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# ASPECTS OF PROTEIN SYNTHESIS IN 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

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## Abbreviations

The abbreviations used in this thesis are as laid down in the Biochemical Journal Instructions to Authors (revised 1978) with the following additions :

| | |
|---|---|
| BHK-21/C13 | Baby hamster kidney cells, clone 13. |
| BS-C-1 cells | African green monkey kidney cells. |
| HSV | Herpes simplex virus. |
| PRV | Pseudorabiesvirus. |
| EBV | Epstein-Barr virus. |
| EHV | Equine herpesvirus. |
| VSV | Vesicular somatitis virus. |
| EMC virus | Encephalomyocarditis virus. |
| p.f.u. | Plaque forming unit. |
| SDS | Sodium dodecyl sulfate. |
| EDTA | Ethylene diamine tetraacetate. |
| TCA | Trichloroacetic acid. |
| DMSO | Dimethylsulfoxide. |
| PPO | 2,5'-diphenyloxazole. |
| TEMED | N,N,N',N', tetramethylethylene diamine |
| HEPES | N-2-Hydroxyethylpiperizine-N'-2-ethane sulfonic acid. |
| DTT | Dithiothreitol. |
| MDL | Messenger dependent lysate. |
| SS | Single stranded. |
| DS | Double stranded. |

## Summary

Inhibition of protein synthesis has been studied in cells infected with PRV. Infection of HeLa, C13 and BS-C-1 cells with PRV showed that in the case of HeLa and C13 cells inhibition was rapid and substantial but for B-SC-1 cells it was gradual and apparently limited. Moreover, C13 and BS-C-1 cells showed some stimulation of protein synthesis at 1 hr post-infection with subsequent inhibition. Because of the simple inhibition kinetics, HeLa cells were mostly used for further studies.

Cell-free translation studies were carried out using mRNA from PRV infected cells. Immediate-early mRNA's (mRNA made in the infected cells before any viral protein synthesis) and late mRNA's were isolated from HeLa cells infected with PRV for 5 hrs in the presence and in the absence of Cycloheximide respectively. Hybridisation data for the poly(A) containing RNA showed the presence of, at least, 5% of viral transcripts in the Immediate-early mRNA preparation. Cell-free translation of this preparation in the Messenger Dependent reticulocyte lysate system showed the presence of some viral proteins but a large number of host proteins were also synthesized. Simultaneous addition of both viral and host mRNA's did not result in the preferential translation of viral mRNA in the cell free system and both cytoplasmic and polysomal late poly(A) RNA's allowed the synthesis of the same spectrum of proteins.

$K^+$ dependence studies in the MDL system did not reveal any difference in the optima for the translation of host, IE and late mRNA's though polyacrylamide gel electrophoresis of the translation products showed that at least one host and one viral protein were slightly resistant to high $K^+$ concentrations in

the system. Studies in the wheat germ extract showed an identical $K^+$ optima for all these mRNA's. Again, protein synthesis *in vivo* under hypertonic conditions showed that synthesis of both host and IE proteins were susceptible to elevated salt concentrations to the same extent.

When protein synthesis was studied in the presence of inhibitor of elongation rate, viral mRNA's were not apparently more efficient than host mRNA's in initiating protein synthesis. So there appears no selectivity in the inhibition of host protein synthesis.

Investigations of the inhibition of protein synthesis revealed that both PRV and HSV-1 caused marked polysome disaggregation in the absence of viral protein synthesis. There was an increase in the extent of polysome disaggregation by PRV when the number of infecting virus particles was increased. Moreover, heat and u.v. inactivated PRV caused substantial disaggregation of host polysomes.

In similar studies, both normal, infective and inactivated PRV caused an almost equal extent of inhibition of protein synthesis at 8 hrs post infection. By 24 hrs post infection, infective virus produced cytopathic effects whereas inactivated viruses did not. Again, infective viruses caused about 80% inhibition of RNA synthesis by 8 hrs post infection whereas by that time inactivated viruses caused only 25% inhibition.

In the cell-free translation system, exogenous mRNA translation was inhibited by both normal and inactivated PRV and this inhibition was not due merely to addition of exogenous proteins. The extent of inhibition increased with the addition

of increasing amounts of both normal and inactivated PRV. So inhibition of protein synthesis is caused by the virus particles which causes polysome disaggregation and might act in some other steps of protein synthesis.

By 6 hr post infection there was induction of an RNase activity which under cell-free conditions selectively degraded host mRNA. No mRNase activity was associated with either normal or inactivated PRV.

These experiments revealed that in PRV infected HeLa cells, translatable host mRNA's are present when IE proteins are being made. There is no evidence of any selective translation of viral mRNA either in the intact cell or in cell-free systems. The only evidence for selectivity resides in the mRNase which degrades host mRNA but not viral mRNA. Again in the intact cell or in cell-free systems, inhibition of protein synthesis is caused by the virus particles themselves and does not involve infected cell protein synthesis. This inhibition also involves polyribosome disaggregation by the virus particles.

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# Chapter 1. Introduction.

## 1.1. General introduction.

The consequence of productive infection by animal viruses is the inhibition of host macromolecule synthesis. This inhibition is reflected in the gradual cessation of host protein synthesis with concomitant synthesis of virus specific proteins in the infected cell. Though early in infection the infected cells show selectivity in translating viral mRNA's, late in infection the translation machinery often loses its activity until the infected cells show a cytopathic effect and eventual lysis. Knowledge about the mechanism of inhibition is scanty and indeed the mechanism is likely to vary from virus to virus and perhaps even from one host to another. However, various suggestions have been put forward to explain inhibition. It could be due to structural protein component(s) of the infecting virus which renders the translation system inactive. Again it could be the result of changes in the intracellular ion concentration following changes in the membrane permeability after infection, or again inhibition might be due to a modification of some component(s) of the translation system so that viral mRNA is selectively translated. In some cases it has been suggested that there is induction of RNase activity in the infected cells, which selectively cleaves host mRNA's. A number of these possibilities are discussed later in this thesis.

## 1.2. Viruses

Viruses are infectious pathogenic entities depending on the host cell metabolism for propagation (Lwoff, 1957). The infectious

extracellular particle is called the virion. It consists of a nucleic acid, DNA or RNA, enclosed in a protein coat (the capsid). In some viruses the capsid may become surrounded by a membraneous material, the envelope. Thus, viruses are entities whose genomes are elements of nucleic acid which replicate inside living cells using the cellular synthetic machinery and cause the synthesis of new particles which can transfer the viral genome to other cells (Luria et al., 1978).

1.3. Herpesvirus.

In 1975 the International Committee for Taxonomy of viruses defined herpesviruses as those having the following characteristics:

The virion consists of a capsid, 120 - 150mm in diameter, surrounded by a lipid containing envelope. Buoyant density (CsCl) of the virion is 1.27 - 1.29 $g/cm^3$. The capsid is icosadeltatetrahedral with 162 hollow capsomers and with a buoyant density (CsCl) of 1.305 $g/cm^3$. The capsid surrounds a core containing DNA wrapped round a protein spool. There are about 33 protein species in the virion with molecular weight in the range of 25,000 to 290,000 .

The genome consists of a linear ds DNA with both terminal reiteration and internal repetition of terminal sequences. G + C content varies from 33 to 74%. The molecular weight is in the range of 92 - 135 x $10^6$ .

Virus multiplication begins in the nucleus and is completed by the addition of a glycoprotein membrane as the virus passes through the inner lamella of nuclear membrane into the endoplasmic reticulum. Margination of chromatin and intranuclear inclusion

bodies are characteristics (Fenner, 1976).

At present about 70 viruses fulfil the criteria for inclusion within the herpesviruses. They are diverse in their host and disease associations and also in biochemical properties. Indirect evidence exists for the involvement of Epstein-Barr Virus (EBV) [Epstein, 1978; The et al., 1978] and Herpes Simplex Virus, Type 2 (HSV-2) [Nahmias et al., 1970] in human cancer.

### 1.3.1. Pseudorabies virus (PRV): Clinical symptoms.

Pseudorabies or Aujeszky disease was among the earliest viral disease recognized (Aujeszky, 1902). The infecting virus, Pseudorabies virus (PRV), is the only member of the herpesvirus to cause natural infection in swine (Andrews, 1962). It normally infects swine, cattle, dog, sheep and cats (Gustafson, 1970). The virus has a wide cell culture host range, replicating in rabbit, lamb, dog and monkey kidney cells, in HeLa cells and in chicken embryo cells (Kaplan, 1969). In cell monolayers PRV causes granulation and clumping of the cells with eventual lysis.

Naturally occurring PRV infection is acquired by droplet infection or ingestion (Gustafson, 1970). Infection is latent in the adult swine but severe in newborn, with about 100% mortality in newborn compared to nil in adult. Virus multiplication occurs in tonsillar and pharyngeal tissues from which it reaches brain via olfactory nerves (Gustafson 1970, McFerran & Dow, 1965). Moreover, leukocytes attracted to the infected area carry the virus to different organs, especially to placenta from whence it invades the foetus causing abortion (Gustafson, 1970).

Signs of PRV infection includes high fever, anorexia, depression, slight coughing and vomit ing, with diarrhoea in some and constipation in others. The disease is mostly characterized by symptoms referable to the central nervous system. The name "Pseudorabies" comes from its clinical similarities to rabies.

Recovery from naturally occurring disease confers a high degree of immunity. Antibody titres attain maximum in three weeks after recovery and are maintained for at least 18 months.

### 1.3.2. Structure of the herpesviruses

Most of studies on the structure of the herpesviruses have been carried out on Herpes Simplex Virus (HSV). However, some structural studies on PRV are also discussed. The virus consists of an innermost core of DNA and protein, surrounded by capsid, tegument and, at the outermost side, envelope (Roizman & Furlong, 1974). The virion consists of protein, DNA, lipid and polyamines.

#### 1.3.2.1. Core.

The core consists of DNA wrapped round a protein plug. Epstein (1962) observed that the central region of the virion is sensitive to DNase. Again, Furlong *et al.*, (1972), using the technique of selective extraction of uranyl ions bound to DNA in a stained preparation, showed that the electron dense region making up the toroid contains DNA. The core also contains spermine in addition to protein and DNA (Roizman, 1978). Olshevsky and Becker (1970) identified two core proteins in HSV,

and Kaplan & Ben-Porat (1970) reckoned three PRV proteins to be associated with the core. However, Hyman (1980) has found four proteins bound tightly but non-covalently to HSV-DNA and these proteins are bound at four specific sites, that is near the ends and within the internal inverted terminal repeats (Wu et al., 1979).

1.3.2.2. Capsid.

The capsid is a moderately electron dense structure separated from the core by an electron translucent shell. It consists of 162 capsomers arranged in 2-, 3- and 5-fold symmetry in the form of an icosadeltatetrahedron (Wildy et al., 1960). The electron translucent region between the core and the capsid is called the pericore. The nature of the material in the pericore is unknown and it could be an extension of the protein plug on which the DNA is wound.

1.3.2.3. Tegument.

The tegument is defined as the structure located between the capsid and the envelope (Roizman & Furlong, 1974) and is said to be a fibrous coat around the capsid (Morgan et al., 1968; Schwartz & Roizman, 1969). The polypeptides comprising the tegument are present in most virions, but the amount seen is variable from virion to virion, even in the same cell (Fong et al., 1973).

1.3.2.4. Envelope.

The envelope is a trilaminar membrane with spikes projecting

from the outer surface (Fong et al., 1973). It is composed of glycoproteins, glycolipids and lipids (Morgan et al., 1954). Though the envelope is derived from the host cell membrane (Watson & Wildy, 1963), it contains virus specific proteins (Ben-Porat & Kaplan, 1972; Spear & Roizman, 1972). The envelope of PRV appears to be derived in large measure from the inner nuclear membrane from areas which are newly synthesized after infection (Ben-Porat & Kaplan, 1971; 1972). The relative abundance of different envelope proteins differ in different herpesviruses, at least with respect to the degree of glycosylation (Keller et al., 1970).

### 1.3.3. DNA of the herpesviruses.

The DNA is linear, double-stranded and with molecular weight 95 - 150 x $10^6$ daltons (Wilkie, 1973; Mossmann & Hudson, 1973; Delius & Clements, 1976; Stevely, 1977; Geelan et al., 1978; Stinski et al., 1979). Single stranded nicks occur in the DNA so that upon alkali denatur ation there appear DNA single strands of fragments as well as of unit length (Wilkie, 1973; Roizman et al., 1974; Lee et al., 1971, Graftstrom et al., 1975; Wadsworth et al., 1975). It is not known whether these alkali-labile regions correspond to true single-stranded nicks or gaps, or to the insertion of ribonucleotides which have been reported to be linked covalently to HSV DNA (Hirsch & Vonka, 1974).

The G + C content varies from 33 moles % in Rabbit lymphoma virus DNA (Goodheart 1972) to 74 moles % in PRV DNA (Russell & Crawford, 1964). HSV DNA contains a terminal sequence which is repeated internally in inverted forms flanking two unique

sequences of 10 x $10^6$ [illegible] (Short or S sequence) and 75 x $10^{10}$ [illegible] (Long or L sequence) [Sheldrick & Berthelot, 1974]. Analysis of the HSV genome with restriction endonuclease (Hayward et al., 1975; Clements et al., 1976), partial denaturation mapping (Delius & Clements, 1976) and nucleic acid hybridisation studies (Wilkie & Cortini, 1976) has shown that the S and the L unique sequences are relatively inverted in different DNA molecules within the population, leading to four genomic arrangements. PRV DNA has a single set of palindromic sequences of 5.4 x $10^6$ [illegible], which surround a short unique sequence of about 10 x $10^6$ [illegible]. Inversion of this short unique sequence gives rise to two isomeric forms. PRV DNA has a molecular weight of 95 x $10^6$ [illegible] (Stevely 1977; Ben-Porat et al., 1979).

The mechanism responsible for the random orientation of short and long unique sequences in HSV is not known, although available evidence favours a high frequency of legitimate recombination through internal inverted repetition, rather than independent replication and joining of the long and the short unique sequences (Roizman, 1978).

### 1.3.4. Structural proteins.

High resolution polyacrylamide gel electrophoresis showed the presence of 30 polypeptides of molecular weight range of 25,000 to 280,000 in the Herpes Simplex Virion (Spear & Roizman, 1972; Heine et al., 1974; Cassai et al., 1975). Pseudorabies virion contains at least 20 polypeptides (Stevely, 1975). A number of HSV-1 and HSV-2 polypeptides are processed

following synthesis and this modification involves phosphorylation, glycosylation and possibly other modifications resulting in increased apparent molecular weight (Pereira et al., 1977; Fenwick & Roizman, 1977). Herpes Simplex Virion contains glycosylated and phosphorylated polypeptides.

### 1.3.5. Other components.

In addition, the virion contains spermine and spermidine in the ratio of 1.6 ± 0.2 : 1.0. This is sufficient to neutralize half of the viral DNA phosphate groups. (Gibson & Roizman, 1971).

### 1.3.6. Infectious unit.

Naked nucleocapsids are not infectious (Stein et al.,1970; Rubenstein & Kaplan, 1975). The reason is not known, probably without envelope the virus cannot adsorb to the cell surface. Viral DNA, native as well as alkali denatured, is infectious (Lando & Ryhiner, 1969; Sheldrick et al., 1973).

## 1.4. Reproductive Cycle.

Virus multiplication starts with the attachment of the virus particles to the host cell surface. Enveloped particles adsorb more readily than the naked particles. There follows the penetration of the virion into the cell. This could be either by pinocytosis (Epstein et al., 1964) or by fusion of envelope to the cell membrane (Morgan et al., 1968). Viral DNA is rapidly uncoated in the cytoplasm, from which it enters the

nucleus to direct virus specific RNA synthesis. After a rapid synthesis of virus specific macromolecules infectious virus particles are assembled and released from the cells (Watson, 1973).

The reproductive cycle depends on the nature of the host and the nature of the virus; the multiplicity of infection; temperature of incubation and the nutritional property of the medium (Roizman, 1978). At 37°C with 50 p.f.u./cell in HSV-1 infected HEp-2 cells the cycle lasts for 17 hrs (Roizman & Furlong, 1974) and in PRV infected BHK-21 cells at 50 p.f.u./cell, the cycle is shorter, 12 hrs (Tyler et al., 1974).

### 1.4.1. Viral RNA synthesis.

After infection the first step towards replication is the synthesis of virus specific RNA. Viral RNA is transcribed from the viral DNA in the nucleus (Wagner & Roizman, 1969a, 1969b; Roizman et al., 1970), and it follows the same mechanism that occurs in uninfected cells. Transcription is carried out by host RNA polymerase II (Preston & Newton, 1976; Costanzo et al., 1977; Ben-Zeer & Becker, 1977). No virus induced RNA polymerase activity has been detected (Lowe, 1978). A small amount of viral RNA synthesis, at times late in infection, is resistant to $\alpha$-amanitin, which may be due to synthesis by RNA polymerase III (Ben-Zeer et al., 1976) and the possibility of virus induced modification of the host enzyme is not ruled out.

#### 1.4.1.1. Characteristics of viral RNA : structure.

Posttranscriptional modification of viral RNA is almost

identical to that of cellular mRNA. Total nuclear transcripts are larger than polysome associated viral mRNA's (Stringer et al., 1977). Cytoplasmic viral mRNA has as a 5'-terminal "Cap" as is found in most eukaryotic mRNA's (Bartkoski & Roizman, 1976; 1978; Moss et al., 1978). Early viral transcripts have an internal methylated base in the form of $m^6A$, which is missing from late mRNA's (Bartkoski & Roizman, 1976; 1978). Another post-transcriptional modification is the polyadenylation of mRNA in the nucleus at the 3' end (Bachenheimer & Roizman, 1972; Ben-Porat et al., 1972). However viral mRNA lacking a poly(A) tail but having the same sequence as poly(A) containing RNA ,has been found on the polysomes (Stringer et al., 1977). Cytoplasmic poly(A) containing RNA represents most, if not all, of the viral RNA sequence present in the cytoplasm (Silverstein et al., 1976).

1.4.1.2. Characteristics of viral mRNA : synthesis.

The expression of herepesvirus genome during productive infection is temporaly regulated (Kozak & Roizman, 1974; Swanstrom & Wagner, 1974; Swanstrom et al., 1975). Immediate-early (IE) mRNA's are synthesized in cells very early after infection and also accumulate in infected cells when protein synthesis is inhibited from the beginning of the infection. Early mRNA's are synthesized after the initial viral protein synthesis in the infected cells but before viral DNA synthesis and late mRNA's are synthesized after viral DNA synthesis. Some IE mRNA's continue to appear on polysomes as functional mRNA's even late in infection but at a concentration different from the original concentration (Anderson et al., 1980).

Some late mRNA's detected before viral DNA replication also show difference in abundance (Stringer et al., 1977; Anderson et al., 1980; Kozak & Roizman, 1974). It is also found that some host poly(A) RNA is synthesized and associates with the polysomes late in infection (Stringer et al., 1977).

Kozak & Roizman (1974) found that the nuclear IE RNA of HSV-1 is homologous to 50% of the DNA whereas Cytoplasmic IE RNA hybridizes to 10% of the DNA. Late cytoplasmic RNA's are homologous to 43% of the DNA and possess no symmetrical transcripts whereas late nuclear transcripts are homologous to 50% of the DNA and possess symmetrical transcripts. Early after infection nuclear precursor and cytoplasmic poly(A) RNA's are essentially of the same size but late in infection nuclear precursors are larger than cytoplasmic poly(A) RNA's (Holland et al., 1979; Jones & Roizman, 1979).

Clements et al., (1977) found that the genetic complexity of the nuclear and the cytoplasmic IE RNA of HSV-1 is the same, possibly the use of different cell lines or the different hybridisation techniques used are responsible for the disparate results. Both nuclear and cytoplasmic IE RNA's are complementary to non-contiguous regions of the virus genome, which are primarily located at, or near, $TR_L/IR_L$ (terminal and inverted repeats flanking the L unique sequences) and $TR_S/IR_S$ (repeats flanking the S unique sequence) [Clements et al., 1977; Jones et al., 1977; Stringer et al., 1978]. Watson et al., (1979) have identified at least five IE mRNA's in HSV-1 infected cells. The genome location and the direction of transcription of these sequences have also been determined (Anderson et al., 1980;

Clements et al., 1979). A 4.2 kb species transcribed from the $TR_S/IR_S$ region with the 3' end distal to $U_S$ (Small unique sequence) codes for a polypeptide of molecular weight 175,000 and a 2.8 kb species from $TR_L/IR_L$ with the 3' end towards $U_L$ codes for a polypeptide of 120000 molecular weight. Of the three 1.8kb species, one transcribed from $U_S$ near $TR_S$ with the 3' end in the $U_S$ codes for a polypeptide of 68,000 molecular weight, a second one from $U_L$ near $IR_L$ with the 3' end in the $U_L$ codes for a polypeptide of 63,000 molecular weight and the third is transcribed from $IR_S$ region with the 3' end in $U_S$.

In the case of PRV late mRNA is complementary to 50% of the DNA and IE RNA to 10% of the genome. IE mRNA hybridizes to the terminal repeats and given a TR of $9.9 \times 10^6$ daltons (Ben-Porat et al., 1979) IE mRNA is transcribed from 7% of the total complexity of the viral genome (Feldman et al., 1979). Using short term labelling it is found that the restricted transcription is not due to degradation of a larger transcript (Feldman et al., 1979).

Early mRNA is transcribed from regions throughout the genome, but the sequence at or near the junction between $U_S$ and the repeats are particularly well represented. Late mRNA is also transcribed from the whole genome and the middle of $U_S$ is transcribed only late in infection (Feldman et al., 1979). Early and late mRNA's differ only in relative abundance of the transcription from different regions of the genome (Clements et. al., 1977) which explains why the early to late transition is not affected by viral DNA amplification. However, IE protein synthesis is a prerequisite for the IE to early transition and

this could be affected by a modification of the host RNA polymerase or by changing the template activity of the viral DNA. The regulatory role of IE polypeptides has been suggested by their modification and translocation to the nucleus (Pereira et al., 1977; Fenwick et al., 1978) and also by their DNA binding capacity in vitro (Hay & Hay, 1980).

