, 751-762.

Allen,G.P., O'Callaghan,D.J. and Randall,C.C. (1977). Virology 76, 395-408.

Alwine,J.C., Steinhart,W.L. and Hill,C.W. (1974). Virology 60, 342-347.

Anderson,C.W., Lewis,J.B., Atkins,J.F., and Gesteland,R.F. (1974). Proc. Nat. Acad. Sci. U.S.A. 71, 2756-2760.

Aron,G.M., Purifoy,D.J.M. and Schaffer,P.A. (1975). J. Virol. 16, 498-507.

Atkins,J.F., Lewis,J.B., Anderson,C.W. and Gesteland,R.F. (1975). J. Biol. Chem. 250, 5688-5695.

Aujeszky,A. (1902). Zentrabl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. 1. Orig. 32, 352-357.

Aviv,H., Boime, I. and Leder, P. (1971). Proc. Nat. Acad. Sci. U.S.A. 68, 2303-2307.

Aviv, H. and Leder, P. (1972). Proc. Nat. Acad. Sci. U.S.A. 69, 1408-1412.

Bachenheimer,S. and Roizman, B. (1972). J. Virol. 10, 875-879.

Baltimore, D. (1969).in "Biochemistry of Viruses" (Levy,H.B. ed) pp. 101-176 Marcel Dekker, New York.

Bartkoski,M. and Roizman, B. (1976). J. Virol. 20, 583-588.

Bartkoski, M. and Roizman, B. (1978). Virology 85, 146-156.

Benne, R., Edman, J., Traut, R.R. and Hershey,J.W.B. (1978). PNAS 75, 108-112.

Benne,R., and Hershey, J.W.B. (1978). J. Biol. Chem. 253, 3078-3087.

Benveniste,K., Wilczek,J., Ruggieri, A. and Stearn, R. (1976). Biochemistry 15, 830-835.

Bernstein,R.G. (1954). Science 120, 459-460.

Ben-Porat,T. and Kaplan,A.S. (1965). Virology 25, 22-29.

Ben-Porat,T. and Kaplan,A.S. (1973) in "The Herpesviruses" (Kaplan,A.S.ed) pp. 163-220. Academic Press, London and New York.

Ben-Porat, T., Kervina,M. and Kaplan,A.S. (1975). Virology 65, 355-362.

Ben-Porat,T., Rakusanova,T. and Kaplan,A.S. (1971). Virology 46, 890-899.

Ben-Porat,T., Shimono,H. and Kaplan,A.S. (1969). Virology 37, 56-61.

Ben-Zeev,A., Asher,Y. and Becker, Y. (1976). Virology 71, 302-311.

Ben-Zeev,A. and Becker,Y. (1977). Virology 76, 246-253.

Birnstiel,M.L., Spiers,J., Purdom,I., Jones,K. and Loening,U.E. (1968). Nature (London) 219, 454-463.

Bishop,J.O., Morton,J.G., Rosbash,M. and Richardson,M. (1974). Nature (London) 250, 199-204.

Bittlingmaier,K., Schneider,D. and Falke, D. (1975). Biochem. Biophys. Acta 407, 384-391.

Block,R. and Haseltine,W.A. (1974).in "Ribosomes" (Nomura,M., Tissieres,A. and Lengyel,P. eds). pp. 747-761. Cold Spring Harbor Laboratory, New York.

Boezi,J.A., Lee,L.F., Blakesley,R.W., Koenig,M. and Towle,H.C. (1974). J. Virol. 14, 1209-1219.

Boime,I. and Leder, P. (1972). Arch. Biochem. Biophys. 153, 706-713.

Bollum,F.J. (1968). in "Methods in Enzymology" Vol. XII B (Grossman,L., and Moldave, K., eds). pp. 169-171. Academic Press, New York.

Brawerman,G. (1975). Annu.Rev. Biochem. 43, 621-642.

Busby,D.W.G., House,W. and Macdonald,J.R. (1964). in "Virological Techniques" pp. 120. Churchill, London.

Carrasco,L. (1977). FEBS Letts. 76, 11-15.

Carrasco,L. (1978). Nature (London) 272, 694-699.