Thus strict transcriptional control is observed in herpesvirus infected cells. It is possible that the controls might operate at the level of the selection of which transcribed sequences are transported to the cytoplasm as mRNA's.

### 1.4.2. Viral protein synthesis.

Viral mRNA's after being synthesized in the nucleus appear in the cytoplasm and are translated to give virus specific proteins (Sydiskis & Roizman, 1966; 1967). After infection there is an initial decline in protein synthesis due to disaggregation of host polysomes, followed by an increase during which viral proteins are synthesized and finally an irreversible decline. The gradual switch-over from host specific to virus specific protein synthesis is reflected in the formation of rapidly sedimenting virus specific polysomes (Sydiskis & Roizman, 1966; Ben-Porat et al., 1971).

Though herpesvirus does not cause complete shut down of host protein synthesis, virus specific proteins can be detected by using the following criteria : (a) changes in electrophoretic or kinetic profiles of the proteins synthesized after infection; (b) stimulation in the rate of synthesis after infection,and

(c) immunoprecipitation by virus specific antigens (Honess & Roizman, 1973). About 52 virus specific proteins have been detected in HSV-1 infected cells and a similar number is found in HSV-2 infected cells (Honess & Roizman 1973; Powell & Courtney 1975; Bookout & Levy, 1980). Analysis of the proteins of five herpesviruses - HSV-1, HSV-2, PRV ; BMV (Bovine Melalitis Virus) and EAV (Equine Abortion Virus) - shows diversity in the electrophoretic mobilities (Killington et al., 1977), indicating that the protein composition of herpesvirus is diverse.

1.4.2.1. Regulation of Protein Synthesis.

Protein synthesis in herpesvirus infected cells is highly controlled. Kinetically, virus specific proteins in HSV-1 infected cells have been differentiated into three groups - $\alpha$, $\beta$ and $\gamma$ (Honess & Roizman, 1974). Using metabolic inhibitors a model of sequential ordering and coordinate regulation of the synthesis of virus specific proteins has been proposed (Honess & Roizman 1974; 1975). $\alpha$-polypeptides synthesized immediately after infection are responsible for the switching-on of $\beta$-polypeptide synthesis which, in turn, shut off $\alpha$-polypeptide synthesis. A similar scheme has been proposed for $\beta$ and $\gamma$-polypeptide synthesis. $\alpha$- and $\beta$-polypeptides contain both structural and non-structural proteins but $\gamma$-polypeptides are all structural. IE polypeptides in PRV infected cells, analogous to the $\alpha$-polypeptides of HSV-1, do not include structural proteins (Ben-Porat et al., 1975).

In this regulatory process, it has been found that functional $\alpha$-polypeptides are required to induce $\beta$-polypeptide synthesis

and different α-polypeptides appear to be responsible for the regulation of the synthesis of different β-polypeptides (Pereira et al., 1977). At the same time functional β-polypeptides are required to inhibit α-polypeptide synthesis. Though γ-polypeptide synthesis coincides with viral DNA replication, some γ-polypeptides are synthesized before viral DNA synthesis but in reduced amounts (Powell et al., 1975; Honess & Watson, 1977). It has been proposed that transcription of progeny DNA is necessary for optimal γ-polypeptide synthesis (Roizman 1978).

Similar regulation of protein synthesis has been observed in HSV-2 infected cells (Powell & Courtney, 1975) and Murine Cytomegalovirus (MCMV) infected cells (Moon et al., 1979). In the case of PRV IE proteins synthesized after withdrawal of a Cycloheximide block imposed from the beginning of infection correspond to α-polypeptides (Ben-Porat et al., 1975). So it is possible that this cascade regulation of protein synthesis is a common property of the herpesvirus.

1.4.2 2. Modification and translocation of viral proteins.

Modification of herpesvirus specific proteins does not involve rapid posttranslational cleavage as observed in picornavirus infected cells (Honess & Roizman, 1973). Phosphorylation of a large number of structural and non-structural proteins of HSV (Pereira et al., 1977; Marsden et al., 1978; Bookout & Levy 1980) and of structural proteins of PRV (Stevely 1975) has been reported. Glycosylated polypeptides have been found in purified Herpes Simplex virions (Roizman, 1978). Kaplan & Ben-Porat (1976)

have shown a non-structural sulfated glycoprotein in PRV infected RK-cells.

Translocation of structural proteins to the nucleus (Spear & Roizman, 1968; Ben-Porat et al., 1969) is related to capsid formation. Translocation occurs through the nuclear membrane and is likely to be determined by the affinity of the polypeptides for various constituents in the nucleus (Fenwick et al., 1978). Almost all the α-polypeptides and a few β-polypeptides from HSV-1 and HSV-2 infected cells are found in the nucleus (Bookout & Levy 1980; Pereira et al., 1977; Cabral et al. 1980) and most of them have DNA binding properties (Bookout & Levy, 1980; Fenwick et al., 1978; Hay & Hay, 1980). Since α-polypeptides control translation of β-polypeptides and β-polypeptides can, in turn, regulate the translation of both α- and γ-polypeptides, so the DNA binding capacity of α- and some β-polypeptides could have a function in transcriptional control. VP 175 is found to be arranged in the nucleus in a marginated pattern in HSV-1 infected cells (Cabral et al., 1980). Though the significance of this finding in HSV-1 infected cells is unknown, a similar binding of H1 parvovirus antigen to peripheral nucleolar chromatin has been suggested to be responsible for early morphological disruption and eventual destruction of the nucleus (Singer 1976). Nucleolar disaggregation in HSV-1 infected cells has also been documented (Schwartz & Roizman, 1969).

### 1.4.3. Viral DNA Synthesis.

Protein synthesis in the infected cell is necessary to initiate viral DNA synthesis, but once DNA synthesis is established

inhibition of protein synthesis has little effect on it (Cheng et al., 1975). New DNA polymerase activities have been reported in cells infected with HSV, PRV, MCMC, EAV (see Allen et al., 1977) Vericella Zoster Virus (VZV) [Miller & Rapp, 1977] and EBV (Muller et al., 1977). After infection the activity of cellular DNA polymerase $\alpha$ declines, coinciding with the inhibition of cellular DNA synthesis (Muller et al., 1977). HSV-DNA synthesis appears to be catalyzed by the virus coded DNA polymerase (Muller & Zahn, 1979), since the change in the activity of the enzyme parallels viral DNA synthesis. HSV-DNA synthesis is suggested to be promoted by an RNA initiator which is removed from the mature DNA by RNase H (Muller et al., 1980). PRV also uses its own DNA polymerase for DNA synthesis (Halliburto & Andrew, 1976). PRV DNA replication occurs in the form of a concatamer (Jean & Ben-Porat 1976; Ben-Porat & Veach, 1980; Ladin et al., 1980). Maturation is accomplished by a capsid or non-capsid protein(s) (Ladin et al. 1980). A few other DNA related enzymes have been described in herpesvirus infected cells e.g. Thymidine kinase and alkaline DNase, but their importance to viral DNA synthesis is not clear (Ben-Porat & Kaplan, 1973).

### 1.4.4. Assembly of the virion and release from the cells.

After virus specific macromolecules are synthesized core and capsid are assembled in the nucleus (Roizman & Furlong, 1974), but the actual flow of events leading to assembly is not clear. Perdue et al., (1976) have suggested that DNA is inserted into capsid without a preformed core. Ladin et al., (1980) have

also produced evidence that the capsid proteins are assembled first into an empty shell, into which DNA is encapsidated. Though the envelope has been thought to be acquired by budding through the cytoplasmic membrane, there are good reasons to believe that the membrane is acquired by budding through the inner nuclear membrane (Watson, 1973; Rodriguez & Dubois-Dalcq, 1978). Release could be through budding into a channel which occur via reverse phagocytosis (Morgan et al., 1959) or even by cell lysis (Watson et al., 1964).

## 1.5. Alterations in cell structure and function.

The eventual consequence of productive infection by HSV is cell death (Roizman, 1978). Though the actual cause of cell death is obscure, the symptoms involved are a drastic reduction or even ceassation of host DNA, RNA and protein synthesis and changes in the membrane structure.

### 1.5.1. Structural alterations.

The infected cell nucleus exhibits gross changes in the structure of the nucleolus, the distribution of chromatin and the composition of the nuclear membrane. The nucleolus is often displaced towards the nuclear membrane (Schwartz & Roizman, 1969). A diagnostic feature is the displacement and condensation of chromatin at the nuclear membrane early in infection There is substantial chromosome breakage in the infected cells. Cytoplasm shows little that can be related to herpesvirus infection, except that there is an increase in the length and

frequency of the number of microvilli (see Roizman, 1978). Changes in transmembrane potential observed in HSV infected cells may reflect leakiness of the infected cell membrane (Fritz & Nahmias, 1972).

1.5.2. Effects on cellular RNA synthesis.

Infection of cells with herpesviruses leads to a gradual decrease in the rate of RNA synthesis, as revealed by low levels of $^{3}$H - uridine incorporation into acid precipitable material (Ben-Porat & Kaplan, 1973; Roizman et al., 1970). The decrease in the RNA synthesis involves both a reduction in the synthesis of 45 S precursor RNA as well as an abortive processing to 28 S and 18 S rRNA (Roizman et al., 1970).

The effect of herpesvirus infection on host mRNA synthesis is not well characterized. However, Stringer et al.,(1977) have shown the presence of some host mRNA's on polysomes even late in infection. Their functional ability is reflected by the presence of newly synthesized host proteins late in infection (Honess & Roizman, 1973; Powell & Courtney, 1975). In the case of PRV host mRNA's having a low turn-over rate are synthesized at times late in infection. But they are not functional though considerable amounts are found in the cytoplasm of the infected cells (Rakusanova et al., 1972). So it appears that in herpesvirus infected cells functional host mRNA synthesis is not inhibited completely.

Few data on the mechanism of inhibition of host RNA synthesis and processing are available. Sasaki et al., (1974) have noted the presence of an inhibitor of nucleolar RNA polymerase in HSV-infected cells but its role is unknown.

Lowe (1978) has found unaltered levels of RNA polymerase II activities in HSV-1 infected BHK cells even at 8 hrs post-infection. An alternative to the presence of an inhibitor is that an increased concentration of HSV-1 DNA in the nucleus with preferential affinity of RNA polymerase II for the viral genome may lead to reduced host RNA synthesis.

### 1.5.3. Effects on cellular protein synthesis.

In HSV infected cells the inhibition of host protein synthesis may first be detected at 2 hrs post-infection. The time of complete inhibition depends on the nature of the virus and the nature of the host cells. Cessation of host protein synthesis is accompanied by a decrease in the number of polyribosomes and a concurrent drop in amino acid incorporation (Honess & Roizman, 1973). The mechanism of inhibition of host protein synthesis is not clear Inhibition of RNA synthesis could be a contributing factor. A few possible mechanisms of inhibition of host protein synthesis following infection will be discussed later in the Introduction (1.7).

### 1.5.4. Effects on cellular DNA synthesis.

Inhibition of host DNA synthesis has been observed in cells infected with PRV (Kaplan & Ben-Porat 1963), HSV-1 (Roizman & Roane, 1964). EAV (O'Callaghan *et al.*, 1968) and EBV (Nonoyama & Pagano, 1972; Gergely *et al.* 1971). Kaplan (1973) has suggested that inhibition of host protein synthesis contributes to the inhibition of host DNA synthesis. Another possible explanation is that virus specific proteins may bind to cellular

DNA (1.4.2.2) and thus prevent host DNA replication.

## 1.6. Protein synthesis in eukaryotes.

In eukaryotic cells transcription and translation occur in distinct cellular compartments, which require proper transport, stabilization and alignment of the macromolecules taking part in protein synthesis. Moreover, the involvement of a large number of ribosomal proteins, initiation factors and other components required for protein synthesis makes this a very complex process.

### 1.6.1. Mechanism of translation.

The mechanism of protein synthesis involves three different processes - initiation, elongation and termination.

#### 1.6.1.1. Initiation.

Initiation covers the events of protein biosynthesis which lead to the formation of the 80 S initiation complex consisting of one 80 S ribosome and one mol of initiator tRNA (Met. $tRNA_f$) bound to the ribosomal P site and mRNA (see Bielka & Stahl 1978). Met $tRNA_f$ forms part of a ternary complex consisting of equimolar amounts of eIF-2 (initiation factor 2), GTP and the tRNA before being able to bind to a 40 S ribosomal subunit to form the quarternary 40 S initiation complex. eIF-1 and eIF-3 stabilize this complex and contribute to the subsequent binding of mRNA. mRNA binding depends on eIF-4A, -4B, -4C and -4D together with ATP hydrolysis. This 40 S initiation complex with mRNA associates with the 60 S ribosomal subunit in the presence of eIF-5 and GTP to yield the 80 S initiation complex. GTP

hydrolysis during 60 S subunit binding releases all initiation factors.

1.6.1.2. Elongation.

The elongation process in protein biosynthesis has been reviewed by Weissbach & Ochoa (1976) and Clark (1980). Binding of aminoacyl tRNA to the A site is preceeded by the formation of a ternary complex consisting of 1 mol each of aminoacyl tRNA, elongation factor, EFI and GTP. Binding involves GTP hydrolysis and the GDP-EFI complex released can be recycled. Peptide formation is catalyzed by peptidyl transferase. This is followed by translocation of peptidyl tRNA from the A site to the P site and this process requires EF-2 and GTP hydrolysis. The number of ribosomes in the polysome is determined by the length of the mRNA, the availability of ribosomes and tRNAs, and the initiation efficiency of the mRNA. Under saturating conditions, one ribosome can bind to 40-100 nucleotides (Legon, 1976).

1.6.1.3 Termination.

Termination involves the release of the completed peptide chain from the mRNA-ribosome complex and this process requires release factor (RF) and GTP. RF binds to ribosome when the termination codon is in the A site. It is interesting that here codon recognition is by protein, not by tRNA. GTP dependent binding of RF to the A site seems to invoke esterase activity at the peptidyl transferase centre, resulting in the hydrolysis of peptidyl tRNA. After GTP hydrolysis RF is released, the

ribosome dissociates into subunits and initiation can occur again (Caskey, 1980).

1.6.2. Recognition during initiation.

In prokaryotic systems there is an interaction between nucleotide sequence near the 3' terminus of 16 S rRNA and purine rich sequences preceeding the AUG codon. Moreover, 1F-2, 1F-3 and ribosomal proteins, S1 and S12 can produce changes in the rRNA or mRNA configurations and by affecting their interactions can determine the affinity of ribosomes for a given cistron (Revel, 1977). It is possible that in eukaryotes such RNA-RNA and RNA-protein interactions play a role in mRNA recognition by ribosomes.

Though capped mRNA's bind more readily to ribosomes (Canaani et al., 1976) uncapped mRNA's are also found to be translated efficiently in the cell-free systems. After analysis of the nucleotide sequences between cap and the AUG codon, it has been proposed (Steitz & Jakes, 1975) that in eukaryotes, as in bacteria (Shine & Dalgarno, 1975), a short sequence of mRNA near the 5' end of AUG base pairs with a complementary region near the 3' end of 18 S rRNA. Both (1979) has shown that such interaction is possible on either side of the AUG codon but that the required codon sequences occur more consistently on the 3' end of AUG. Such interactions may increase the rate and stability of mRNA binding to ribosomes and thus help initiation.

Kozak (1978, 1979) has suggested a scanning mechanism in which the 40 S subunit binds to the 5' end of the mRNA and subsequently migrates to the interior of the chain, stopping

when the first AUG codon is encountered. Base-pairing within the 5' region of mRNA could bring cap and AUG codon into optimal alignment, thus helping initiation (Ahlquist et al., 1979). The high degree of secondary structure which has been demonstrated in many mRNA's (Van et al., 1976; Brentani et al., 1977; Heindall et al., 1978)may facilitate selection of the correct 5' terminal AUG codon, because extensive migration of the 40 S subunit occurs in mRNA having no secondary structure (Kozak, 1980). Moreover, differences in the affinities for initiation factors (1.7.3) and differences in the secondary structure of the mRNA's may contribute to variation in the translation efficiency among mRNA's.

### 1.6.3. Regulation of Protein biosynthesis.

Cells could regulate protein biosynthesis in a number of ways, for example by changes in the level of initiator tRNA, by regulating the number of active 40 S ribosomal subunits or by sequestering the mRNA in cytoplasmic RNP (Gross et al., 1973; Rosbash & Ford, 1974).

It is important to point out that different mRNA's differ in their rate of polypeptide chain initiation. A kinetic treatment of initiation and elongation allowed Lodish (1974) to propose that any non-specific reduction in the rate of polypeptide chain initiation at, or before binding mRNA will result in preferential inhibition of translation of mRNA's with lower rate constants for chain initiation. So with changes in the overall rate of chain initiation, translation of different mRNA's would appear to be affected differently. The theory also suggests that any increase in initiation will favour the weakest binding

mRNA while an increase in elongation will favour the strongest binding mRNA.

In several systems the amount of protein synthesized is not directly related to the amount of mRNA present. For example, in rabbit reticulocytes the $\alpha$ and the $\beta$ globin chains are present in the ratio of 1:1 but $\alpha$ mRNA is present in excess of $\beta$-mRNA. Since the elongation rate in both cases is the same the differences in translation must be due to differences in the initiation rate (Lodish, 1971). It is not clear whether these differences can be attributed to differences in mRNA structure, as in the case of RNA phage RNA (Revel, 1977). However, addition of eIF-4A and eIF-4B change $\alpha$ globin mRNA translation relative to $\beta$-mRNA without increasing the overall rate of translation. This effect could not be due to an increase in the concentration of the overall rate limiting factor but must influence the efficiency of mRNA in initiation complex formation (Kabat & Chappell 1977). Reviewing a variety of results Revel & Groner (1978) propose, contrary to Lodish, that mRNA's do not have unique initiation constants. They claim that the efficiency of mRNA recognition by ribosomal machinery is due to the overall kinetic parameters of the initiation system.

In the rabbit reticulocyte system it has been found that deprivation of haem results in a decline in the rate of peptide chain initiation and this effect is due to the phosphorylation of the small subunit of eIF-2 by a cyclic AMP independent kinase (see Farrell et al., 1977). It is possible that phosphorylation prevents interaction of eIF-2 with one or more cofactors such as eIF-2 stimulatory protein (ESP) [deHaro & Ochoa, 1978, deHaro et al., 1979], stabilisation factor (Ranu & London

1979), co-eIF-2A (Dasgupta et al., 1978) and co-eIF-2B (Das et al., 1979) which are necessary for normal functioning. It seems likely that control of the rate of initiation is a very complex phenomenon. It is possible that more than one step in the initiation process is subjected to control and also that several different control systems may work together. For instance, ds RNA in the cell free extracts can cause the phosphorylation of eIF-2$\alpha$ (Leven & London, 1978) as well as induce an endonucleolytic activity which degrades mRNA (Clemens & Williams 1978; Williams et al., 1979) [see 1.8] . Such control mechanisms, though mainly examined in the context of interferon, may be part of a more general control system limiting the rate of polypeptide synthesis.

## 1.7. Inhibition of protein synthesis in virus infected cells.

Inhibition of cellular protein synthesis and selective translation of animal virus mRNA's during the course of productive infection have been observed in cells infected with adenovirus (Levine & Ginsberg, 1967), herpesvirus (Sydiskis & Roizman, 1966), vaccinia virus (Hanafusa, 1960) mengovirus (Baltimore et al., 1963), poliovirus (Penman & Sumners, 1965) and VSV (vesicular somatitis virus) [Yaoi et al., 1970; Wartz & Younger, 1972] . In the case of herpesvirus, in some systems, a decrease in protein synthesis which occurs early in infection has been noted and is due to a decrease in the synthesis of cellular proteins. This is followed by an increase in the overall rate of synthesis as virus specific proteins are being made and finally there is an irreversible decrease (Ejercito et al., 1968). With only a

few exceptions little is known about the mechanism of inhibition. The following are possibilities that could explain inhibition of protein synthesis after virus infection.

### 1.7.1. Inhibition by RNA degradation.

In poliovirus infected cells cellular mRNA's released from the decaying polyribosomes are unable to reenter the ribosomes (Willems & Penman, 1968), but can still be isolated and translated in a cell free system (Abreu & Lucas-Lenard, 1976). No host mRNA degradation has been observed in cells infected with vaccinia virus (Rosemond-Hornbeak & Moss, 1975), VSV (Nishioka & Silverstein, 1977) or picornavirus (Willems & Penman 1968; Colby et al., 1974; Fernandez-Munoz & Darnell, 1976). On the other hand in HSV-1 infected Friend erythroleukemia cells considerable degradation of host mRNA occurs (Nishioka & Silverstein 1977; 1978a; 1978b) and degradation of host mRNA's has also been observed in polyoma transformed cells infected with HSV-1 (Pizer & Beard, 1976). It is further suggested that an early viral protein, possibly an $\alpha$ polypeptide, is required in HSV-1 infected FL cells for the degradation of host mRNA to occur (Nishioka & Silverstein, 1978b).

### 1.7.2. Inhibition by membrane leakiness and hypertonicity.

In poliovirus, VSV and reovirus infected cells, the mRNA's translated after exposure of the cells to hypertonic medium are predominantly viral (Nuss et al., 1975). A number of susceptible cell lines show membrane leakiness after viral infection. These include mouse cells infected with EMC virus (Farnham &

Epstein 1965), HeLa cells infected with poliovirus (Carrasco & Smith, 1976), BHK cells infected with Semliki Forest virus (SFV), mouse cells 3T6 infected with mengovirus and CV1 cells infected with SV-40 (Contreras & Carrasco, 1979). Based on both *in vivo* and *in vitro* experimental findings it has been suggested that changes in membrane permeability due to binding of a picornavirus coat protein(s) cause changes in the intracellular ion concentration (high $Na^+$ ions) which favour viral mRNA translation but inhibit host protein synthesis (Carrasco, 1977). Changes in membrane leakiness with distortion of monovalent ion gradients, loss of ATP and some other components necessary for translation have been observed in EAT cells infected with mengovirus (Egberts *et al.*, 1978), in BHK cells infected with Sindbis virus (Garry *et al.*, 1979) and in Sendai virus infected HeLa cells (Fuchs *et al.*, 1978). But in the case of HSV-2 infected vero cells inhibition of both host and viral protein synthesis occurs at high salt concentrations (Fenwick & Walker 1978). Again in PRV infected HeLa cells host proteins and late viral proteins are equally sensitive to high salt concentrations (Stevely & McGrath, 1978).

### 1.7.3. Inhibition due to changes in initiation factors.

It has been suggested that in EMC virus infected cells direct competition between host and viral mRNA's for eIF-4B is responsible for inhibition of host protein synthesis (Golini *et al.*, 1976). In picornavirus infected cells inhibition of host protein synthesis occurs at the level of initiation (Leibowitz & Penman, 1971). Using a cell free translation system

it appears that discrimination is caused by initiation factors. But which initiation factor is the initial target of virus infection is controversial. While inactivation of eIF-4B has been demonstrated in poliovirus infected cells (Rose et al., 1978; Padilla et al., 1978) eIF-2 is the target in EMC virus infected cells (Kempfer et al., 1978). There are also reports that the effects of infection could be at the level of eIF-4A (Blair et al., 1977). Again, the cap structure at the 5' end of most eukaryotic mRNA's facilitates the initiation of translation (Shatkin, 1976). Poliovirus induced inhibition of translation of capped mRNA can be reversed by a protein (mol. wt. 24,000) found in association with eIF-3 and eIF-4B of rabbit reticulocyte ribosomes (Rose et al., 1978; Sonenberg et al., 1980) and this protein is identical to cap binding protein (CBP). CBP is essential for the translation of capped mRNA (Sonenberg et al., 1980). It appears that poliovirus infection leads to the inactivation of some crucial property of CBP, so that capped host mRNA translation is inhibited but uncapped poliovirus mRNA is translated (Rose et al., 1978; Sonenberg et al., 1980; Trachsel et al., 1980). It is also found that in reovirus infected mouse L cells there is cap independent translation possibly as a result of inhibition of CBP as in poliovirus infected HeLa cells (Skup & Millward, 1980). No information is available on the effects of herpesvirus infection on initiation factors.

### 1.7.4. Inhibition by general effects on initiation.

In HSV-1 infected cells inhibition of protein synthesis is

accompanied by a decrease in functional polyribosomes. Inhibition appears to occur at the level of peptide chain initiation (Silverstein & Engelhardt, 1979). Nonetheless, it has been found that host mRNA's enters polyribosomes late in infection even though host protein synthesis is severely curtailed (Stringer et al., 1977). It has been claimed that some translational control process selectively represses host protein synthesis. If true, this could be the result of modification of the mRNA at some initiation site, perhaps at the site for ribosomal binding or initiation of translation, or it could be due to binding of some virus specific proteins to mRNA reducing its translatability. Further possibilities include destruction of tRNA molecules or modification of the ribosomal subunits or a combination of several of the above. Phosphorylation of ribosomal subunits in vaccinia virus infected cells has been reported (Kaerlin & Horak, 1976) though its relation to inhibition of protein synthesis is not clear. In haem-depleted reticulocyte lysate, the inhibition of protein synthesis is accompanied by phosphorylation of ribosomal proteins (see Farrell et al., 1977). Phosphorylation of ribosomal protein has been observed in HSV-2 infected vero cells and it appears that phosphorylation is not responsible for suppression of host protein synthesis (Fenwick & Walker, 1979). Phosphorylation of ribosomal proteins S6 and S16/S18 in PRV infected BHK-21 cells and S6 in HSV-1 infected BHK-21 cells has also been observed (I. Kennedy, Personal Communication). Early after infection of AGMK cells with SV-40 there is an alteration to the ribosomes, probably involving the induction of a new initiation factor and this

allows regulation of the species of mRNA to be translated (Nakajima & Oda 1975).

1.7.5. Inhibition by structural component of the virus.

Using metabolic inhibitors and also u.v. irradiated virus it has been shown that macromolecule synthesis is not required to inhibit protein synthesis in vaccinia virus infected cells (Rosemond-Hornbeak & Moss, 1975). In VSV infected cells inhibition of host RNA and protein synthesis occurs even when highly irradiated virus is used (Huang & Wagner, 1965; Wagner & Huang, 1966; Yaoi et al., 1970) and it appears that inhibition is due to u.v. resistant component(s) of the virus. Selective inhibition of host macromolecule synthesis occurs in cells infected with Frog Virus 3 inactivated by heat, u.v. irradiation and $\gamma$-irradiation (Goorha & Granoff, 1976; Raghow & Granoff, 1979). There are also reports that in cells infected with VSV (Baxt & Bablanian, 1976), Vaccinia virus (Esteban & Metz, 1973), reovirus (Shaw & Cox, 1973) and SFV (Wengler & Wengler, 1976) inhibition of host protein synthesis is caused by a component(s) of the infecting virus. Intact and heat disrupted poliovirus causes greater inhibition than an equivalent amount of virus RNA in reticulocyte lysate and inhibition is independent of viral protein synthesis (Racereskis et al., 1976). The formatio of 40 S initiation complex is inhibited in cytoplasmic extracts derived from cells infected with vaccinia virus in the presence of cordycepin and also in reticulocyte lysates incubated with purified cores (Person et al., 1980) indicating that inhibition of host protein synthesis is mediated by a virion associated

component(s). Loss of ability to disaggregate polyribosomes using u.v. irradiated HSV-1 has been reported (Sydiskis & Roizman, 1967) and it has been suggested that this inability could be due to the inactivation of sensitive component(s) required for inhibition when a high u.v. dose is used. Ben-Porat et al., (1971) have suggested that breakdown of host polyribosomes in PRV infected RK cells requires the synthesis of infected cell proteins. However, in HSV-2 infected vero cells inhibition occurs although no virus specific proteins are synthesized. In these experiments, infection of whole cells with u.v. irradiated virus and infection of cytoplasts provided the evidence for the lack of requirement for viral protein synthesis (Fenwick & Walker, 1978).

## 1.8. The Interferon system.

Interferons are polypeptides which are mainly glycosylated and are secreted by the virus infected cells. The interferons promote the establishment of an antiviral state in infected cells (Finter, 1973). Treatment of animal cells in culture with interferon reduces their ability to support virus multiplication.

Recent studies have shed some light on the mechanisms involved in interferon action. Interferon induced enzymatic activities and their role in the antiviral state have been reviewed by Baglioni (1979). It has been found that exposure of cells to interferon results in an increase in the activity of an enzyme, 2', 5' oligo nucleotide polymerase (2,5 oligo A polymerase) which synthesizes a series of oligo nucleotides, pppA $(2'p5'A)_n$ from ATP (Kerr & Brown, 1978). This 2,5 oligo A

polymerase activity has been studied by Baglioni et al., (1979) and Minks et al., (1979). Baglioni et al., (1979) have observed a correlation between the induction of 2,5 oligo A polymerase and the inhibition of viral RNA synthesis in EMC virus infected HeLa cells. A similar observation has been made on VSV replication in chick embryo cells (see Baglioni,1979). In interferon treated, VSV- and MLV (Murine Leukemia Virus) - infected cells 2,5 oligo A polymerase binds to the virion core. The enzyme is inactive in the virion but following infection and uncoating it becomes active and causes destruction of the virion (Wallace & Revel, 1980). Association between cellular and viral components as a part of interferon induced antiviral state has also been observed in that uncoated reoviruses isolated from interferon treated infected cells, inspite of having ds RNA and coat proteins, are unable to synthesize full size mRNA's (Galster & Lengyl, 1976).

Infection of interferon treated cells with mengovirus increase RNase activity in a subcellular fraction known to contain viral replicating complex (see Baglioni, 1979). It has been observed that pppA $(2'p5'A)_n$ synthesized by 2,5 oligo A polymerase activates an endonuclease which indiscriminately cleaves both cellular and viral mRNA's, free or polyribosomes bound (Clemens & Williams 1978, Baglioni et al., 1978). But it has been suggested that different mRNA;s may be cleaved at different rates (Ratner et al., 1977, Epstein & Samuel, 1978). It seems probable that this endonuclease cleaves at specific nucleotides as does a restriction endonuclease.

Though accumulation of viral mRNA is inhibited in interferon treated infected cells, it is not yet clear whether this inhibition is due to an impairment of transcription, translation or cleavage of the viral transcripts. There is a postulate that interferon treatment decreases the probability of initiating viral mRNA transcription but premature termination or even degradation has not been ruled out (Marcus & Sekellick, 1978). Nelson & Baglioni (1979) have demonstrated with cell free extracts that in interferon treated cells there are enzyme activities which can "shave" single-stranded nascent viral mRNA from the double-stranded core of replicative intermediate, but this has not been observed in intact cells.

It has been known that both synthetic and viral ds RNA's induce synthesis and secretion of interferon in animal cells (see Torrence & De Clercq 1977). Farrell et al., (1977) have found that ds RNA can inhibit initiation in reticulocyte lysates by phosphorylation of eIF-2$\alpha$ by a C yclic AMP independent kinase which is antigenically different from the protein kinase found after haeme depletion in reticulocyte lysates (1.6.3). This kinase mediates its effects via phosphorylation of a 65,000 mol. wt. ribosome associated protein (Ohtsuki et al., 1980). But activation of this enzyme would lead to general inhibition since all proteins are initiated via eIF-2. Probably activation of kinase by ds RNA represents a pathway of inhibition of virus replication different from that of 2',5' oligo A polymerase/ endonuclease (Farrell et al., 1978). If the latter enzymes fail to act, the kinase may block protein synthesis before structural proteins are produced in sufficient amounts for virus assembly.

A further suggestion has been made that discrimination between host and viral specific protein synthesis in interferon treated infected cells could be due to tailoring of tRNA's for host mRNA (Falcoff et al., 1976; Zilberstein et al., 1976). This sequestering of tRNA could limit its availability for certain mRNA's.

## 1.9. Aim of the Project

Pseudorabies virus (PRV) Pig Herpesvirus 1 causes inhibition of host protein synthesis after infection. Compared to other herpesviruses there is little published work on the mechanisms behind the aspect of PRV infection. In this project a number of aspects of protein synthesis in PRV infected cells were studied in an attempt to explain the mechanism by which the virus inhibits protein synthesis. These aspects are as follows :

A. Though PRV is known to have a wide cell culture host range, it was decided to investigate whether the virus caused a similar inhibition of protein synthesis in three different cell lines - HeLa, C13 and BS-C-1.

B. Hypertonic conditions of the medium do not offer any selective advantage to the synthesis of either HSV-2 specific proteins (Fenwick & Walker, 1978) or PRV specific proteins at times late in infection (Stevely & McGrath, 1978). Again in vitro translation studies do not show any selective advantage of late PRV mRNA translation at elevated $k^+$ concentrations (McGrath

& Stevely, 1980). In the case of these viruses inhibition of protein synthesis occurs early after infection. So studies were carried out to investigate whether Immediate-early mRNA have any selective advantage in hypertonic conditions, both in vivo and in vitro.

C. There are reports that some viruses including HSV-2 cause inhibition of host protein synthesis by some component(s) of the infecting virus particles, that is without the synthesis of any new virus protein (1.7.5). Here three different types of experiments were carried out to study the effects of PRV particles on host protein synthesis.

(a) U.V. and heat inactivated viruses were used to study their inhibitory effects on RNA and protein synthesis.

(b) Host polyribosome disaggregation was studied in cells infected in the presence of inhibitors of protein synthesis as well as in cells infected with inactivated PRV.

(c) Particle effects were also studied by adding normal as well as inactivated PRV to cell-free translation systems.

D. Host mRNA degradation has been reported in Friend erythroleukemia cells infected with HSV-1 (1.7.1). Investigations were carried out to study mRNase activity in PRV infected HeLa cells.

## Chapter 2. Isolation and Characterisation of Poly(A) containing RNA.

### 2.1. Introduction

In growing cells the active mRNA's are bound to the polyribosomes and so the isolation of polyribosomes provides a convenient source of translateable mRNA's. In herpes-virus infected cells, at times late in infection polyribosomal RNA's are mainly viral (Stringer et al., 1977). Early after infection Immediate-early (IE) mRNA's are found in the infected cells. Under normal circumstances the concentration of IE mRNA is very small. But if protein synthesis is inhibited in the infected cells during infection, IE mRNA accumulates in a greater abundance and becomes bound to polyribosomes when the inhibitor of protein biosynthesis is removed (Rakusanova et al., 1971). So the isolation of polyribosomal RNA from HeLa cells infected for 5 hrs in the presence and in the absence of Cycloheximide makes it possible to isolate bot IE and late viral mRNA's. Since cytoplasmic host and viral mRNA's are polyadenylated at the 3' end (Rakusanova et al., 1972; Bachenheimer & Roizman, 1972; Brawerman, 1976), isolation of poly(A) containing RNA from infected and mock-infected cells allows isolation of viral and host mRNA's respectively.

### 2.2. Isolation of polyribosomes.

Polyribosomes were isolated from HeLa cells, mock-infected or infected with PRV in the presence or in the absence of cycloheximide as described in Materials and Methods. The use of heparin during the preparation inhibited RNase activities, giving a better yield. However, given the variation in cell growth which does occur from time to time, the yield varied from 40-60 $A_{260nm}$ units per 1000 x $10^6$ cells.

The typical profiles of mock-infected and infected cell polyribosomes on sucrose gradients are shown in Fig. 2.2.1.

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

### 2.3.1. Extraction of polyribosomal RNA.

The polyribosomal fractions from sucrose gradients were collected and the polyribosomes were pelleted by high speed centrifugation. The RNA was extracted from the pellets by phenol: chloroform:isoamylalcohol, using a pH 9.0 extraction buffer, as described in Materials and Methods. From the polyribosomal RNA, poly(A) containing RNA was separated by affinity chromatography on oligo [dT] cellulose columns.

### 2.3.2. Affinity Chromatography.

Poly(A) containing RNA's can be isolated by virtue of their affinity for oligo-[dT]- cellulose (Aviv & Leder, 1972; Edmonds & Caramela, 1969) or poly(U)-sepharose (Adesnik *et al.*, 1972; Lindberg & Persson, 1972). Though a greater and purer yield of poly(A) containing RNA is obtained with poly(U)-sepharose chromatography (Bishop *et al.*, 1974; Vass, 1975) the presence of formamide and SDS in the elution buffer makes this technique less attractive. These denaturing agents are very difficult to remove from the final preparation without losing mRNA, whose concentration is already very small and moreover, any trace amount of these agents inhibits *in vitro* translation studies. Thus the oligo-[dT]- cellulose chromatographic technique was used to isolate poly(A) containing RNA

After dissolving the polyribosomal RNA in loading buffer the poly(A) containing RNA was bound to oligo [dT] cellulose by passing the preparation through a preequilibrated column, as described

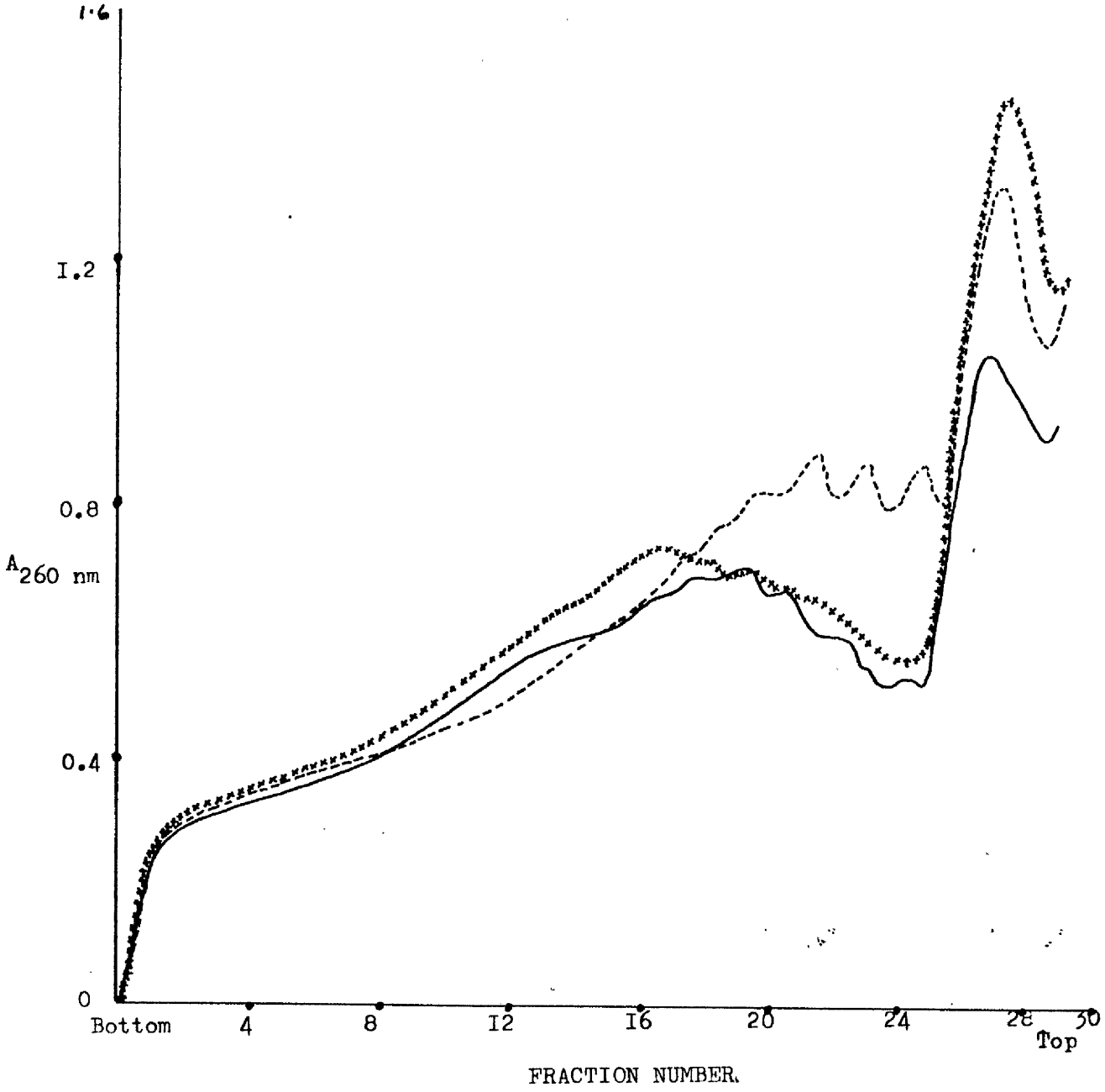
Fig. 2.2.1. Polyribosome profiles in mock-infected and PRV infected HeLa cells.

Confluent monolayers of HeLa cells were mock-infected or infected with PRV at 50 p.f.u./cell in the presence and in the absence of Cycloheximide (50µg/ml). At 5 hrs post infection Cycloheximide treated cells were washed to remove Cycloheximide. After 15 min incubation in normal medium, cells were harvested, homogenized and the cytoplasmic extracts were layered on 15 -30% (w/v) sucrose gradients in RSB. The gradients were centrifuged at 27,000 r.p.m. for 110 min in 4°C in the Beckman SW27 rotor and analysed, thereafter, using a Gilford 2000 recording spectrophotometer which monitored absorbance at 260nm.

———————— Mock-infected cell polyribosomes.

----------Immediate-early polyribosomes (PRV infected)

xxxxxxxxxxxxxxxx Late polyribosomes (PRV infected).


1·6
I.2
0.8
$A_{260}$ nm
0.4
0
Bottom
4
8
I2
I6
20
24
28
30
Top
FRACTION NUMBER.

in Materials and Methods. Loosely bound RNA's, mostly non-poly(A) containing ribosomal RNA's, were washed off by intermediate buffer. Bound poly(A) containing RNA was eluted by elution buffer of low ionic strength. Reprecipitation with ethanol removed traces of SDS present in the preparation. The mRNA activity of the preparation was studied by translation in the MDL system (see Chapter 3).

## 2.4. RNA-DNA hybridisation.

Poly(A) containing RNA was hybridized to PRV DNA bound to nitrocellulose filter discs to determine the presence of virus specified RNA. HeLa cells, mock-infected or infected with PRV to prepare IE mRNA, were labelled with inorganic $^{32}$P-phosphate (5mCi/burler) in phosphate free medium for 3 hrs. Two burlers of mock-infected and infected cells were labelled and three burlers of unlabelled cells of each type were added. This treatment allowed the isolation of a reasonable amount of labelled poly(A) containing RNA.

Poly(A) containing RNA was dissolved in 0.1 x SSC and aliquots were hybridized to PRV DNA bound to nitro-cellulose filter discs, as described in Materials and Methods. The data (Table 2.4.1) shows the presence of virus specified transcripts in the Immediate-early mRNA preparation.

## 2.5. Discussion.

Extraction of polyribosomal RNA from virus infected cells and isolation of poly(A) containing RNA allows a convenient method of obtaining viral mRNA's. In PRV infected cells, at

Table 2.4.1.

Hybridisation of poly(A) containing RNA from mock-infected and PRV infected (IE) cells to PRV DNA bound to nitrocellulose filters.

| Poly(A) containing RNA | Input | | Bound | % |
|---|---|---|---|---|
| | µg | cpm | cpm | Bound |
| Mock-infected HeLa | 0.5 | 13,000 | 140 | 1.07 |
| IE RNA | 0.5 | 4,000 | 260 | 6.5 |
| IE RNA | 1.0 | 8,000 | 540 | 6.75 |

Each of the filter discs contained 5µg PRV DNA.

times late in infection, the polyribosomal poly(A) containing RNA's are mostly viral. In this laboratory it has been shown by McGrath (1978) that at 5 hrs post-infection at least 50% of the transcripts present in the infected cell polysomes are viral. For the IE preparations made, the proportion of viral transcripts is at least 5%. Since the hybridisation does not reach the saturation limit, the proportion of the virus genome represented in the transcripts cannot be estimated. The preparation of IE mRNA clearly contains a large number of host mRNA's (see also in vitro translation studies, Chapter 3).

Hybridisation in the presence of increasing amount of viral DNA allows determination of the amount of viral RNA present. In the present case the data donot give such a clear idea but the presence of some viral RNA in the preparation is evident.