Carrasco,L. and Smith,A.E. (1976). Nature (London) 264, 807-809.

Caspar,D.L.D., Dulbecco,R., Klug,A., Lwoff,A., Stoker,G.P. Tournier,P. and Wildy,P. (1962). Cold Spring Harb. Symp. Quant. Biol. 27, 49.

Cheng,Y., Goz,B., and Prusell,W.H. (1975). Biochem. Biophys. Acta 390, 253-263.

Chopra,H.C., Shibley,G.A. and Walling,M.J. (1970). J. Microscop. 9, 167-176.

Civelli,O., Vincent.A., Buni,J-F., Scherrer,K. (1976). FEBS Letts. 72, 71-76.

Clements,J.B., Watson,R.J. and Wilkie,N.M. (1977). Cell 12, 275-285.

Clemens,M.J. and Williams,B.R.G. (1978). Cell 13, 565-572.

Cohen,S.S. (1971). "Introduction to Polyamines". Prentice Hall, New Jersey.

Cohen,J.C., Perdue,M.L., Randall,C.C., and O'Callaghan,D.J. (1975). Virology 67, 56-67.

Cohen,J.C., Perdue,M.L., Randall,C.C. and O'Callaghan,D.J. (1977). Virology 76, 621-633.

Costanzo,F., Campadelli-Fiume,G., Foá-Tomasi,L., and Cassai,E. (1977). J. Virol. 21, 996-1001.

Cox,G.S., Weintraub,B.D., Rosen,S.W., and Maxwell,E.S. (1976). J. Biol. Chem. 251, 1723-1730.

Daggleman,H. and Beard,P. (1976). "Animal Virus Course Manual" p.54. Cold Spring Harbor Laboratory, New York.

Darlington,R.W. and Granoff,A. (1973). in "The Herpesviruses" (Kaplan,A.S. ed). pp. 93-132. Academic Press, London and New York.

David,A.E. (1978). J. Gen. Virol. 39, 149-160.

Davies,J.W. and Kaesberg,P. (1973). J. Virol. 12, 1434-1441.

Davies,J.W. & Kaesberg,P. (1974). J. Gen. Virol. 25, 11-20.

deHaro,C. & Ochoa,S. (1978). Proc. Nat. Acad. Sci. U.S.A. 75, 2713-2716.

deHaro,C., Datta,A.& Ochoa,S. (1978). Proc. Nat. Acad. Sci. U.S.A. 75, 243-247.

Dimmock,N.J. and Watson,D.H. (1969). J. Gen. Virol. 5, 499-509.

Durham,A.C.H. (1977). Nature (London) 267, 375-376.

Durham,A.C.H. (1978). Abstracts of the Fourth International Congress for Virology held at The Hague, The Netherlands. p. 178.

Egberts,E., Hackett,P.B. & Traub,P. (1977). J. Virol. 22, 591-597.

Ejercito,P.M., Kieff,E.D. and Roizman,B. (1968). J. Gen. Virol. 3, 357.

Epstein,M.A. (1962). J. Expt. Med. 115, 1-11.

Epstein,M.A. & Holt,S.J. (1963). Nature (London) 198, 509-510.

Erickson,J.S. (1976). Virology 71, 598-601.

Farrell,P.J., Balkov,K., Hunt,T. Jackson,R.J. and Trachsel,H. (1977). Cell 11, 187-200.

Fenner,F. (1976). Intervirology 7, 1-116.

Fenwick,M.L. and Roizman,B. (1977). J. Virol. 22, 720-725.

Fenwick,M.L., Walker,M.J. & Petkevich,J.M. (1978). J. Gen. Virol. 39, 519-529.

Fong,CK.Y., Tenser,R.B., Hsiung,G.D. & Gross,P.A. (1973). Virology 52, 468-477.

Francoeur,A.M. and Stanners,C.P. (1978). J. Gen. Virol. 39, 551-554.

Frenkel,N. and Roizman,B. (1972). Proc. Nat. Acad. Sci. U.S.A. 69, 2654-2658.

Frenkel,N., Silverstein,S., Cassai,E. and Roizman,B. (1973). J. Virol. 11, 886-892.