# Chapter 3. Translation of mRNA in the Messenger Dependent Lysate (MDL) System.

## 3.1. Introduction

In general it has been found that mRNA from one cell type can be translated in cell extracts or intact cells of a different type without a requirement for specific factors or components. Numerous eukaryotic cell-free systems efficiently and faithfully translate exogenous mRNA's. Cell-free translation systems have been reported from Krebs II ascite cells (Kerr et al., 1972; Swan et al., 1972), rat and mouse liver cells (Sampson & Borghetti 1972; Sampson et al., 1972), HeLa cells (McDowell et al., 1972), reticulocytes (Berns et al., 1972; Pelham & Jackson 1976), wheat germ (Roberts & Paterson, 1973; Zagorski, 1978) and rye embryo cells (Carlier & Peumans, 1976). Some of these systems have a high concentration of endogenous mRNA's and added mRNA can only be translated to the extent that it can compete with these endogenous mRNA's. Rabbit reticulocyte lysates provide a complete eukaryotic cell-free system which can reproducibly and faithfully translate a wide range of exogenous mRNA (Pelham & Jackson, 1976) and pretreatment with micrococcal nuclease makes the preparation low in endogenous mRNA. Compared with the wheat germ extract system which produces a high proportion of incomplete translation products, this unfractionated messenger dependent lysate (MDL) system is capable of synthesizing proteins of very high molecular weight (Benoff & Nadal-Ginard, 1979; Pelham, 1979). At the same time it is easier and more convenient to prepare the MDL system than most others which have been described. Therefore,

this system was the one mainly used to study the cell free translation of PRV infected cell mRNA's.

## 3.2. mRNA activity of viral and host mRNA's

An mRNA is an RNA species which can serve as a template for protein synthesis. In an efficient translation system it has the ability to stimulate incorporation of radioactive amino acids into TCA precipitable material.

Poly(A) containing RNA, isolated from mock-infected and PRV infected cells, was translated in the MDL system. The translation system contained all the components described in Materials and Methods. The $K^+$ concentration was kept at 110mM and $Mg^{++}$ concentration at 2.2mM. Incubation was at 30°C for 60 min (any changes from this standard condition are noted in the figure legends) after which the reaction was stopped and the TCA precipitable material was counted to determine the radioactivity incorporated. The poly(A) containing mRNA's showed significant mRNA activity in stimulating radioactive amino acid incorporation into TCA precipitable material. Table 3.2.1 gives the results obtained for two different MDL preparations.

Incorporation of TCA precipitable radioactivity was studied as a function of time. After incubation at 30°C, the reaction was stopped at appropriate times by quick transfer on to ice. 5µl aliquots were counted in duplicate to determine acid precipitable radioactivity. The results (Fig. 3.2.1.) show increasing incorporation up to 60 min and this time was chosen as the standard incubation time.

Table 3.2.1.

mRNA activity of exogenous mRNA in the Messenger Dependent Lysate (MDL) System.

| mRNA | Concentration of mRNA (μg/ml) | MDL -1 (a) | | MDL -2 (b) | |
|---|---|---|---|---|---|
| | | cpm/5μl | stimulation (χ endogenous) | cpm/μl | stimulation (χ endogenous |
| Endogenous | - | 4,200 | - | 20,420 | - |
| HeLa mRNA | 5.2 | 18,900 | 4.5 | 246,630 | 12.0 |
| Immediate-early (IE) mRNA | 8.0 | 26,880 | 6.4 | 182,320 | 8.9 |
| Late mRNA | 7.8 | - | - | 184,000 | 9.0 |

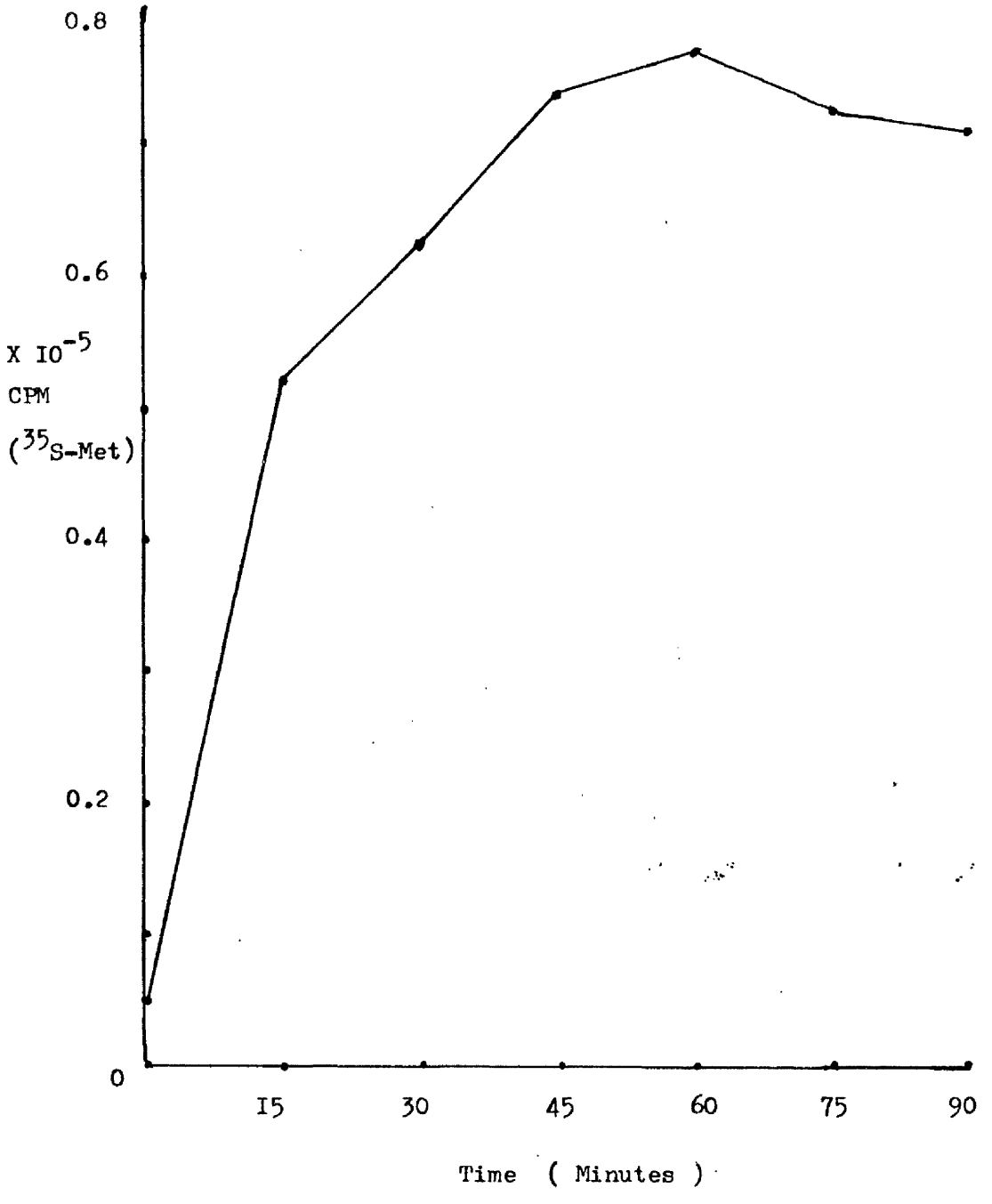
(a) The assay mixture contained 50μl MDL-1 (prepared in the lab) plus 7.5μl of a solution containing the specified amount of the appropriate mRNA and 20 μCi $^{35}$S-methionine.

(b) The 25μl assay mixture contained 10μl of MDL-2 (commercially prepared), a specified amount of the appropriate mRNA and 13μl of a mastermix containing all necessary components plus 40 μCi $^{35}$S-methionine.

After 60 min incubation at 30°C, aliquots (5μl from the assay mix with MDL-1 and 1μl from the assay mix with MDL-2) were counted in duplicate by the filter disc method to determine the TCA precipitable radioactivity. $K^+$ concentration was 110mM and $Mg^{++}$ 2.2mM.

Fig. 3.2.1. Kinetics of $^{35}$S-Methionine incorporation into proteins in the MDL system, specified by HeLa mRNA.

The 57.5µl assay mixture contained 50µl of the MDL and 20µCi $^{35}$S-Methionine plus 6µg/ml HeLa mRNA. Incubation was at 30°C. At appropriate times 5µl aliquots were counted in duplicate by the filter disc method to determine the TCA precipitable radioactivity.


0.8
0.6
X IO-5
CPM
(35S-Met)
0.4
0.2
0
I5
30
45
60
75
90
Time ( Minutes )

## 3.3. Characterisation of translation products.

One important use of *in vitro* translation studies is to characterise the proteins specified by the mRNA. The mRNA isolated as Immediate-early mRNA from PRV infected cells contained a large number of host mRNA's and so the translation products would contain a number of host proteins. However, it was possible to detect virus specific proteins by SDS-polyacrylamide gel electrophoresis and autoradiography.

$^{35}$S-Methionine containing translation products were denatured with SDS (2%, w/v), β-mercaptoethanol (5%, v/v), glycerol (20%, v/v) and heated for 2 min in a boiling water bath, and then applied onto the polyacrylamide gel (Materials and Methods). Autoradiography of the dried gel (Fig. 3.3.1) showed a large number of host proteins along with a number of Immediate-early viral proteins (Track C). The molecular weight of those polypeptides which appear to be virus specified are given in Table 3.3.1. The late mRNA specified products also contained some host proteins (Fig. 3.3.1, Track D), but the major products are viral. The molecular weight of various products are again given in Table 3.3.1.

Since after infection the translation apparatus is said to be adapted to synthesize mainly viral proteins, it is possible that viral mRNA's have some selective advantage over host mRNA's during translation. An attempt was made to study whether simultaneous addition of host and viral mRNA's resulted in preferential translation of viral mRNA's. Analysis of the translation products shows no obvious selective advantage for late mRNA (Fig 3.3.2) over host mRNA in the MDL system.

Fig. 3.3.1. Products of exogenous mRNA translation in the Messenger Dependent Lysate (MDL) System.

HeLa mRNA (10.2μg/ml), IE mRNA (9.4μg/ml) and Late mRNA (12.4μg/ml) were translated in the MDL System, as described in Materials and Methods. Translation products were labelled with $^{35}$S-Methionine (1500μCi/ml). Samples were processed by denaturing with SDS (2% w/v), β-mercaptoethanol (5% v/v) and heating in boiling water bath for 2 min. Appropriate amounts of sample were applied on to a SDS-polyacrylamide gel containing 10% (w/v) acrylamide and 0.3% (w/v) bis-acrylamide. After electrophoresis at 20mA overnight the gel was dried and exposed to X-ray film for autoradiography. Molecular weight markers were used to determine the molecular weight of unknown proteins.

| | | Counts applied. |
|---|---|---|
| Track A | Endogenous | 50,000 |
| B | HeLa mRNA | 220,000 |
| C | IE mRNA | 307,000 |
| D | Late mRNA | 387,000 |

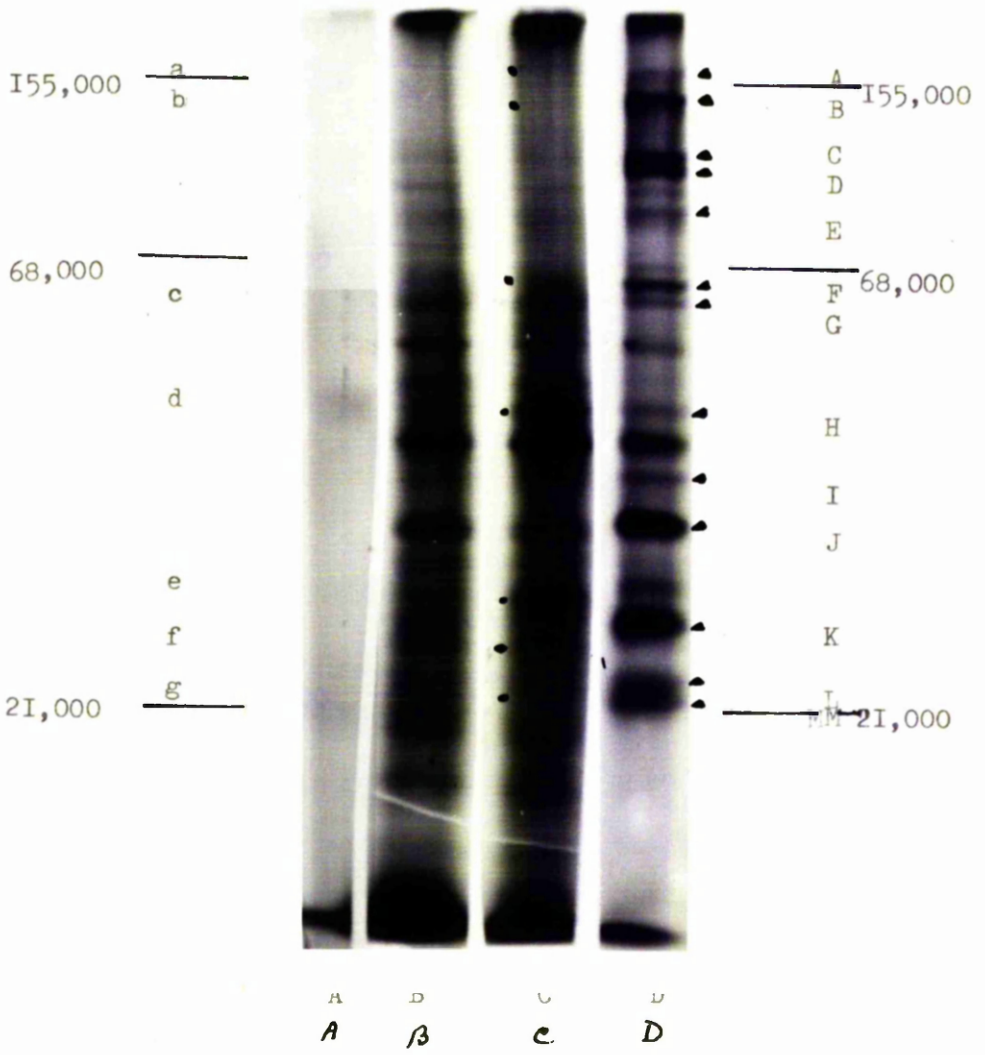

155,000
a
b
68,000
c
d
e
f
g
21,000
A
B
C
D
E
F
G
H
I
J
K
L
155,000
68,000
21,000
A
B
C
D

Table 3.3.1.

Polypeptides synthesized by virus specific mRNA's in the MDL System.

| Nature of the mRNA | Polypeptides | Molecular weight $\chi 10^{-3}$ |
|---|---|---|
| IE mRNA | a | 177 |
| | b | 112 |
| | c | 63 |
| | d | 52 |
| | e | 36 |
| | f | 28 |
| | g | 25 |
| Late mRNA | A | 177 |
| | B | 112 |
| | C | 94 |
| | D | 89 |
| | E | 79 |
| | F | 63 |
| | G | 60 |
| | H | 50 |
| | I | 39 |
| | J | 37 |
| | K | 31 |
| | L | 26 |
| | M | 23 |

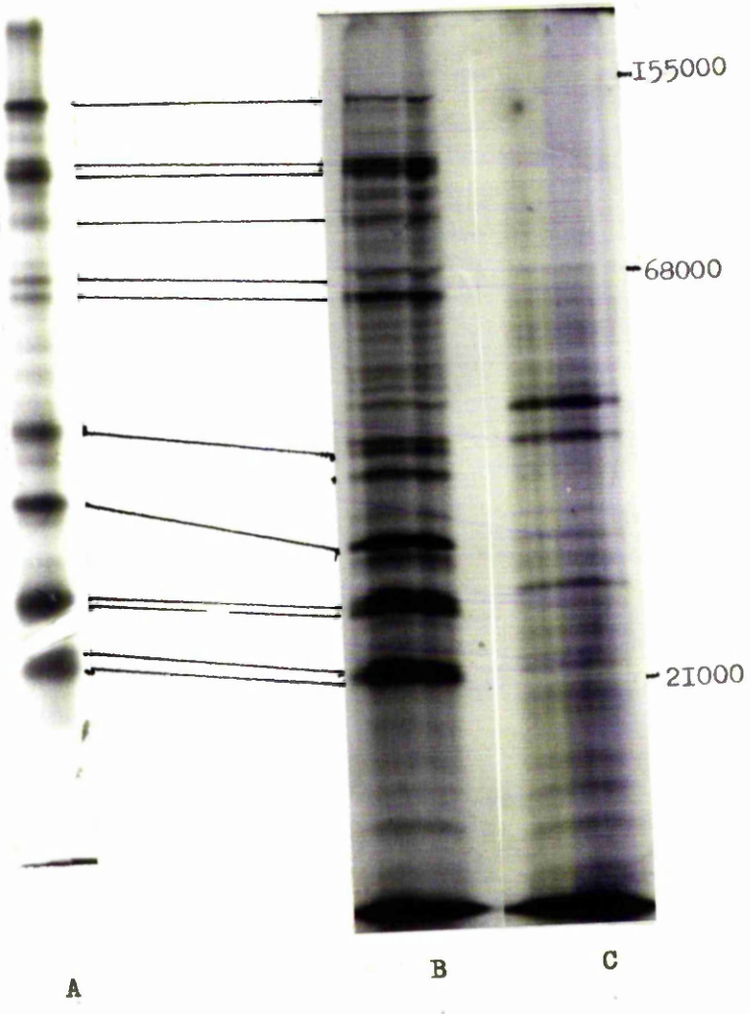
HeLa mRNA, IE mRNA and Late mRNA were translated in the MDL System (Materials and Methods) and the translation products were analyzed by polyacrylamide gel electrophoresis and autoradiography. Using marker proteins of known molecular weight the molecular weight of virus specific proteins were determined.

Fig. 3.3.2. Products of exogenous mRNA's translated in the MDL System.

Each assay mixture contained the specified mRNA's in addition to $^{35}S$ - methionine (1000$\mu$Ci/ml). After 60 min incubation at 30°C, the translation products were processed and analyzed by polyacrylamide gel electrophoresis and autoradiography.

Tracks

A, Late mRNA ( 8.6 µg/ml ).
B, Late mRNA ( 7.2 µg/ml ) and HeLa mRNA (8.4 µg/ml ).
C, HeLa mRNA ( 8.4 µg/ml ).


155000
68000
21000
A
B
C

It has been suggested that in some systems the virus mRNA's may displace host mRNA's from the polyribosomes (Ben-Porat et al., 1971). If this is so, then mRNA isolated from the whole cytoplasm should give different translation products from mRNA isolated from the polyribosomes only. Poly(A) containing RNA was isolated from the cytoplasm of cells infected for 5 hrs with PRV. Analysis of the translation products of cytoplasmic and polyribosomal poly(A) containing RNA shows no significant difference in the pattern of the proteins synthesized (Fig. 3.3.3)

## 3.4. Discussion.

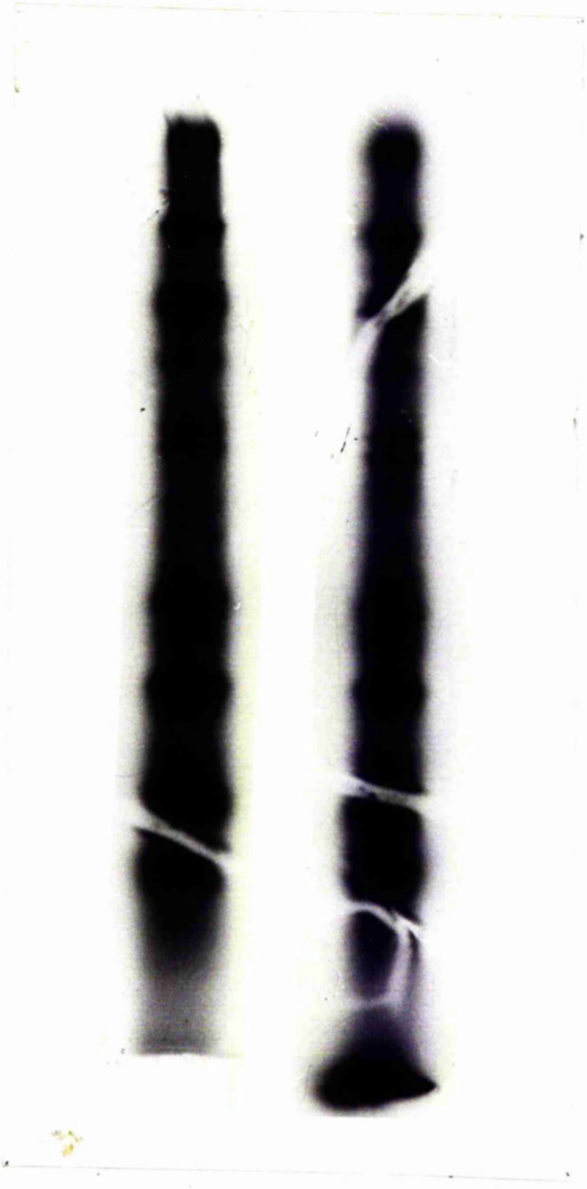
The two MDL systems - one prepared in the lab and one commercially prepared, give similar stimulation of protein synthesis by adding mRNA. The translation products of the IE mRNA preparation show the presence of a large number of host proteins. It has been claimed that in Cycloheximide treated HSV-1 infected cells IE proteins are mainly synthesized after withdrawal of the drug (Roizman et al., 1974). However, other results show the synthesis of a number of host proteins as well (Preston, 1979). Our finding for PRV is similar to that of Preston (1979) in that we find host mRNA's on the polyribosomes when IE proteins are being made. The hybridisation data also suggested the presence of a large amount of host mRNA in the IE mRNA preparation. Still it is possible to detect at least seven IE polypeptides in the translation products of the IE mRNA preparation. The situation is different with late mRNA's. The translation products show mainly viral proteins, with a few host proteins.

Fig. 3.3.3. Polypeptides synthesized in the MDL System specified by polyribosomal and Cytoplasmic Late mRNA.

The assay mixture contained polyribosomal Late mRNA (9.2μg/ml) and Cytoplasmic late mRNA (13.2μg/ml) in addition to all the necessary components and $^{35}$S-methionine (1200μCi/ml). After 60 min incubation at 30°C, the translation products were processed and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Track A Polysomal late mRNA

Track B Cytoplasmic late mRNA.



A B

During infection the infected cells are said to preferentially synthesize viral proteins. Simultaneous addition of almost equal amounts of host and viral mRNA's does not show preferential translation of viral mRNA's. So it can be argued, by this criterion, that any selectivity of translation does not reside in the mRNA.