Friedmann,A., Coward,J.E., Rosenkranz,H.S. and Morgan,C. (1975). J. Gen. Virol. 26, 171-181.

Fritsch,C., Mayo,M.A., & Hirth,L. (1977). Virology 77, 722-732.

Fujiwara,S. and Kaplan,A.S. (1967). Virology 32, 60-68.

Furlong,D., Swift,H. & Roizman,B. (1972). J. Virol. 10, 1071-1074,

Gerber,P. and Hoyer,B.H. (1971). Nature (London) 231, 46-47.

Gentry,G.A. and Randall,C.C. (1973). in "The Herpesviruses" (Kaplan,A.S. ed.) pp. 45-92. Academic Press, London and New York.

Gergely,L., Klein,G. & Ernberg,I. (1971a). Int. J. Cancer 7, 293-302.

Gergely,L., Klein,G. & Ernberg,I. (1971b). Virology, 45, 10-21.

Gey,G.O., Coffman,W.D. and Kubicek,M.T. (1952). Cancer Res. 12, 264-265.

Gibson,W. and Roizman,B. (1971). Proc. Nat. Acad. Sci. U.S.A. 68, 2818-2821.

Gibson,W. and Roizman,B. (1972). J. Virol. 10, 1044-1052.

Gibson,W. and Roizman,B. (1974). J. Virol. 13, 155-165.

Gill,T.J. (1970). Immunochemistry 7, 997-999.

Gillespie,D. and Spiegelman,S. (1965). J. Mol. Biol. 12, 829-842.

Gilmore-Hebert,M.A. & Heywood,S.A. (1976). Biochem. Biophys. Acta 454, 55-66.

Golini,F., Thach,S.S., Birge,C.H., Safer,B., Merrick,W.C. and Thach,R.E. (1976). Proc. Nat. Acad. Sci. U.S.A. 73, 3040-3044.

Gostling,J.V.T. and Bedson,S.P. (1956). Brit. J. Exp. Pathol. 37, 434-446.

Grummt,F. and Grummt,I. (1976). Eur. J. Biochem. 64, 307-312.

Grummt,F., and Speckbacher,M. (1975). Eur. J. Biochem. 57, 579-585.

Gupta,P. and Rapp,F. (1978). Virology 84, 199-202.

Gurdon,J.B., Lingrel,J.B. and Marbaix,G. (1973). J. Mol. Biol. 80, 539-551.

Gustafson,D.P. (1970).in "Diseases of Swine" (Dunne,H.W. ed.). 3rd edition pp. 337-355. Iowa State University Press, Ames.

Hackett,P.B., Egberts,E. and Traub,P. (1978). Eur.J. Biochem. 83, 353-361.

Hamada,C. and Kaplan,A.S. (1965). J. Bacteriol. 89, 1328-1334.

Harris,T.J.R. & Wildy,P. (1975). J. Gen. Virol. 28, 299-312.

Harwood,R., Grant,M.E. and Jackson,D.S. (1975). FEBS Letts. 57, 47-50.

Hastie,N.D., and Held,W.A. (1978). Proc. Nat. Acad. Sci. U.S.A. 75, 1217-1221.

Hay,J., Moss,H., Jamieson,A.T. and Timbury,M.C. (1976). J. Gen. Virol. 65-73.

Hayes,L.C., Plapp,F.V., Titzer,L.L. and Chiga,M. (1975). Biochem. Biophys. Res. Commun. 65, 258-264.

Heine,J.W., Honess,R.W., Cassai,E. and Roizman,B. (1974). J. Virol. 14, 640-651.

Helentjaris,T. & Erhenfeld,E. (1978). Abstracts of the Fourth International Congress for Virology held at The Hague, The Netherlands. p. 314.

Herpesvirus Study Group (1975). J. Gen. Virol. 20, 417-419.

Higgins,T.J.V. and Spencer,D. (1977). Plant Physiol. 60, 655-661.

Hochberg,E. and Becker,Y. (1968). J. Gen. Virol. 2, 231-241.

Holmes,I.H. and Watson,D.H. (1963). Virology 21, 112-123.

Honess,R.W. and Roizman,B. (1973). J. Virol. 12, 1347-1365.

Honess,R.W. and Roizman,B. (1974). J. Virol. 14, 8-19.

Honess,R.W. and Roizman,B. (1975a). Proc. Nat. Acad. Sci. U.S.A. 72, 1276-1280.

Honess,R.W. and Roizman,B. (1975b). J. Virol. 16, 1308-1326.

Honess,R.W. and Watson,D.H. (1977). J. Gen. Virol. 37, 15-37.

House,W. and Wildy,P. (1965). Lab. Pract. 14, 594-595.

Huang,E-S (1975). J. Virol. 16, 1560-1565.

Hunt,T. and Jackson,R.J. (1974). in "Modern Trends in Human Leukemia". (Neth,R., Galla,R.C., Speigelman,S. and Stohlman,F. eds.) pp. 300-307. J.F. Lehmanns, Verlag, München.

Hunter,A.R.., Farrell,P.J., Jackson,R.J., and Hunt,T. (1977). Eur. J. Biochem. 75, 149-157.

Hurst,E.W. (1934). J. Exp. Med. 59, 729-745.

Inglis,S.C., McGeoch,D.J., and Mahy,B.W.J. (1977). Virology 78, 522-536.

Jackson,R.J. (1975) in "MTP International Review of Science". Biochem. Series One 7, (Arnstein,H.R.V. ed.). pp. 89-135. Butterworths, London.

Jacquemont,B. and Roizman,B. (1975). J. Virol. 15, 707-713.

Jean,J.H., Ben-Porat,T. and Kaplan,A.S. (1974). Virology 59, 516-523.

Johnson,F.B., Blacknow,N.R. and Hoggan,M.D. (1972). J. Virol. 9, 1017-1026.

Jones,R.A., Larkins,B.A. and Tsai,C.Y. (1977). Plant Physiol. 59, 525-529.

Kabat,D. and Chappell,M.R. (1977). J. Biol. Chem. 252, 2684-2690.

Kaempfer,R., Rosen,H. and Israeli,R. (1978). Proc. Nat. Acad. Sci. U.S.A. 75, 650-654.

Kaplan,A.S. (1969). Virology Monogr. 5, "Herpes Simplex and Pseudorabies Viruses". Springer-Verlag, Wien and New York.

Kaplan,A.S. (1973). Cancer Res. 33, 1393-1398.

Kaplan,A.S. and Ben-Porat,T. (1976). Virology 70, 561-563.

Kaplan,A.S., Shimono,H. and Ben-Porat,T. (1970). Virology 40, 90-101.

Kaplan,A.S. and Vatter,A.E. (1959). Virology 7, 394-407.

Katz,F.N., Rothman,J.E., Lingappa,V.R., Blobel,G. and Lodish,H.F. (1977). Proc. Nat. Acad. Sci. U.S.A. 74, 3278-3782.

Kedes,L.H. (1976). Cell 8, 321-331.

Kemp,M.C., Cohen,J.C., O'Callaghan,D.J. and Randall,C.C. (1975). Virology 68, 467-482.

Kemper,W.M., Berry,K.W., and Merrick,W.C. (1976). J. Biol. Chem. 251, 5551-5557.

Kemper,B. and Stolarsky,L.(1977). Biochemistry 16, 5676-5680.

Kennedy,D.R. and Heywood,S.M. (1976a). Prog. Nuc. Acid Res. Mol. Biol. 19, 477-484.

Kennedy,D.R. & Heywood,S.M. (1976b). FEBS Letts. 72, 314-318.

Kennedy,D.R., Siegel,E. and Heywood,S.M. (1978). FEBS Letts. 90, 209-214.

Killington,R.A., Yeo,J., Honess,R.W., Watson,D.H., Halliburton,I.W. and Munford,J. (1977). J. Gen. Virol. 37, 297-310.

Koch,G., Oppermann,H., Bilbello,P., Koch,F. and Nuss,D. (1976). in "Modern Trends in Human Leukemia II". (Neth,R., Gallo,R., Mannweiler,K. and Moloney,W.C. eds.). pp. 541-555. J.F. Lehmanns, München.