Normally, translatable mRNA's become bound to polyribosomes. In an attempt to investigate the presence of translatable mRNA in the cytoplasm free from polyribosomes, it is found that polyribosomal and cytoplasmic poly(A) RNA's specify the same proteins. This indicates that in PRV infected cells all translatable mRNA's are not preferentially bound to polyribosomes.

# Chapter 4. Protein synthesis in cell-free systems at different $K^+$ concentrations.

## 4.1. Introduction

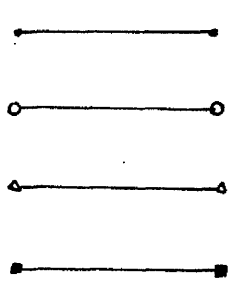
Changes in the intracellular ion concentrations after virus infection and their possible correlation with selective inhibition have been discussed in 1.7.1. In a cell-free translation system it has been shown that late PRV mRNA has no selective advantage over host mRNA at elevated $K^+$ concentrations (McGrath & Stevely, 1980). Since in herpesvirus infected cells inhibition of protein synthesis occurs early after infection and in MCMV infected cells Immediate early proteins have been shown to be resistant to high salt concentrations, it was of interest to investigate whether IE PRV mRNA has any advantage over host mRNA at elevated $K^+$ concentrations.

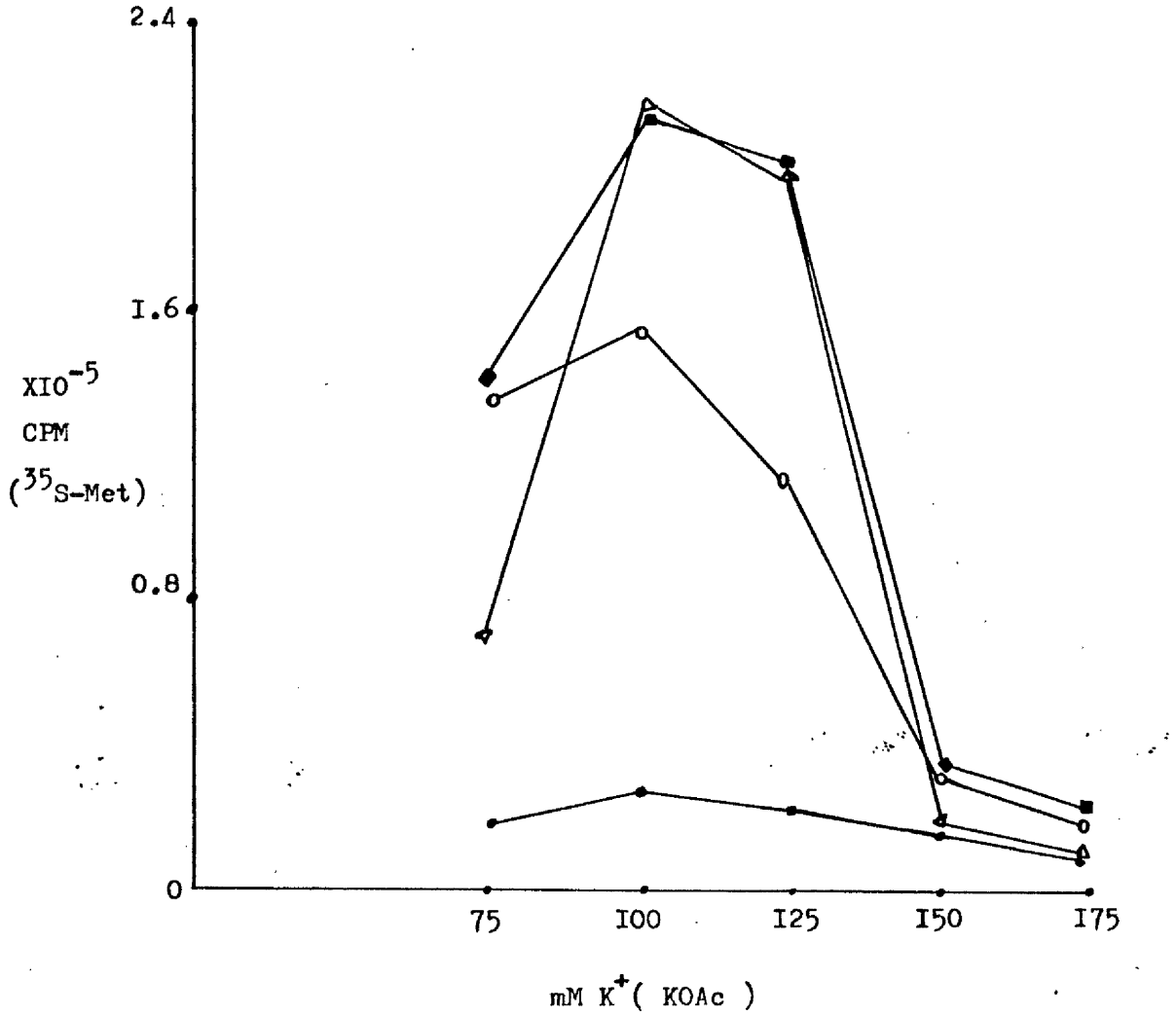
## 4.2. $K^+$ optima for translation of viral mRNA's in the cell-free translation systems.

The optimal concentration of $K^+$ required for the translation of host and viral mRNA's in the MDL system and in the wheat germ extract system was determined by varying the concentration of exogenous $K^+$ added to the assay mixture. The assay mixture in the MDL system contained 2.2mM $Mg^{++}$ and appropriate concentrations of KCl or KOAc. The reaction rate was followed by determining the TCA precipitable radioactivity at different $K^+$ concentrations. Fig 4.2.1. shows the translation of HeLa, IE and Late mRNA's at different KOAc concentrations in the MDL System. All three types of mRNA showed the same $k^+$ optima in the range of 100-110mM.

Fig. 4.2.1. Effects of different concentration of $K^+$ on exogenous mRNA translation in the MDL system.

The assay mixture containing HeLa mRNA (10.2μg/ml), IE mRNA (9.6μg/ml) and late mRNA (12.2μg/ml) were varied with respect to $k^+$ concentration by the addition of different concentrations of KOAc. Mixtures containing 1000μCi $^{35}$S-Methionine per ml was incubated at 30°C for 60 min. 1μl aliquots were counted, in duplicate, by filter disc method to determine the TCA precipitable radioactivity.




2.4
1.6
0.8
0
XIO$^{-5}$
CPM
($^{35}$S-Met)
75
I00
I25
I50
I75
mM K$^{+}$( KOAc )

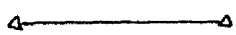
When KCl was used instead of KOAc as the source of $k^+$ both HeLa and IE mRNA s showed a $K^+$ optima in the same region (Fig. 4.2.2). At $K^+$ concentrations above 125mM all classes of mRNA showed a gradual inhibition of translation.

Analysis of the translation products by polyacrylamide gel electrophoresis and autoradiography showed that viral proteins apparently did not have any selective advantage over host proteins at higher $K^+$ concentrations (Fig. 4.2.3). To clarify the effects of high concentrations of $K^+$ on protein synthesis the gel autoradiograph was traced and portions of the gel tracing containing a few prominent protein bands are shown in Fig. 4.2.4. From the tracing it appeared that one IE polypeptide (, Molecular Weight 31,000) showed a gradual decrease in synthesis when the $K^+$ concentrations were changed from 100 to 150mM as did host proteins and . On the other hand, another IE polypeptide (, Mol. wt. 52,000) and a host protein showed some resistance to the effects of elevated $K^+$ concentrations. The areas corresponding to these five proteins were computed and setting 100mM $K^+$ value as 100% the areas at other salt concentrations are given in Tables 4.2.1 and 4.2.2. While it is recognized that the darkening of the film is not linear with respect to radioactivity it is, nonetheless, reasonable to take the areas as a rough guide to relative incorporation. The tables show the impression gained as to relative resistance by visual observation to be correct.

A commercial wheat germ extract system gave an almost identical $K^+$ (KOAc) optima for HeLa, IE and Late mRNA translation in the region of 100 - 115mM (Fig. 4.2.5).

Fig. 4.2.2. Translation of exogenous mRNA's in the MDL System at different KCl concentrations.

The MDL translation system was programmed with HeLa mRNA (10.2μg/ml) and IE mRNA (9.6μg/ml) with 400μCi $^{35}$S-Methionine per ml. The concentration of $k^+$ was varied by adding different concentrations of KCl. After 60 min incubation at 30°C, 5μl aliquots were counted, in duplicate, to determine the TCA precipitable radioactivity.


HeLa mRNA.


IE mRNA preparation.

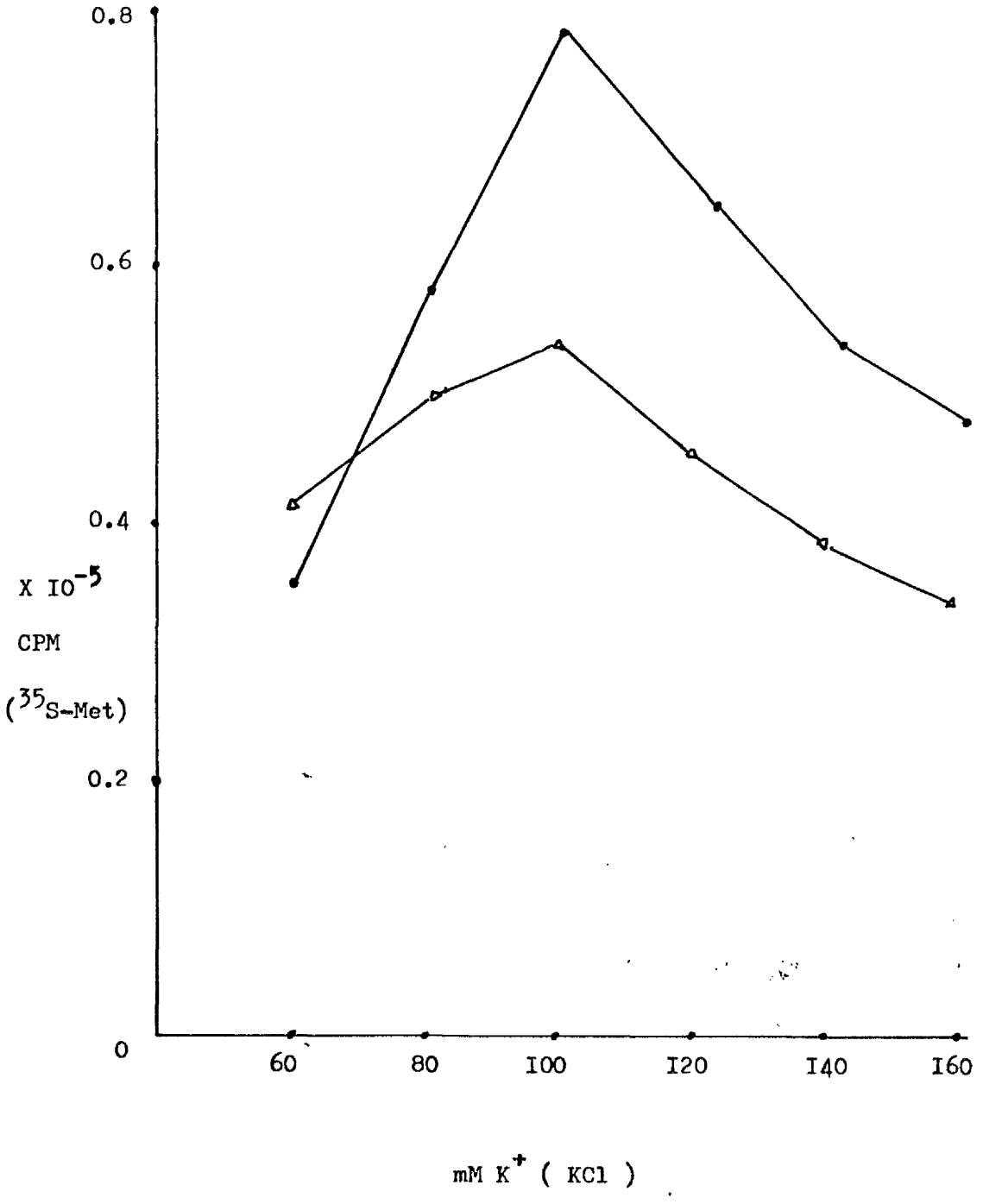

0.8
0.6
0.4
0.2
0
X IO-5
CPM
(35S-Met)
60
80
I00
I20
I40
I60
mM K+ ( KCl )

Fig. 4.2.3. Protein synthesis in the MDL System at elevated $K^+$ concentrations.

The assay mixtures containing HeLa mRNA (10.2µg/ml), IE mRNA preparation (9.6µg/ml) and 1000µCi $^{35}$S-Methionine per ml plus different concentrations of KOAc were incubated at 30°C for 60 min. The translation products were processed and analyzed by polyacrylamide gel electrophoresis and autoradiography.

An equal amount of protein was applied on each gel track.

Tracks 1 to 5 HeLa mRNA's; Tracks 6 to 10 IE mRNA's ;
Tracks 1 and 6 50mM $K^+$; Tracks 2 and 7 75mM $K^+$ ;
Tracks 3 and 8 100mM $K^+$ ; Tracks 4 and 9 125mM $K^+$ ; and
Tracks 5 and 10 150mM $K^+$.

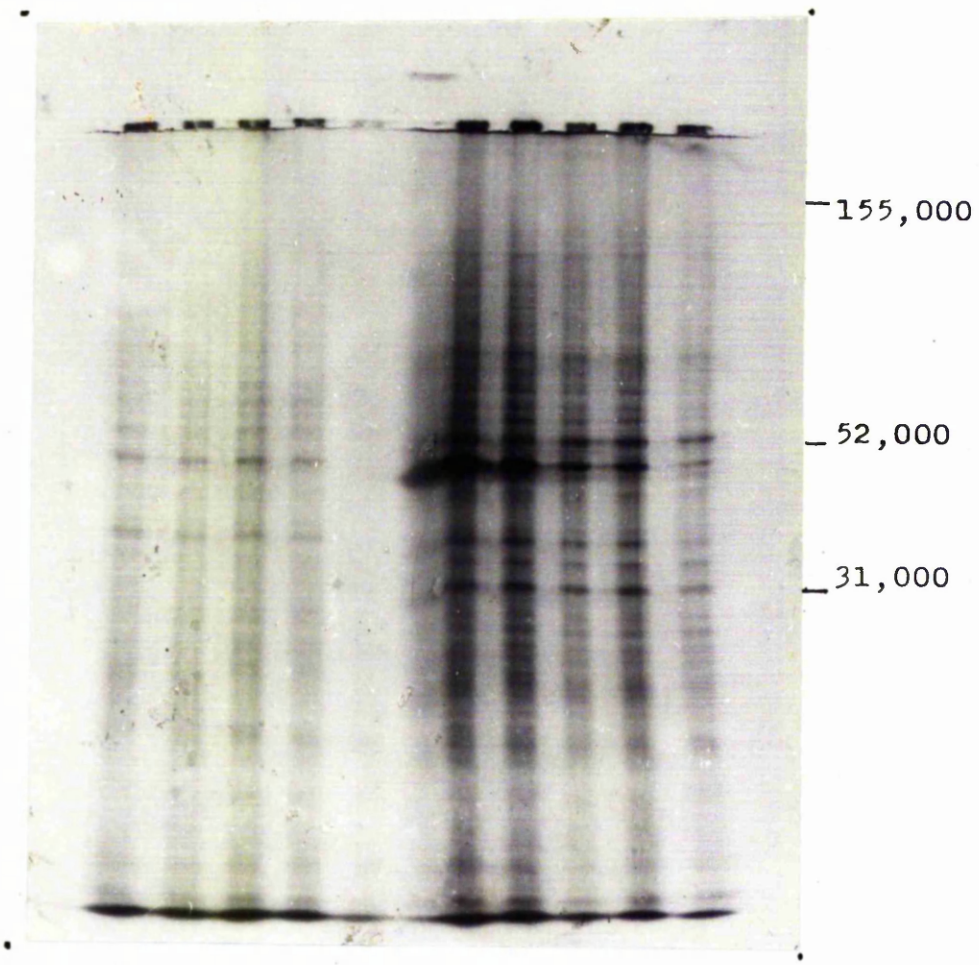

155,000
52,000
31,000
50 75 100 125 150
mM K+
HeLa mRNA
50 75 100 125 150
mM K+
IE mRNA

Fig. 4.2.4. Tracing of portions of the gels containing translation products specified by IE mRNA preparation at different salt conc. in the MDL System.

Gel tracks from Fig. 4.2.3. containing translation products specified by IE mRNA preparation at 100mM, 125mM and 150mM $K^+$ concentrations were scanned and traced.

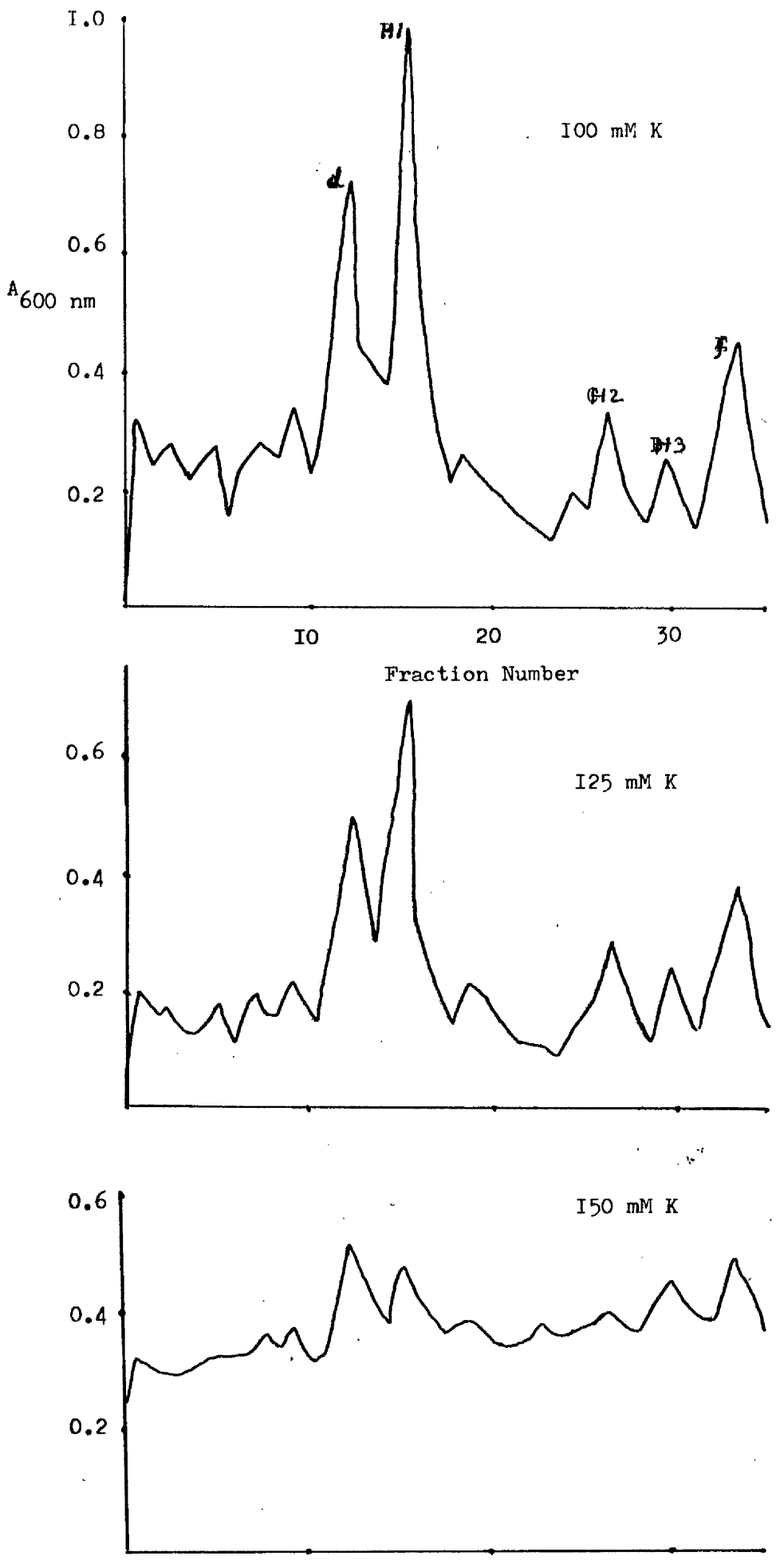

I.0
0.8
0.6
A600 nm
0.4
0.2
H1
d
H2
H3
F
100 mM K
10
20
30
Fraction Number
0.6
0.4
0.2
125 mM K
0.6
0.4
0.2
150 mM K

Table 4.2.1.

Percent incorporation of $^{35}$S-Methionine at various $K^+$ concentrations in the MDL System.

| Nature of the polypeptides | 100mM $K^+$ | 125mM $K^+$ | 150mM $K^+$ |
|---|---|---|---|
| α, Immediate-early polypeptide | 100 | 70 | 33 |
| H1 Host polypeptide | 100 | 78 | 20 |
| H2 Host polypeptide | 100 | 133 | 34 |
| H3 Host polypeptide | 100 | 128 | 160 |
| β, Immediate-early polypeptide | 100 | 103 | 44 |

Considering the 100mM $K^+$ value as 100 percent, the percent incorporation of $^{35}$S Methionine in a few polypeptides specified by IE mRNA preparation at higher $K^+$ concentrations, as traced in Fig. 4.2.4. was measured.

Table 4.2.2.

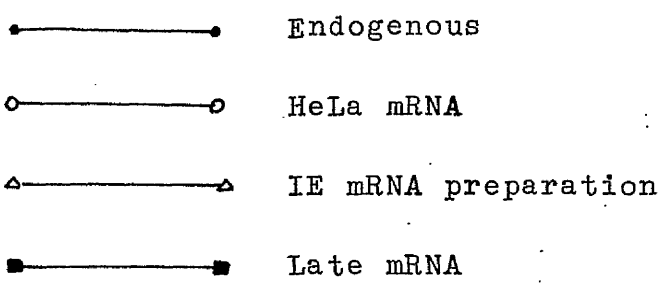
Relative incorporation of $^{35}$S-Methionine at different $K^+$ concentrations in the MDL System.