Konecki,D., Kramer,G., Pinphanichakam,P., and Hardesty,B. (1975). Arch. Biochem. Biophys. 169, 192-198.

Kozak,M. and Roizman,B. (1974). Proc. Nat. Acad. Sci. U.S.A. 71, 4322-4326.

Kozak,M. and Roizman,B. (1975). J. Virol. 15, 36-40.

Kyner,D., Zabos,P. and Levin,D.H., (1973). Biochem. Biophys. Acta. 324, 386-396.

Laemmli,U.K. (1970). Nature (London) 227, 680-684.

Ledbetter,M.L.S. & Lubin,M. (1977). Exp. Cell Res. 105, 223-236.

Lee,L.F. (1972). J. Virol. 10, 167-170.

Lee-Huang,S., Sierra,J.M., Naranjo,R., Filipowitz,W. and Ochoa,S. (1977). Arch. Biochem. Biophys. 180, 276-287.

Lindberg,U. and Persson,T. (1972). Eur. J. Biochem. 31, 246-254.

Lodish,H.F. (1974). Nature (London) 251, 385-388.

Lodish,H.F. (1976). Annu. Rev. Biochem. 45, 39-72.

Lodish,H.F. and Desalu,O. (1973). J. Biol. Chem. 248, 3520-3527.

Lowe,P.A. (1978). Virology 86, 577-580.

Ludwig,H. and Rott,R. (1975). J. Virol. 16, 217-221.

Luria,S.E. and Darnell,J.E. (1967). in "General Virology" 2nd Edition. John Wiley & Sons Inc., New York, London, Sydney.

Luthe,D.S. and Petersen,D.M. (1977). Plant Physiol. 59, 836-841.

Lwoff,A. (1957). J. Gen. Microbiol. 17, 239-253.

McKercher,D.G. (1973). in "The Herpesviruses" (Kaplan,A.S. ed.). pp. 427-493. Academic Press, London and New York.

McMillen,J. and Consigli,R.A. (1977). J. Virol. 21, 1113-1120.

Macpherson,I.A. and Stoker,M.G.P. (1962). Virology, 16, 147-151.

Manservigi,R., Spear,P.G. and Buchan,A. (1977). Proc. Nat. Acad. Sci. U.S.A. 74, 3913-3917.

Marcu,K. and Dudock,B. (1974). Nucleic Acid Res. 1, 1385-1397.

Marsden,H.S., Crombie,J.K. and Subak-Sharpe,J.H. (1976). J. Gen. Virol. 31, 347-372.

Mathews,M.B. (1972). Biochem. Biophys. Acta 272, 108-118.

Mathews,M.B. and Korner,A. (1970). Eur. J. Biochem. 17, 328-338.

Mathews,M.B. & Osborn,M. (1974). Biochem. Biophys. Acta. 340, 147-152.

Maurer,R.A., Stone,R. and Gorski,J. (1976). J. Biol. Chem. 251, 2801-2807.

Mendecki,J., Lee,S.Y. and Brawerman,G. (1972). Biochemistry 11, 792-798.

Miller,R.L., Glaser,R. and Rapp,F. (1977). Virology 76, 494-502.

Miller,R.L. and Rapp,F. (1977). J. Gen. Virol. 36, 515-524.

Morgan,C., Ellison,S.A., Rose,H.M. & Moore,D.I. (1954). J. Exp. Med. 100, 195-202.

Morgan,C., Rose,H.M., Holden,M. and Jones,E.P. (1959). J. Exp. Med. 110, 643-656.

Moss,B., Gershowitz,A., Stringer,J.R., Holland,L.E. and Wagner,F.K. (1977). J. Virol. 23, 234-239.

Nakaniski,S., Taii,S., Hirata,Y., Matsukura,S., Imura,H. and Numa,S. (1976). Proc. Nat. Acad. Sci. U.S.A. 73, 4319-4323.

Nazerian,K. (1974). J. Virol. 13, 1148-1150.

Nishioka,Y. and Silverstein,S. (1977). Proc. Nat. Acad. Sci. U.S.A. 74, 2370-2374.

Nonoyama,M., and Pagano,J.S. (1972). J. Virol. 9, 714-716.