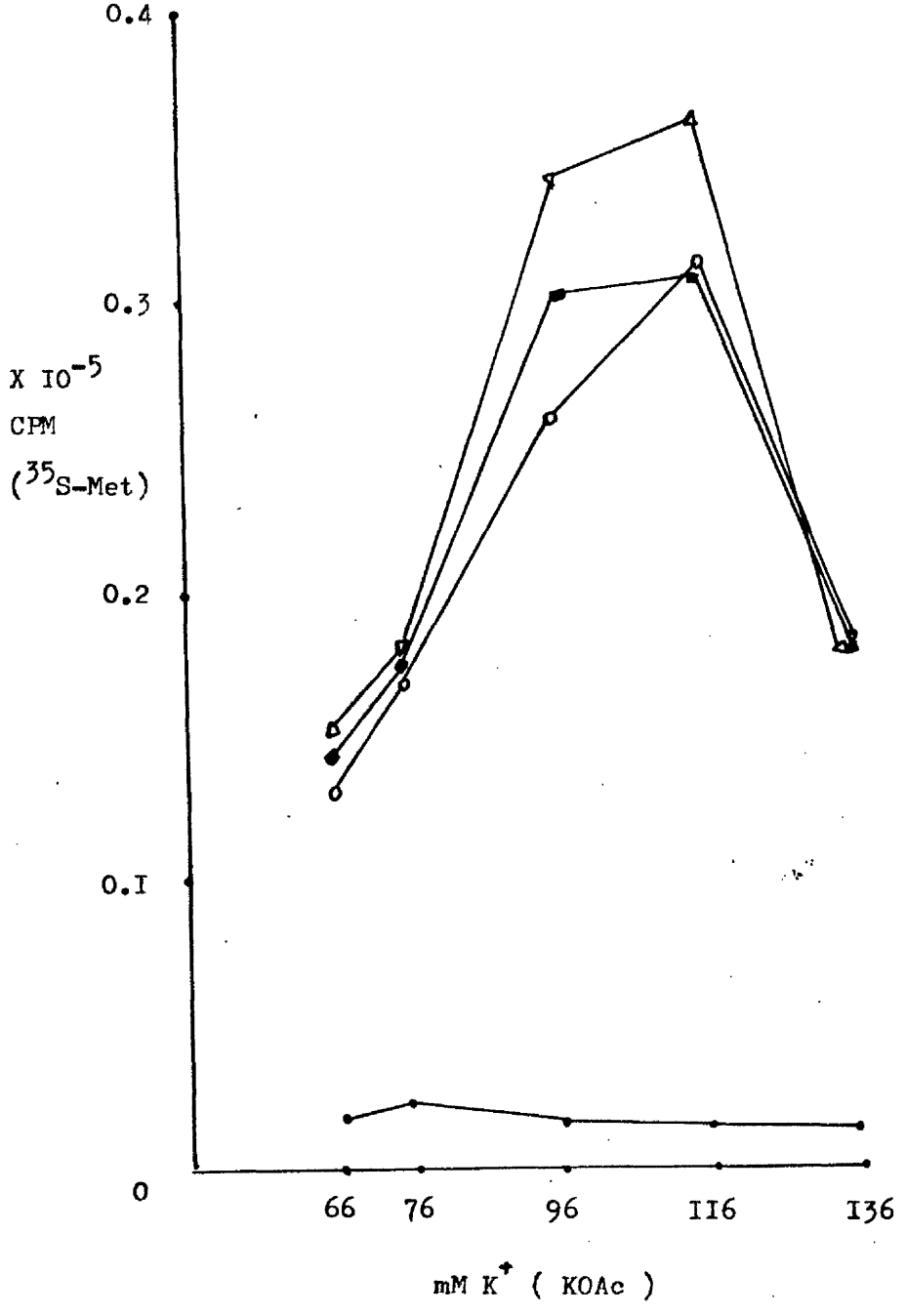
| Nature of the polypeptide | % of the total incorporation. 100mM $K^+$ | 125mM $K^+$ | 150mM $K^+$ |
|---|---|---|---|
| α, Immediate-early polypeptide | 46 | 43 | 58 |
| H1 Host protein | 54 | 57 | 42 |
| Total | 100 | 100 | 100 |
| H2 Host protein | 25 | 29 | 14 |
| H3 Host protein | 16 | 18 | 43 |
| β, Immediate-early polypeptide | 59 | 53 | 43 |
| Total | 100 | 100 | 100 |

The percent incorporation of $^{35}$S-Methionine in the polypeptides was measured at three different $K^+$ concentrations.

Fig. 4.2.5. Exogenous mRNA translation in wheat germ extract system at different $K^+$ concentrations

A commercially available wheat germ extract system was used to translate HeLa mRNA (8.4μg/ml), IE mRNA (7.5μg/ml) and Late mRNA (7.8μg/ml) at different KOAc concentrations. The assay mixture containing 750μCi $^{35}$S-Methionine per ml was incubated at 25°C for 60 min. 1μl aliquots were counted, in duplicate, to determine TCA precipitable radioactivity.




0.4
0.3
X IO$^{-5}$
CPM
($^{35}$S-Met)
0.2
0.I
0
66
76
96
II6
I36
mM K$^{+}$ ( KOAc )

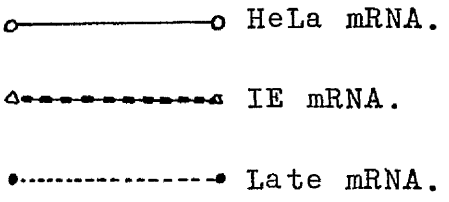
But a wheat germ extract system prepared in this lab showed an optimal $K^+$ concentration 66-76mM for HeLa and Late PRV mRNA's whereas IE mRNA's showed an optimum in the range of 84-92mM (Fig. 4.2.6).

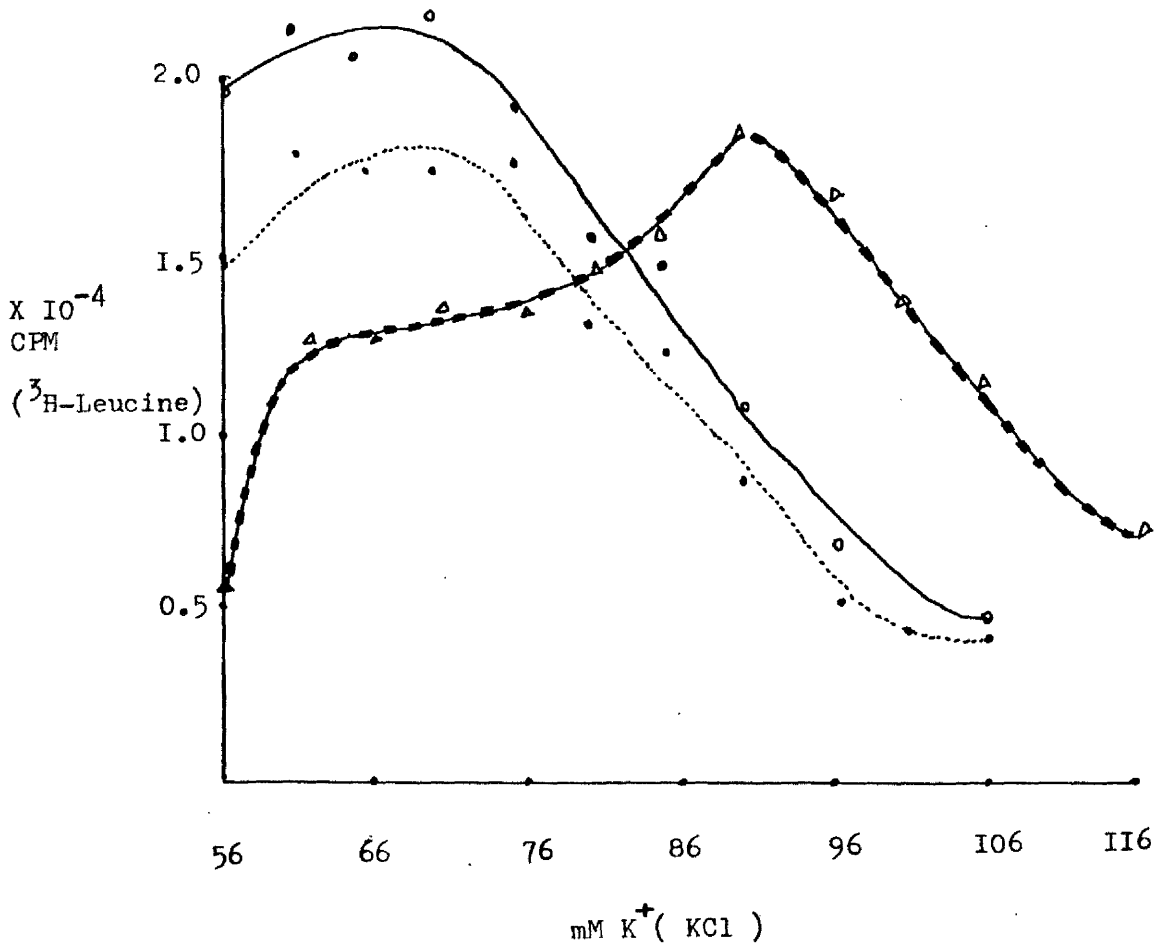
## 4.3. Discussion.

Proteins do vary in their resistance to high salt concentrations in cell-free systems but PRV IE mRNA's as a class are not especially resistant. There has been a suggestion that in the wheat germ extract system inhibition of protein synthesis at higher KCl concentrations is not due to high $K^+$ concentration but is due to the higher $Cl^-$ concentration (Weber et al., 1977), because the optimal $K^+$ is higher if KOAc is used instead of KCl. But in the MDL system the $K^+$ optima did not change when KCl was used instead of KOAc. Since the IE mRNA preparation contains only 5% virus specific RNA, it is possible that their high $K^+$ optima is overshadowed by the $K^+$ optima for the large number of host mRNA's. Analysis of the translation products showed no obvious resistance by any IE proteins at higher $K^+$ concentration. With a commercial wheat germ extract system, these messages again show an identical $K^+$ optima for translation. But extracts made in this lab showed a lower $K^+$ optima for HeLa and Late PRV mRNA's compared to IE mRNA. The reason for this different behaviour in the two wheat germ extract systems is not clear. It is possible that the extract systems prepared from different batches of wheat germ might differ in their properties. It is also possible that in the wheat germ extract system prepared in the lab there is a limiting amount of some factor(s) required

Fig. 4.2.6. Effects of different concentrations of $K^+$ on the translation of exogenous mRNA's in the wheat germ extract system.

The wheat germ extract system prepared in the laboratory was used to translate HeLa mRNA (8.4μg/ml), IE mRNA (7.5μg/ml) and Late mRNA (7.8μg/ml) at different KCl concentrations. (Late mRNA was a gift from B.M. McGrath). The reaction mixture containing 100μCi $^3$H-Leucine per ml was incubated at 25°C for 60 min. 5μl aliquots were counted, in duplicate, to determine TCA precipitable radioactivity.




2.0
I.5
X IO$^{-4}$
CPM
($^{3}$H-Leucine)
I.0
0.5
56
66
76
86
96
I06
II6
mM K$^{+}$( KCl )

for translation, leading to the effects shown. In the MDL as well as in the commercial wheat germ extract system a higher concentration of this factor would result in the failure to show discrimination. For example, it has been reported (Shih et al., 1980) that the wheat germ extract system cannot translate EMC virus mRNA completely under standard conditions unless it is supplemented with a salt wash from reticulocyte ribosomes. Probably the wheat germ extract system lacks some factor(s) without which most of the exogenous messages can be translated but not all. This factor is present in the ribosomal salt wash from reticulocytes. Later batches of our own wheat germ extract system together with the commercial wheat germ system all gave identical optima for all mRNA's and so this finding could not be followed up although it had been obtained previously on a number of occasions. Our gel system at that stage was still being developed and so no autoradiographs are available of the early results. We cannot, therefore, place weight on this work and must conclude that the bulk of the studies indicate no difference between the translation properties of IE, late and HeLa mRNA's.

# Chapter 5. Protein synthesis in cells infected with herpes viruses.

## 5.1. Introduction

Inhibition of cellular protein synthesis and selective translation of viral mRNA's occur during the course of productive infection by many animal viruses, for example, herpesvirus (Sydiskis & Roizman, 1966), vaccinia virus (Hanafusa, 1960), adenovirus (Levin & Ginsberg, 1967), poliovirus (Penman & Summers, 1965) and vesicular somatitis virus, VSV (Yaoi et al., 1970). In Encephalomyocarditis (EMC) virus infected ascites cells, inhibition is said to be due to competition between host and viral mRNA's for elf-4B (Golini et al., 1976). Inactivation of Cap-binding protein in poliovirus infected cells appears to be responsible for inhibition of capped host-mRNA translation (Rose et al., 1978; Trachsel et al., 1980; Sonenberg et al., 1980). Initiation factors isolated from poliovirus infected HeLa cells are defective in the translation of capped mRNA in vitro whereas initiation factors from EMC virus infected HeLa cells are not (Jen et al., 1980).

There is a report of host mRNA degradation in HSV-1 infected Friend erythroleukemia cells (Nishioka & Silverstein 1978b). In HSV-2 infected vero cells inhibition is caused by a component(s) of the infecting virus (Fenwick & Walker 1978). As a first step in the study of inhibition of protein synthesis in PRV infected cells investigations were carried out to find out the extent of inhibition of protein synthesis in different cell lines after herpesvirus infection.

## 5.2. Inhibition of protein synthesis in HeLa cells after herpesvirus infection.

HeLa cells were infected with PRV and protein synthesis in the course of viral infection was examined by pulse-labelling with [$^{35}$S]-methionine. The synthesis rate was determined in terms of the radioactivity incorporated into TCA precipitable material and the result of a typical experiment is shown in Fig. 5.2.1. Inhibition of protein synthesis in the infected cells was rapid and occurred soon after infection. By 6 hrs infection almost 70% inhibition was observed. Analysis of the infected cell lysates by SDS-polyacrylamide gel electrophoresis and fluorography showed few virus specified proteins by 2 hrs after infection (Fig. 5.2.2). The number of virus specified proteins increased between 2 and 5 hrs after infection. By 5 hrs post-infection, the number of host cell proteins decreased markedly.

Virus protein synthesis appeared not to be sensitive to the process that inhibited cellular protein synthesis. This insensitivity was suggested by the observation that when cellular protein synthesis was declining sharply, viral protein synthesis was increasing. This switch-over from host specific to virus specific protein synthesis may indicate a selective inhibition that suppresses cellular protein synthesis without affecting viral protein synthesis.

Kinetic studies of protein synthesis in HeLa cells infected with HSV-1 (KOS) also showed a rapid decline in protein synthesis and by 6 hrs post-infection inhibition was about 70% (Fig. 5.2.3.).

Fig. 5.2.1. Protein synthesis in HeLa cells infected with PRV.

Confluent monolayers of HeLa cells grown on coverslips in 50mm petridishes were infected with PRV at 50 p.f.u./cell. At different times after infection cells were labelled with 15$\mu$Ci [$^{35}$S]- methionine per petridish for 30 min in methionine-free medium. Determination of TCA precipitable radioactivity, after labelling, was carried out as described in Materials and Methods.

100% protein synthesis (Mock-infected cells) ≡ $10.8 \times 10^4$ cpm.

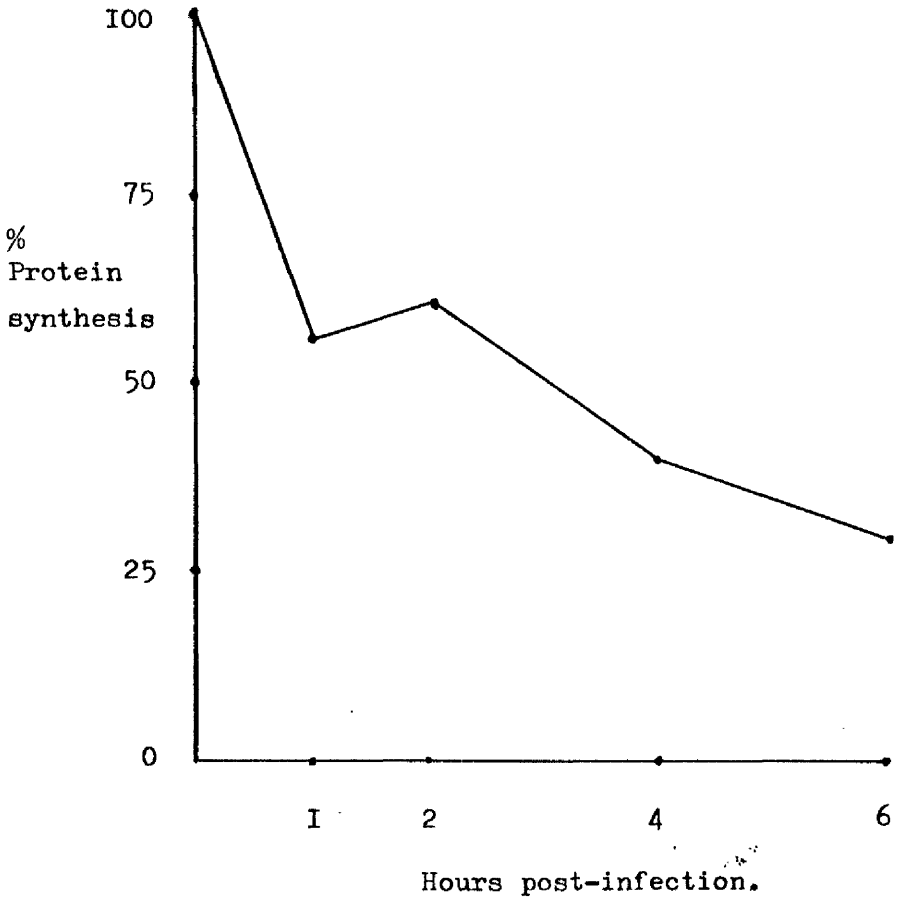

100
75
%
Protein
synthesis
50
25
0
1
2
4
6
Hours post-infection.

Fig. 5.2.2. Polypeptides synthesized in cells infected with PRV.

HeLa cells grown in 90 m.m dia. petridishes were mock-infected or infected with PRV at 50 p.f.u./cell. At different times after infection cells were labelled with 25 $\mu$Ci [$^{35}$S]-methionine per petridish in methionine-free medium for 30 min before being harvested. After lysis, the cell lysates were processed for analysis by SDS-polyacrylamide gel electrophoresis. The gel contained 10% (w/v) acrylamide and 0.3% (w/v) bis-acrylamide. After electrophoresis, the gel was processed for fluorography.

Equal amounts of proteins were applied to each track.

Track 1 : Mock-infected;

Track 2 : 1 hr infected;

Track 3 : 2 hrs infected;

Track 4 : 3 hrs infected;

Track 5 : 5 hrs infected;

Track 6 : 7 hrs infected.

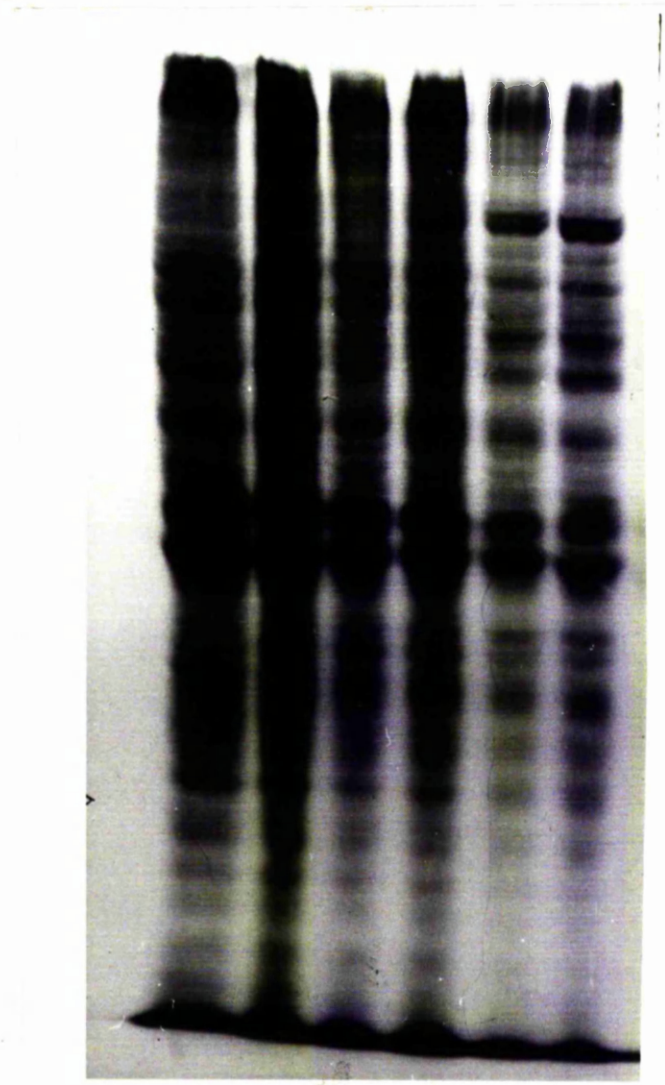
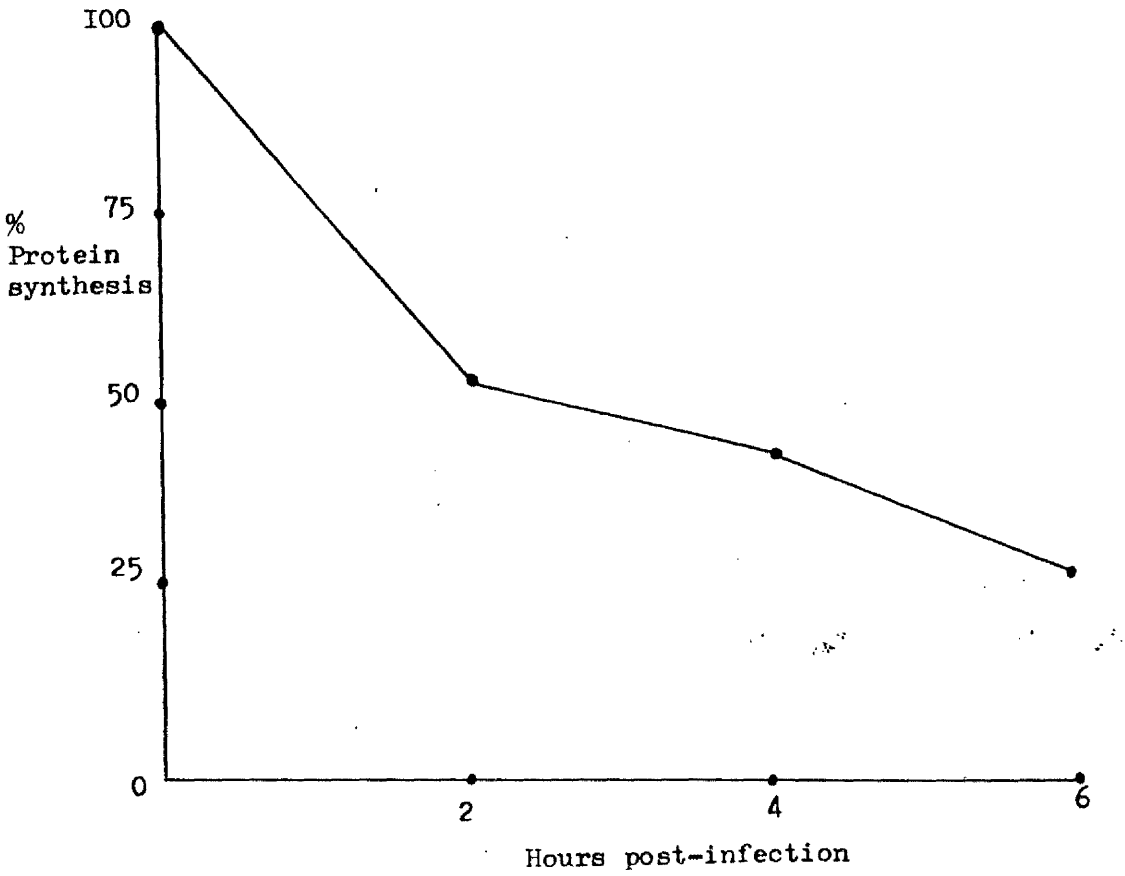

155,000
110,000
68,000
1 2 3 4 5 6

Fig. 5.2.3. Protein synthesis in HeLa cells infected with HSV-1 (KOS).