Nuss,D.L., Oppermann,H. and Koch,G. (1975). Proc. Nat. Acad. Sci. U.S.A. 72, 1258-1262.

Olshevsky,U., Levitt,H.J. and Becker,Y. (1967). Virology, 33, 323-334.

Oppermann,H. and Koch,G. (1976a). J. Gen. Virol. 32, 261-274.

Oppermann,H. and Koch,G. (1976b). Arch. Virol. 52, 123-134.

Orlowski,M. and Sypherd .,P.S. (1978). Biochemistry 17, 569-575.

Ouchterlony,Ö. (1964) in "Immunological Methods". (Ackroyd,J.F. ed.). pp. 55-78. Blackwell Scientific Publications, Oxford.

Pain,V.M. and Henshaw,E.C. (1975). Eur. J. Biochem. 57, 335-342.

Pappenheimer,A.M. (1977). Annu. Rev. Biochem. 46, 69-94.

Paterson,B.M. and Bishop,J.O. (1977). Cell 12, 751-765.

Paterson,B.M., Roberts,B.E. and Kuff,E.L. (1977). Proc. Nat. Acad. Sci. U.S.A. 74, 4370-4374.

Pearson,G., Dewey,F., Klein,G., Henle,G. and Henle,W. (1970). J. Nat. Cancer Inst. 45, 989-995.

Pelham,H.R.B. and Jackson,R.J. (1976). Eur. J. Biochem. 67, 247-256.

Penman,S., Scherrer,K., Becker,Y. and Darnell,J.E. (1963). Proc. Nat. Acad. Sci. U.S.A. 49, 654-662.

Perdue,M.L., Cohen,J.C., Randall,C.C. and O'Callaghan,D.J. (1976). Virology 74, 194-208.

Pereira,L., Wolff,M.H., Fenwick,M.L. and Roizman,B. (1977). Virology, 77, 733-749.

Perry,R.P., Latorre,J., Kelley,D.E. and Greenberg,J.R. (1972). Biochim. Biophys. Acta 262, 220-226.

Pizer,L.I. and Beard,P. (1976). Virology 75, 477-480.

Powell,K.L. and Courtney,R.J. (1974). Virology 66, 217-228.

Powell,K.L., Purifoy,D.J.M. and Courtney,R.J. (1975). Biochem. Biophys. Res. Commun. 66, 262-271.

Preston,C.M. (1977). Virology 78, 349-353.

Preston,C.M. and Newton,A.A. (1976). J. Gen. Virol. 33, 471-483.

Prives,C.L., Aviv,H., Paterson,B.M., Roberts,B.E., Rozenblatt,S., Revel,M., and Winocour,E. (1974). Proc. Nat. Acad. Sci. U.S.A. 71, 302-306.

Purifoy,D.J.M. and Benyesh-Melnick,M. (1975). Virology 68, 374-386.

Rakusanova,T., Ben-Porat,T., Himeno,M., and Kaplan,A.S. (1971). Virology 46, 877-889.

Rakusanova,T., Ben-Porat,T., and Kaplan,A.S. (1972). Virology, 49, 537-548.

Raymondjean,M., Bogdanovsky,D., Bachner,L., Kneip,B. and Schapira,G. (1977). FEBS Letts. 76, 311-315.

Revel,M. & Groner,Y. (1978). Annu. Rev. Biochem. 47, 1079-1126.

Ricciardi,R.P., Goodman,R.M. and Gottlicks,D. (1978). Virology 85, 310-314.

Roane,P.R. and Roizman,B. (1964). Virology 22, 1-8.

Roberts,B.E. and Paterson,B.M. (1973). Proc. Nat. Acad. Sci. U.S.A. 70, 2330-2334.

Rodriguez,M., and Dubois-Dalcq,M. (1978). J. Virol. 26, 435-477.

Roizman,B. (1978). in "The Molecular Biology of Animal Viruses" Vol. 2. (Nayak,D.P. ed.). pp. 769-848. Marcel Dekker, Inc., New York and Basel.

Roizman,B., Bachenheimer,S., Wagner,E.K. and Savage,T. (1970). Cold Spring Harbor Symp. Quant. Biol. 35, 753-771.

Roizman,B., Borman,G.S. & Rousta,M.K. (1965). Nature (London) 206, 1374-1375.

Roizman,B. and Furlong. D. (1974) in "Comprehensive Virology" Vol. 3. (Fraenkel-Conrat,H. & Wagner,R.R. eds.). pp. 229-403. Plenum, New York.

Roizman,B., Kozak,M., Honess,R.W. and Hayward,G. (1974). Cold Spring Harbor Symp. Quant. Biol. 39, 687-701.

Roizman,B. and Spring,S.B. (1967). in "Cross-Reacting Antigens and Neoantigens" (Trentin,J.F. ed.). pp. 85-97. Williams and Wilkins, Baltimore, Maryland.

Rose,J.K., Trachsel,H., Leongi.K. and Baltimore,D. (1978). Proc. Nat. Acad. Sci. U.S.A. 75, 2732-2736.

Rubenstein,A.S. and Kaplan,A.S. (1975). Virology, 66, 385-392.

Rubin,H. (1978). Nature (London) 271, 186-187.

Ruhlig,M.A. and Person.S. (1977). J. Virol. 24, 602-608.

Rupniak,H.T.R. and Quincey,R.V. (1975). FEBS Letts. 58, 99-101.

Sasaki,Y., Sasaki,R., Cohen,G.H. and Pizer,L.I. (1974). Intervirology 3, 147.

Saxton,R.E. and Stevens,J.G. (1972). Virology 48, 207-220.

Schecter,I. and Burstein,Y. (1976). Biochem. J. 153, 543-550.

Schmeckpeper,B.J., Cory,S.M. and Adams,J.M. (1974). Mol. Biol. Reports 1, 355-363.

Schreier,M.H., Erni,B. and Stachelin,T. (1977). J. Mol. Biol. 116, 727-753.

Schwartz,J. and Roizman,B. (1969). Virology 38, 42-49.

Shafritz,D.A., Weinstein,J.A., Safer,B., Merrick,W.C., Weber,L.A., Hickey,E.D. and Baglioni,C. (1976). Nature (London) 261, 291-294.

Sharma,O.K., Beezley,D.N. and Roberts,W.K. (1976a). Biochemistry 15, 4313-4318.

Sharma,O.K., Beezley,D.N. and Borek,E. (1976b). Nature (London) 262, 62-63.

Shatkin,A.J. (1976). Cell 9, 645-653.

Sheddon,W.I.H. and Wildy,P. (1966). Nature (London) 212, 1068-1069.

Silverstein,S., Millette,R., Jones,P. and Roizman,B. (1976). J. Virol. 18, 977-991.

Sim,C. and Watson,D.H. (1973). J. Gen. Virol. 19, 217-233.

Smid,B., Valicek,L. and Necas,O. (1977). Arch. Virol. 53, 167-170.

Soffer,R.L. (1974). Adv. Enzymol. 40, 91-139.

Sonenshein,G.E. & Brawerman,G. (1976). Biochemistry 15, 5501-5506.

Spear,P.G. (1975). in "Oncogenesis and Herpesviruses II" (de The,G., Epstein,M.A. and zur Hausen,H. eds.). pp. 47-61. International Agency for Research on Cancer Publications, Lyon.

Spear,P.G. (1976). J. Virol. 17, 991-1008.

Spear,P.G. and Roizman,B. (1968). Virology 36, 545-555.

Spear,P.G. and Roizman,B. (1970). Proc. Nat. Acad. Sci. U.S.A. 66, 730-737.

Staehelin,T., Trachsel,H., Erni,B., Boschetti,A. and Schreier,M.H. (1975). in "Proceedings of the Tenth FEBS Meeting" Vol. 39. (Raoul,Y., Gen. Ed.). pp. 309-323. North Holland Publishing Company, Amsterdam.

Stanners,C.P., Francoeur,A.M. and Lam,T. (1977). Cell, 11, 273-281.

Stevely,W.S. (1975). J. Virol. 16, 944-950.

Stevely,W.S. (1977). J. Virol. 22, 232-234.

Stringer,J.R., Holland,L.E., Swanstrom,R.I., Pivo,K. and Wagner,E.K. (1977). J. Virol. 21, 289-901.