Confluent monolayers of HeLa cells grown on coverslips in 50mm petridishes were infected with HSV-1 (KOS) at 50 p.f.u./cell. At different times after infection cells were labelled with 15 $\mu$Ci [$^{35}$S] methionine per petridish for 30 min in methionine-free medium. Determination of TCA precipitable radioactivity, after labelling, was carried out according to the procedure described in Materials and Methods.

100% protein synthesis (Mock-infected cells) ≡ $6.5 \times 10^5$ cpm.


100
75
%
Protein
synthesis
50
25
0
2
4
6
Hours post-infection

## 5.3. Inhibition of protein synthesis after PRV infection in other cell lines.

For comparative studies C13 and BS-C-1 cells were infected with PRV to study the kinetics of protein synthesis in these cell lines.

In PRV infected C13 cells the extent of inhibition was followed by determining [$^{35}$S]-methionine incorporation into TCA precipitable material, as had been done for HeLa cells (Fig. 5.3.1 After infection there was an initial 50% stimulation of [$^{35}$S]-methionine incorporation at 1 hr post-infection. A decline in protein synthesis followed thereafter. By 6 hrs post-infection protein synthesis was reduced by 68%. Few virus specific polypeptides appeared in the first 2 hrs of infection, their number increased by 5 hrs post-infection (Fig. 5.3.2).

The kinetics of protein synthesis in BS-C-1 cells infected with PRV are shown in Fig. 5.3.3. Here also there was an initial stimulation of 50% at 1 hr post-infection. But unlike HeLa and C13 cells, inhibition in BS-C-1 cells was slow and by 7 hrs post-infection only 30% inhibition was observed. At 5 hrs post-infection a few virus specific proteins were found (Fig. 5.3.4) but there was little decline in host protein synthesis.

## 5.4. Discussion

Protein synthesis in eukaryotic cells requires specific and selective interactions between initiation factors, mRNA and ribosomal sub units. It is possible that the selectivity of interaction varies in different cell lines. As such, the same

Fig. 5.3.1. Protein synthesis in BHK-21 (C13) cells infected with PRV.

Confluent monolayers of C13 cells grown on coverslips in 50mm petridishes were infected with PRV at a multiplicity of 50 p.f.u./cell. At different times after infection cells were labelled with 15 $\mu$Ci [$^{35}$S]-methionine per petridish for 30 min in methionine-free medium. Determination of TCA precipitable radioactivity was carried out according to the procedure described in Materials and Methods.

100% protein synthesis (Mock-infected cells) ≡
$10.4 \times 10^4$ cpm.

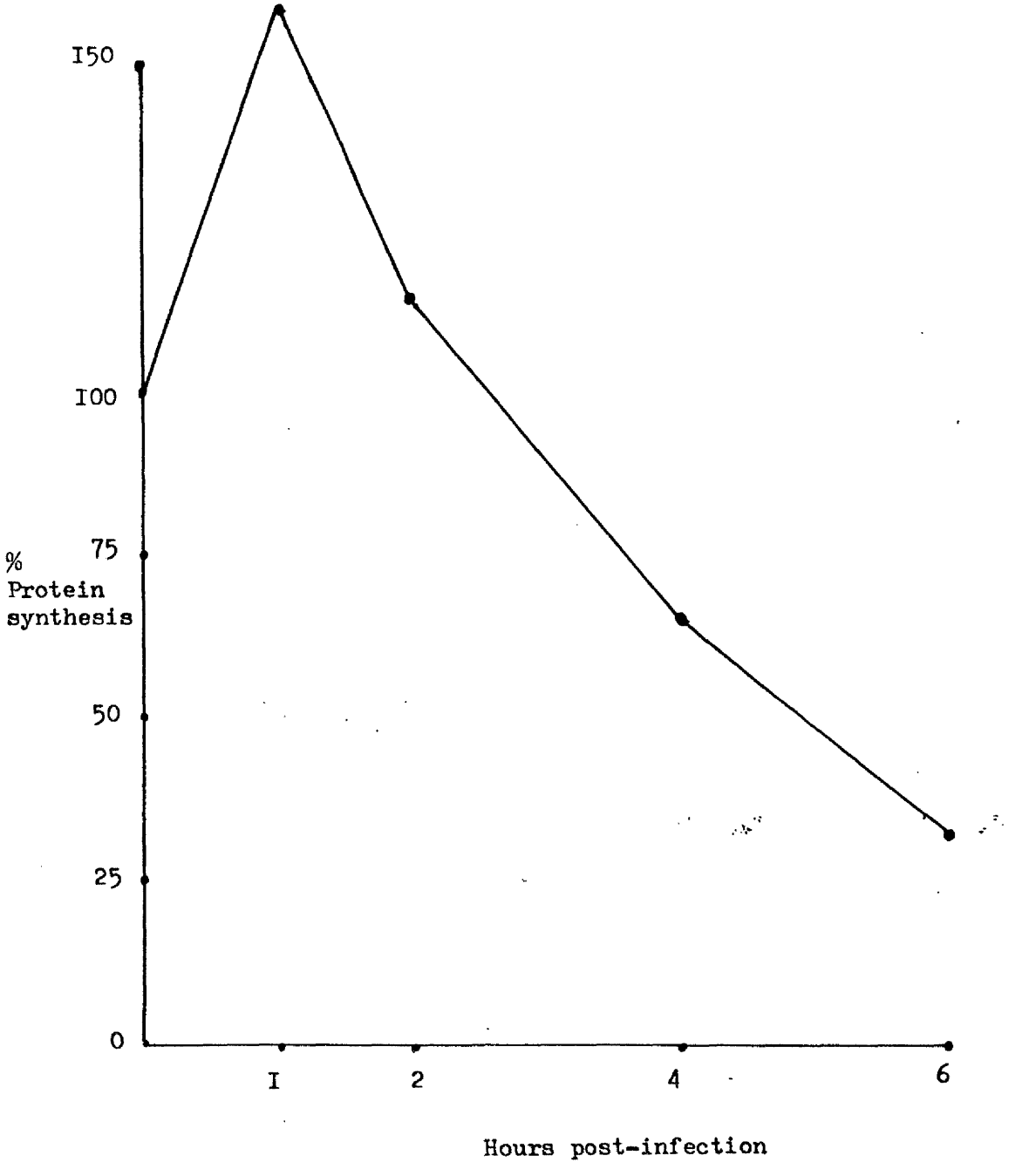

150
100
75
%
Protein
synthesis
50
25
0
1
2
4
6
Hours post-infection

Fig. 5.3.2. Polypeptides synthesized in C13 cells infected with PRV.

C13 cells grown in 90mm dia. petridishes were infected with PRV at 50 p.f.u./cell. Labelling of cells for 30 min at different times after infection, harvesting, lysing and processing of the cell lysates for analysis by SDS-polyacrylamide gel electrophoresis were carried out as previously described. The gel contained 10% (w/v) acrylamide and 0.3% (w/v) bis-acrylamide. After electrophoresis the gel was processed for fluorography.

Equal amounts of proteins are applied to each track.

Track 1 : Mock-infected;

Track 2 : Infected for 1 hr;

Track 3 : Infected for 2 hrs;

Track 4 : Infected for 3 hrs;

Track 5 : Infected for 5 hrs;

Track 6 : Infected for 7 hrs.

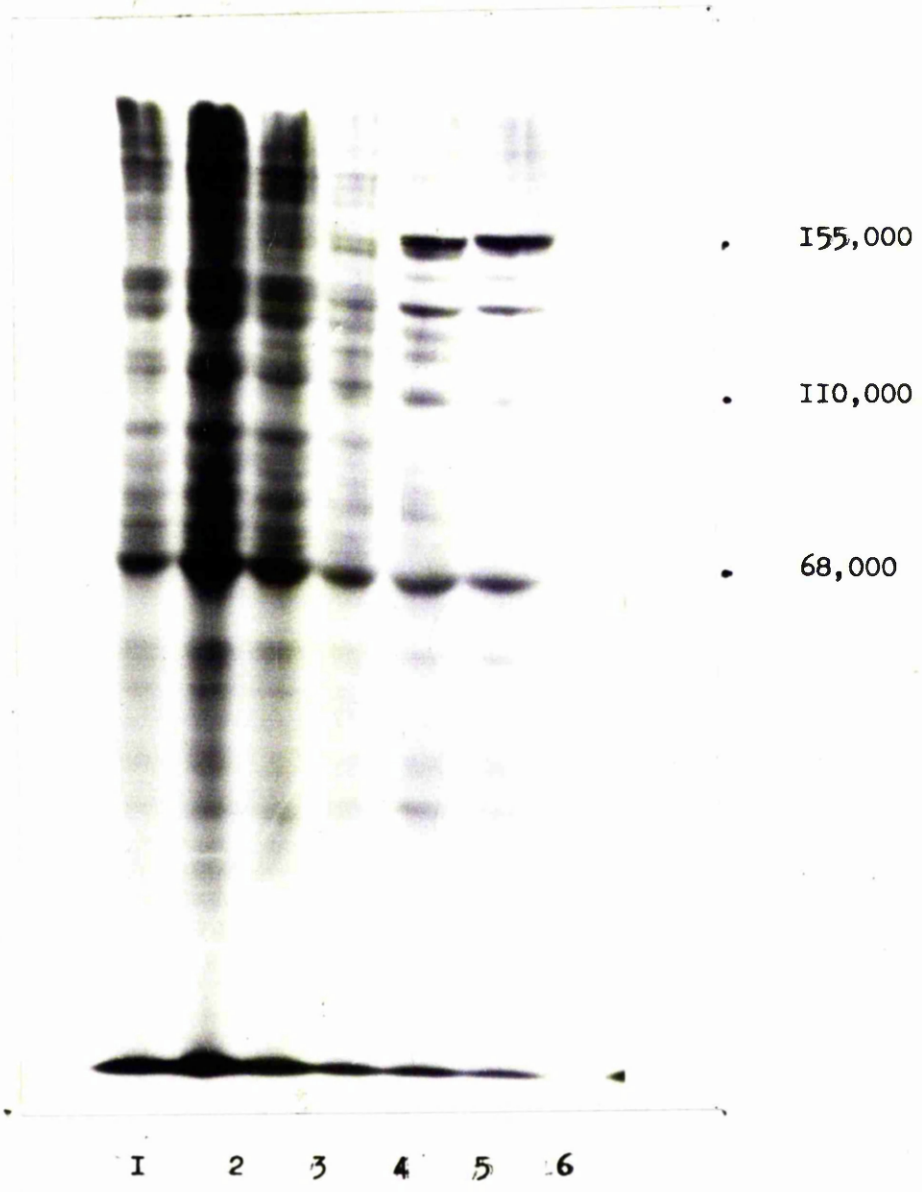

155,000
110,000
68,000
1
2
3
4
5
6

Fig. 5.3.3. Protein synthesis in BS-C-1 cells infected with PRV.

Confluent monolayers of BS-C-1 cells grown on coverslips in 50mm dia. petridishes were infected with PRV at 50 p.f.u./cell. At different times after infection cells were labelled with 15 $\mu$Ci [$^{35}$S] methionine per petridish in methionine-free medium for 30 min. After labelling TCA precipitable radioactivity was determined as described in Materials and Methods.

100% protein synthesis (Mock-infected cells) ≡
$4.2 \times 10^4$ cpm.

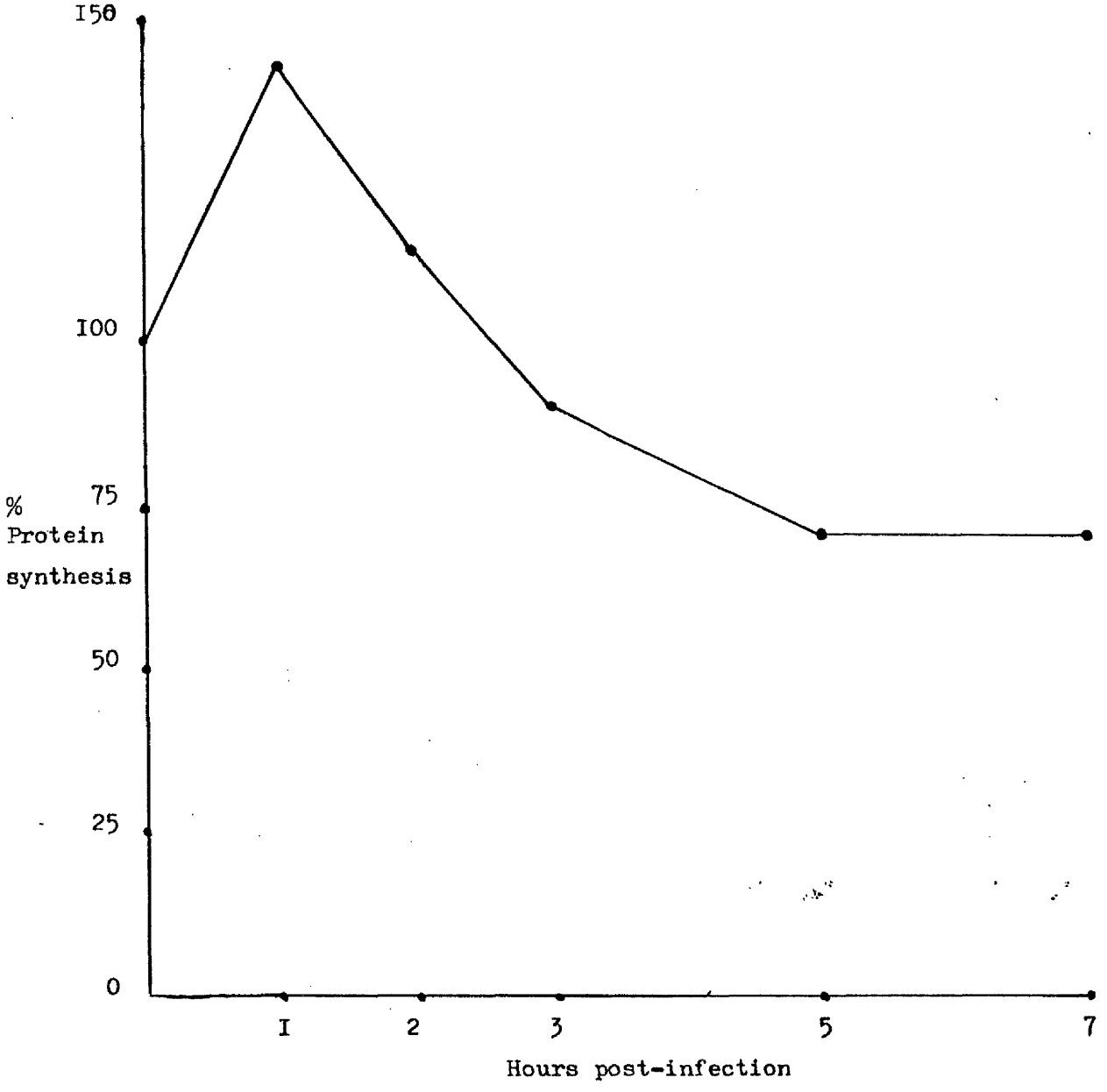

150
100
75
%
Protein
synthesis
50
25
0
1
2
3
5
7
Hours post-infection

Fig. 5.3.4. Polypeptides synthesized in BS-C-1 cells infected with PRV.

Confluent monolayers of BS-C-1 cells grown in 90mm dia. petridishes were infected with PRV at 50 p.f.u./cell. Labelling of cells with [$^{35}$S]-methionine for 30 min at different times after infection, harvesting, lysing and processing of the cell lysates for analysis by SDS-polyacrylamide gel electrophores were carried out as done previously. The gel contained 10% (w/v) acrylamide and 0.3% (w/v) bis-acrylamide. After drying the gel was exposed to X-ray film for autoradiography.

Equal amounts of protein were applied onto each track.

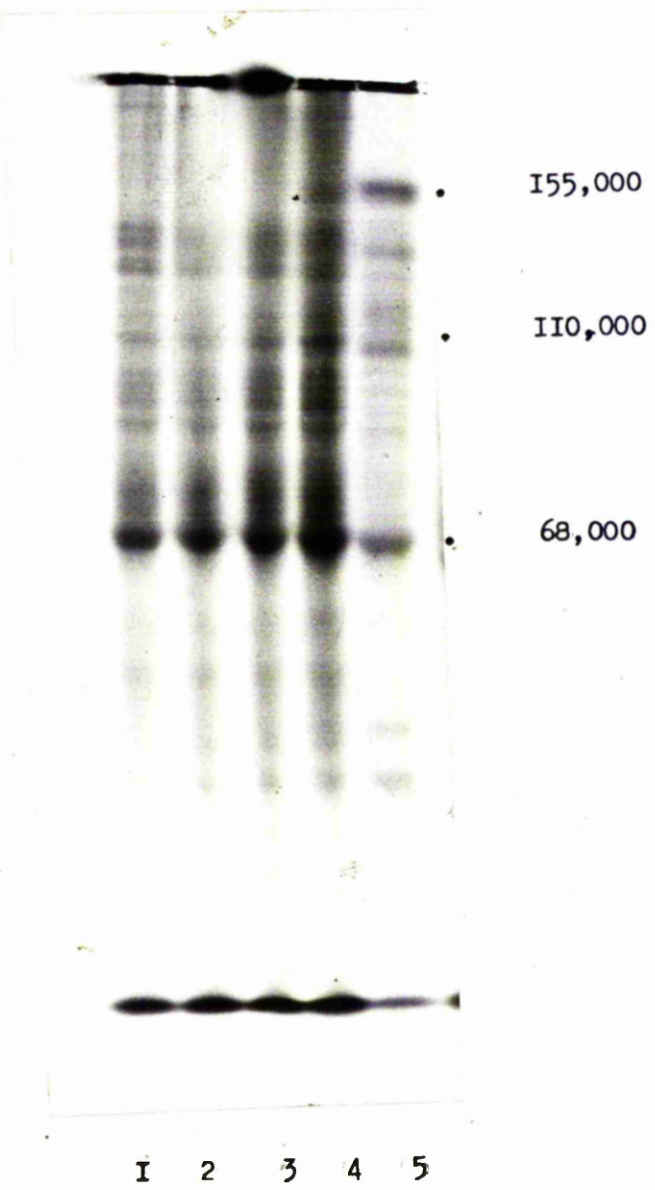
Track 1 : Mock-infected;

Track 2 : 1 hr infected;

Track 3 : 3 hrs infected;

Track 4 : 5 hrs infected;

Track 5 : 7 hrs infected.


155,000
110,000
68,000
1 2 3 4 5

mRNA's may not be translated equally well in different systems. Here it was found that PRV and HSV-1 cause a rapid decline in protein synthesis in HeLa cells. In C13 cells, though inhibition is again rapid, at 1 hr post-infection with PRV there is a stimulation of protein synthesis. Since few virus specific proteins are present even at 2-3 hrs post-infection, this induction is not due to activation of viral protein synthesis. Possibly the cells after infection undergo some form of sensatisation, as a result of which the translation machinery becomes more active than before infection. A similar stimulation is observed in PRV infected BS-C-1 cells. In BS-C-1 cells inhibition is not rapid but gradual. These results emphasize the importance of the host cell in modulating virus infection. In this project most of the experiments were carried out on HeLa cells as this cell line showed a gradual decrease in the protein synthesis after PRV infection, with no stimulation.