Strnad,B.C. and Aurelian,L. (1976). Virology 69, 438-452.

Sun,S.M., Buckbindi,B.U. and Hall,T.C. (1975). Plant Physiol. 56, 780-785.

Swanstrom,R.I., Pivo,K. and Wagner,E.K. (1975). Virology 66, 140-150.

Sydiskis,R.J., and Roizman,B. (1976). Science 153, 76-78.

Sydiskis,R.J. & Roizman,B. (1967). Virology 32, 678-686.

Tabor,H. & Tabor,C.W. (1972). Adv. Enzymol. 36, 203-268.

Thang,M.N., Dandon,L. and Mohier,E. (1976). FEBS Letts. 61, 85-90.

Tournier,P. and Lwoff,A. (1966). in "IXth International Congress for Microbiology" pp. 417-422.

Trachsel,H., Erni,B., Schreier,M.H. and Staehelin,T. (1977). J. Mol. Biol. 116, 755-767.

Tse,T.P.H. and Taylor,J.M. (1977). J. Biol. Chem. 252, 1272-1278.

Tyler,S.J., Hay,J. and Stevely,W.S. (1974). Arch. Gesamte Virusforsch. 44, 84-91.

Vass,J.K. (1975). Ph.D. Thesis, University of Glasgow.

Vaughan,M.H. and Hansen,B.S. (1973). J. Biol. Chem. 248, 7087-7096.

Villa-Komaroff,L., Guttman,N., Baltimore,D. and Lodish,H.F. (1975). Proc. Nat. Acad. Sci. U.S.A. 72, 4157-4161.

Viotti,A., Balducci,C. and Weil,J.H. (1978). Biochem. Biophys. Acta 517, 125-132.

Wagner,A.F., Bugianesi,R.L. and Shen,T.Y. (1971). Biochem. Biophys. Res. Commun. 45, 184-189.

Wagner,E.K. and Roizman,B. (1969a). J. Virol. 4, 36-46.

Wagner,E.K. and Roizman,B. (1969b). Proc. Nat. Acad. Sci. U.S.A. 64, 626-633.

Ward,R.L. and Stevens,J.G. (1975). J. Virol. 15, 71-80.

Warrington,R.C., Wratten,N. & Hechtman,R. (1977). J. Biol. Chem. 252, 5251-5257.

Watson,D.H. (1973a). in "The Herpesviruses" (Kaplan,A.S. ed.). pp. 27-43. Academic Press, London and New York.

Watson,D.H. (1973b). in "The Herpesviruses" (Kaplan,A.S. ed.). pp. 133-161. Academic Press, London and New York.

Watson,D.H. and Wildy,P. (1963). Virology 21, 100-111.

Watson,D.H., Wildy,P. and Russell,W.C. (1964). Virology 24, 523-538.

Waxman,H.S. and Rabinovitz,M. (1966). Biochem. Biophys. Acta. 129, 369-379.

Weber,L.A., Feman ,E.R. & Baglioni,C. (1976). Biochemistry 14, 5315-5321.

Weber,L.A., Hickey,E.D., Maroney,P.A., and Baglioni,C. (1977). J. Biol. Chem. 252, 4007-4010.

Weissbach,H. & Ochoa,S. (1976). Annu. Rev. Biochem. 191-216,

Wheeler,T., Bayley,S.T., Harvey,R., Crawford,L.V. and Smith,A.E. (1977). J. Virol. 21, 215-224.

Wildy,P. (1971). Virology Monog. 7 "Classification and Nomenclature of Viruses" pp. 7-10. S. Karger, Basel.

Wildy,P., Russell,W.C. and Horne,R.W. (1960). Virology 12, 204-224.

Zagorski,W. (1977). Abstracts 11th FEBS Meeting, Copenhagen. A2-5-217-3.

Zambernard,J. and Vatter,A.E. (1966). Virology 28, 318-324.

Zeichner,M. & Breitkereutz,D. (1978). Arch. Biochem. Biochem.188, 410-417.

Zilberstein,A., Dudock,B., Berissi,H. & Revel,M. (1976). J. Mol. Biol. 108, 43-54.