# Chapter 6. Effect of herpes virus on the polyribosome disaggregation in the infected cells.

## 6.1. Introduction

When certain animal viruses infect their host cells there is observed a decrease in the number of functioning polyribosomes concomitant with an abrupt drop in the amino acid incorporation into protein. For example, in Picorna virus infected cells cellular mRNA's, after being released from the decaying polyribosomes, appear to be incapable of reentering the ribosomes (Willems & Penman, 1966). But on the other hand, in the case of the Rhabdovirus, VSV, there is no significant alteration in the polyribosome pattern during the course of infection (Nishioka & Silverstein, 1978a). In HSV-1 infected cells disaggregation of host polyribosomes is followed by the gradual appearance of virus specific polyribosomes which have a higher sedimentation value (Sydiskis & Roizman, 1966). Faster sedimenting virus specific polyribosomes have also been detected in PRV infected RK cells at 2.5 hrs after infection (Ben Porat et al., 1971). The initial decrease in polyribosomes at 2 hr post infection in HSV-1 infected vero cells, possibly due to a decrease in the rate of polypeptide chain initiation, coincides with an overall decrease in protein synthesis at that time. As infection proceeds the polyribosomes increase in size by recruiting released monosomes and protein synthesis also increases. But late in infection the protein synthetic activity of the infected cell polyribosomes declines progressively (Silverstein & Engelherdt, 1979). Fenwick & Walker (1978)

have shown that in HSV-2 infected vero cells the polyribosome disaggregation is caused by the infecting virus particles. It has been reported that as an indication of the inhibition of protein synthesis in PRV infected HeLa cells, the polysome/monosome ratio decreases significantly at 3 hrs post infection (Vass, 1975). Since the evidence of Fenwick & Walker (1978) with HSV-2 is in contrast to the previous results with HSV-1 (Sydiskis & Roizman, 1966) it was of particular interest to study the effect of PRV in the absence of virus protein synthesis.

## 6.2. Polyribosome disaggregation after virus infection.

The disaggregation of polyribosomes in HeLa cells after PRV infection was approached in three different ways.

### 6.2.1. Disaggregation in the presence of Cycloheximide.

Confluent monolayers of HeLa cells were infected with PRV in the presence of cycloheximide (50μg/ml) for 2 hours. Cytoplasmic extracts were analysed by sucrose gradient centrifugation to determine the polyribosome content. The results (Fig. 6.2.1.1) show a marked disaggregation of polyribosomes after infection, under conditions which prevented synthesis of viral proteins. To show more clearly the effects on disaggregation the data from a typical experiment are presented as percent polyribosomal RNA calculated from the polyribosome profiles (Table 6.2.1.1). HSV-1 strain MP17, was found to exhibit a similar inhibitory effect (Table 6.2.1.1). Since cycloheximide at a concentration of 50μg/ml caused more than

Fig. 6.2.1.1. Polyribosome disaggregation in herpes virus infected cells.

Confluent monolayers of HeLa cells were mock-infected or infected with PRV and HSV-1 (MP17 Strain) in the presence of Cycloheximide (50µg/ml). At 2 hrs post infection cells were harvested and the cytoplasmic extracts, made according to the procedure described in Materials and Methods, were analysed by sucrose gradient centrifugation on 15 - 30% (w/v) sucrose in RSB at 27,000 r.p.m. for 110 min at 4°C in the Beckman SW27 rotor. Gradients were then pumped through a Gilford recording spectrophotometer at 260nm. The polyribosome profiles were traced.

———— Mock-infected

..... Infected with PRV, 20 p.f.u./cell.

—·—·— Infected with HSV-1, 20 p.f.u./cell.

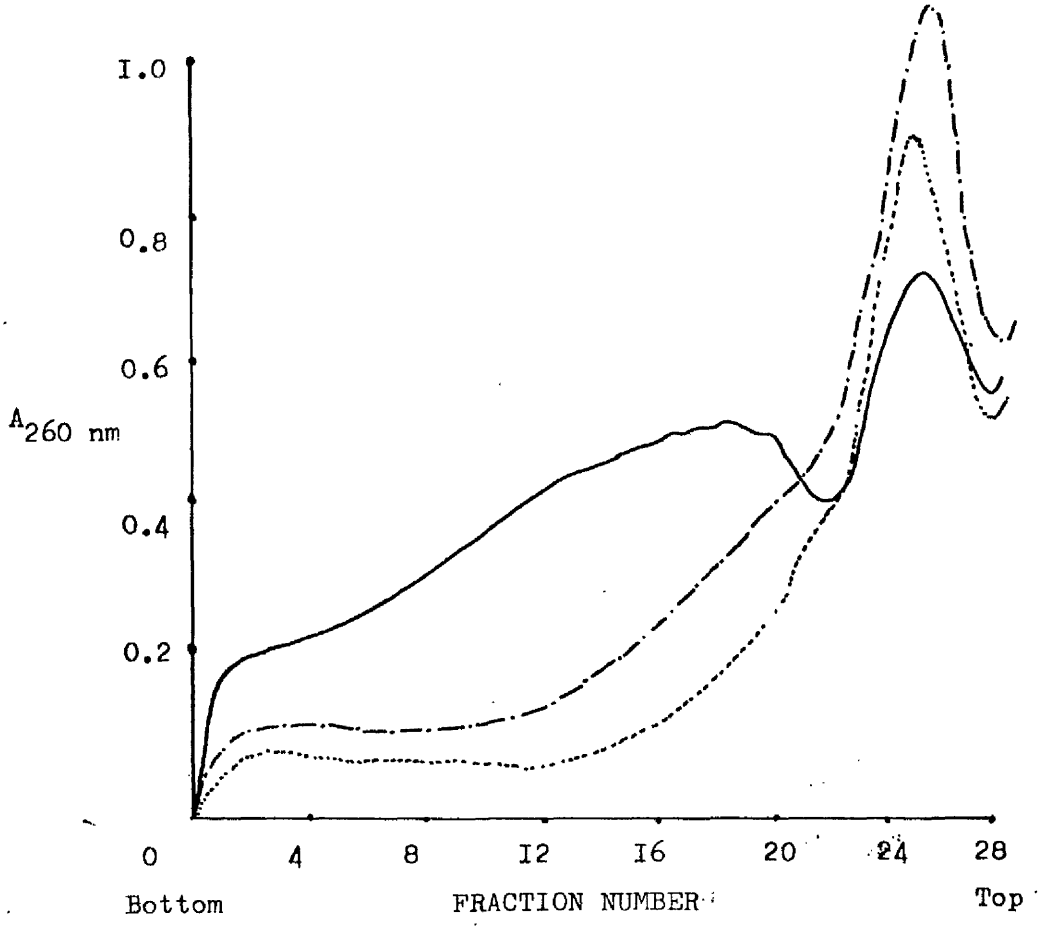

I.0
0.8
0.6
$A_{260\ nm}$
0.4
0.2
0
4
8
I2
I6
20
24
28
Bottom
FRACTION NUMBER
Top

Table 6.2.1.1.

Polyribosome disaggregation after herpes virus infection.

| Nature of the virus | p.f.u./cell | % polyribosomes |
|---|---|---|
| - | - | 70.0 |
| PRV | 20 | 42.0 |
| HSV-1, MP17 | 20 | 47.0 |

Confluent monolayers of HeLa cells were mock-infected or infected with PRV and HSV-1 at a multiplicity of 20 p.f.u./cell in the presence of Cycloheximide (50μg/ml). At 2 hr post-infection, cytoplasmic extracts from both mock-infected and infected cells were analysed by sucrose density gradient centrifugation at 27,000 r.p.m. at 4°C for 110 min in the Beckman SW27 rotor to determine the polyribosome content (Materials and Methods). The percent polyribosomes were determined by cutting and weighing the tracing paper under appropriate areas of the tracing.

The polyribosome area taken was the area under the curve due to polyribosomes with more than 2 ribosomes.

95% inhibition of protein synthesis (Fig. 6.2.1.2) the results indicate that polyribosome disaggregation occurs in cells infected with PRV and HSV-1 even when there is a maximum inhibition of the synthesis of virus proteins.

6.2.2. Effects of multiplicity of infection.

Confluent monolayers of HeLa cells were infected with PRV at different multiplicities in the presence of cycloheximide (50μg/ml). Analysis of the cytoplasmic extracts 2 hrs after infection showed a gradual decrease in the polyribosome content with the use of increasing numbers of infecting virus particles. The data from a typical experiment is shown in Table 6.2.2.1. This is an added evidence supporting the involvement of virus component(s) in polyribosome disaggregation.

6.2.3. Use of inactivated PRV.

HeLa cells were infected with normal and inactivated PRV at a multiplicity of 50 p.f.u./cell. Details of the inactivation of PRV by u.v. irradiation and heat are given in Materials and Methods. At 2 hr post infection cytoplasmic extracts were analysed to determine the polyribosome content. The results of a typical experiment showed that with normal PRV there was little decrease in the polyribosome content but with inactivated virus there was a marked disaggregation (Table 6.2.3.1). Since the measurement is made at only 2 hr post infection the minor decrease after normal PRV infection is expected though at later times disaggregation is marked. The substantial disaggregation by inactivated PRV definitely suggests the involvement of the virus

Fig. 6.2.1.2. Protein synthesis in Cycloheximide treated cells.

Confluent monolayers of HeLa cells grown on cover slips in 50mm petridishes were infected with PRV at 50 p.f.u./cell in the presence of Cycloheximide. At 2 hrs post infection cells were labelled for 30 min with $\mu$Ci $^{35}$S-methionine per petridish in methionine-free medium containing the same concentration of Cycloheximide. After labelling TCA precipitable radioactivity was determined, as described in Materials and Methods.

100% protein synthesis = $0.98 \times 10^5$ cpm.

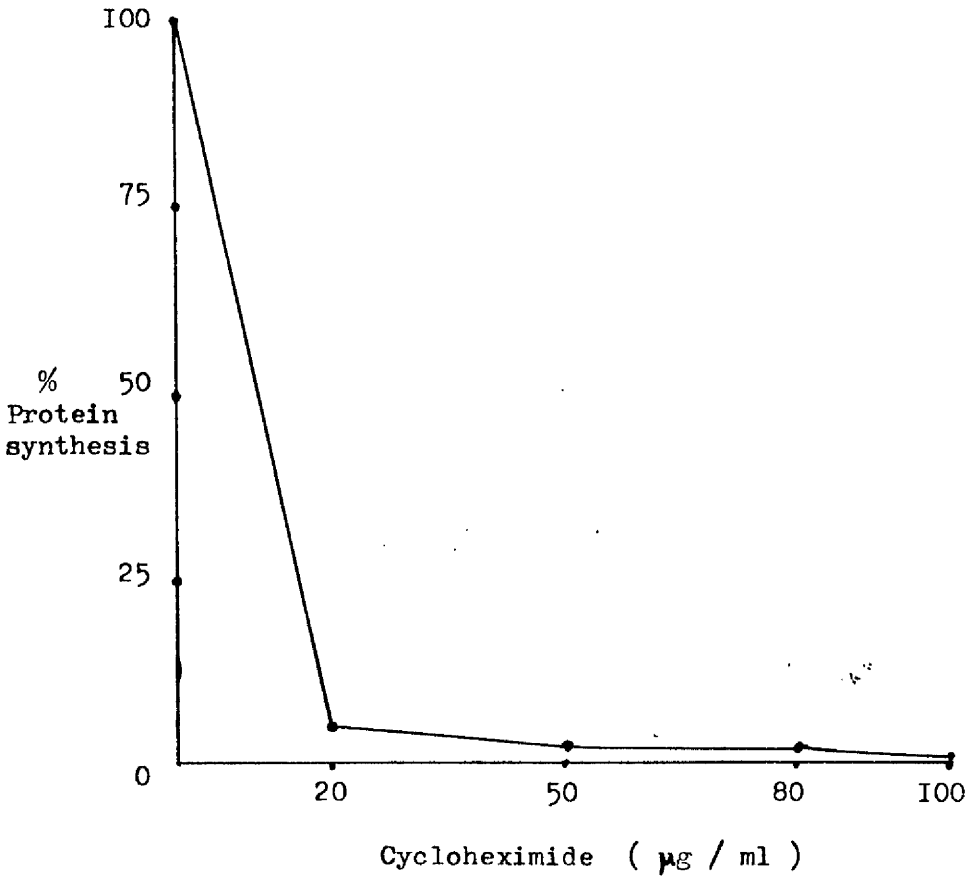

100
75
50
25
0
% Protein synthesis
20
50
80
100
Cycloheximide ( µg / ml )

Table 6.2.2.1.

Polyribosome disaggregation at different multiplicities of infection.

| p.f.u./cell | % polyribosomes |
|---|---|
| 0 | 67 |
| 10 | 40 |
| 20 | 38 |
| 30 | 28 |

Confluent monolayers of HeLa cells were infected with PRV at 10, 20 and 30 p.f.u./cell in the presence of Cycloheximide (50$\mu$g/ml). At 2 hrs post-infection cells were harvested and the cytoplasmic extracts were analysed on sucrose gradient centrifugation to determine the polyribosome content (Materials and Methods). The percent polyribosomes were determined by cutting and weighing of the tracing paper under appropriate area (as in Table 6.2.1.1.).

Table 6.2.3.1.

Polyribosome disaggregation by inactivated virus

| Nature of virus | Multiplicity of Infection | % polysome |
|---|---|---|
| - | - | 67.0 |
| Normal PRV | 50 p.f.u./cell | 60.4 |
| u.v. Irradiated PRV (1000 ergs/$mm^2$) | 50 p.f.u./cell | 37.5 |
| u.v. Irradiated PRV (64000 ergs/$mm^2$) | 50 p.f.u./cell | 42.7 |
| Heat-inactivated PRV ($60^oC$ 60 min) | 50 p.f.u./cell | 35.2 |

Confluent monolayers of HeLa cells were mock-infected or infected with normal and inactivated PRV. At 2 hrs post-infection, cytoplasmic extracts were analysed by sucrose gradient centrifugation at 27,000 r.p.m. for 110 min in the Beckman SW27 rotor to determine the polysome content (Materials and Methods). The percent polysomes were determined by cutting and weighing the tracing paper under appropriate areas of the tracing, as before.

The polysome area taken was the area under the curve due to polysomes with more than 2 ribosomes.

particles in polyribosomes disaggregation.

## 6.3. Discussion.

The experiments described here suggest that the infecting PRV and HSV-1 particles cause polyribosome disaggregation even when infected cell protein synthesis is inhibited almost completely. This indicates that disaggregation does not involve any virus specific proteins synthesized in the infected cells. That the active material(s) is a structural component(s) of the virus is supported by the increase in polyribosome disaggregation which occurs when the number of the infecting PRV particles is increased. Moreover, inactivated PRV also causes substantial disaggregation of polyribosomes. It seems clearer, therefore, that polyribosome disaggregation occurs due to the effects of the infecting virus particles themselves.

# Chapter 7. The effects of hypertonicity on protein synthesis in PRV infected cells

## 7.1. Introduction.

Exposure of HeLa cells to hypertonic medium results in a complete and reversible inhibition of protein synthesis (Saborio et al., 1974). The inhibition occurs at the level of peptide chain initiation and is accompanied by the breakdown of polyribosomes. In polio-, VSV- and reo-virus infected cells the mRNA's that are translated after exposure of cells to hypertonic medium are predominantly viral mRNA's (Nuss et al., 1975, Oppermann & Koch, 1976a). This selective inhibition of host peptide chain initiation by hypertonicity indicates that the initiation of viral mRNA translation is more efficient than that of host mRNA. Exposure of infected cells to hypertonic medium provides a unique tool with which to study early events in the infectious cycle of at least some viruses by permitting the efficient unmasking of virus specific polypeptide synthesis (Nuss et al., 1975) as well as the study of the viral proteins in cells where host protein synthesis is not inhibited after viral infection (Oppermann & Koch, 1976b).

There have been a few similar studies in herpesvirus infected cells. In PRV infected HeLa cells, both HeLa cell protein synthesis and infected cell protein synthesis late after infection, are susceptible to a high NaCl concentration, (Stevely & McGrath, 1978). Again in HSV-2 infected vero cells host proteins and viral proteins are equally sensitive to an increased concentration of NaCl in the medium (Fenwick &

Walker, 1978). In the case of MCMV infected cells, though hypertonicity causes selective inhibition of host protein synthesis, inhibition of a few late viral proteins does occur. This system allowed a more careful examination of Immediate-early viral proteins than had previously been possible (Chantler, 1978). From similar findings it was proposed that the translational efficiency of viral mRNA's was greatest for early mRNA's and markedly less for late mRNA's (Oppermann & Koch 1976a). Thus it was of interest to study the effects of hypertonicity on Immediate-early protein synthesis in PRV infected cells.

## 7.2. Immediate-early protein synthesis under hypertonic conditions

Host cell protein synthesis and Immediate-early protein synthesis in PRV infected HeLa cells were compared by determining the incorporation of [$^{35}$S] methionine into TCA precipitable materials after removal of the inhibitor when infected cells were grown for 5 hrs in the presence of Cycloheximide (50$\mu$g/ml). The details of the procedure are described in Materials and Methods. Cells grown on coverslips were infected at 50 p.f.u./cell in the presence of Cycloheximide. At 5 hrs post infection cells were washed six times to remove cycloheximide and incubated for 5 min in normal medium. Then after another 10 min incubation in medium containing different concentrations of NaCl, cells were labelled with [$^{35}$S] methionine for 15 min. The cells were harvested and acid precipitable radioactivity was determined, as described in Materials and Methods.

To obtain a clearer idea of the effects of hypertonicity on the synthesis of host proteins and Immediate-early proteins

the results are plotted in terms of the percent incorporation relative to the incorporation in cells grown in medium containing the normal concentration of NaCl (Fig 7.2.1). It appears that both host proteins and viral proteins are susceptible to high salt concentrations almost to the same extent. IE protein synthesis showed slightly more sensitivity at 50mM added NaCl concentration.

## 7.3. Analysis of proteins synthesized in the infected cells under hypertonic conditions.

It was important to see whether all proteins of the mock-infected and infected cells were equally sensitive to the high NaCl concentration. When IE proteins are being made, cell protein synthesis is not inhibited completely and it has been shown that the Immediate-early mRNA preparation contains a large number of host mRNA's (Table 2.4.1). So it could be that in the infected cells host proteins show greater sensitivity than IE proteins to hypertonic medium. For this reason, host proteins and infected cell proteins were analysed by SDS-polyacrylamide gel electrophoresis and and autoradiography. Procedures for the preparation of cell lysates and electrophoresis are described in Materials and Methods. The autoradiogram (Fig 7.3.1) shows that host and viral proteins are equally sensitive to high salt concentration in the medium. None of the infected cell proteins showed any resistance to hypertonicity.

## 7.4. Discussion.

It has been found that the synthesis of both host cell

Fig. 7.2.1. Effect of different concentrations of NaCl solutions on IE protein synthesis in PRV infected HeLa cells.

HeLa cells grown on coverslips in petridishes were mock-infected or infected with PRV at 50 p.f.u./cell in the presence of Cycloheximide, (50μg/ml). At 5 hrs post infection, cells were washed six times to remove Cycloheximide. Then both mock-infected and infected cells were grown in normal medium for 5 min before being treated with different concentrations of NaCl. After 10 min incubation in NaCl containing medium, cells were labelled with [$^{35}$S]-methionine (15μCi/petridish) for 30 min in methionine free medium again containing NaCl. Cells were harvested and acid precipitable radioactivity was determined. Results are presented here as percent of those obtained with medium containing the normal amount of NaCl.

o——————o Mock-infected cell protein

•——————• IE proteins

100% Mock-infected cell protein = $2.26 \times 10^4$ cpm

100% IE proteins = $1.2 \times 10^4$ cpm.

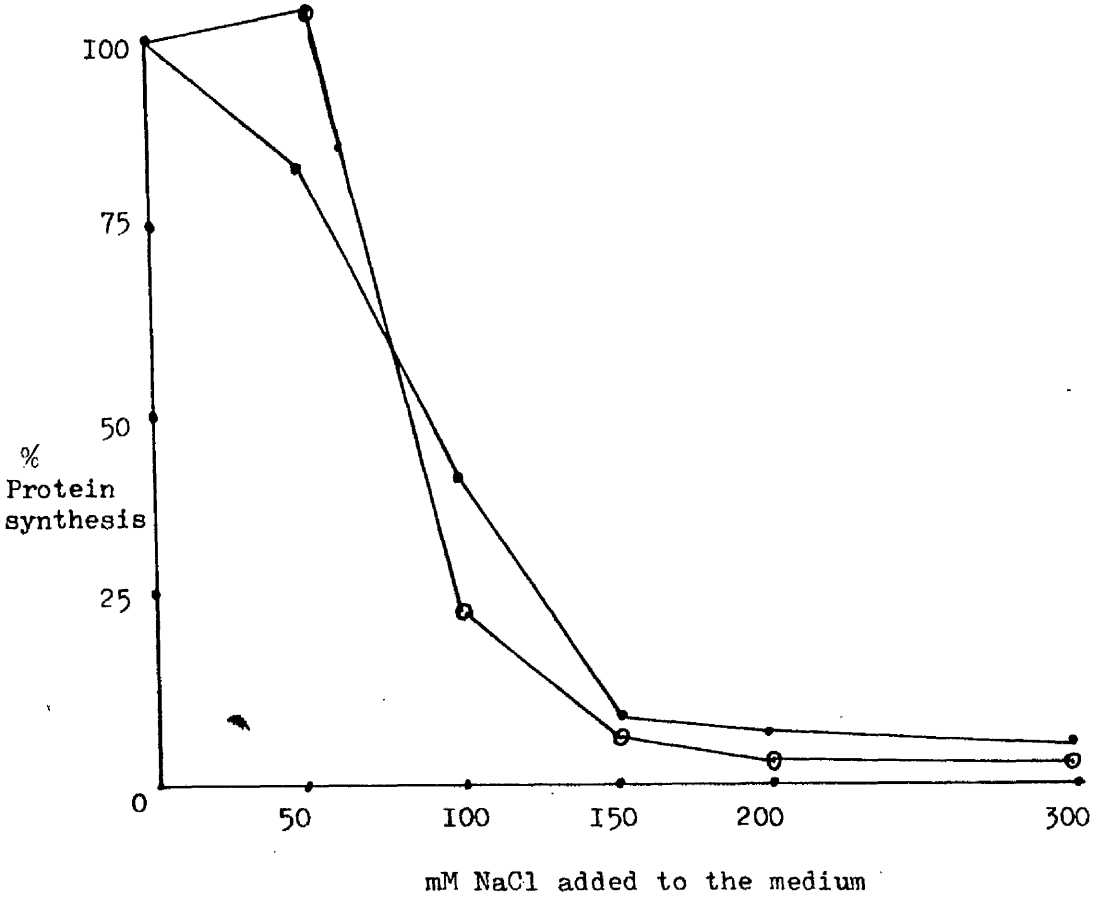
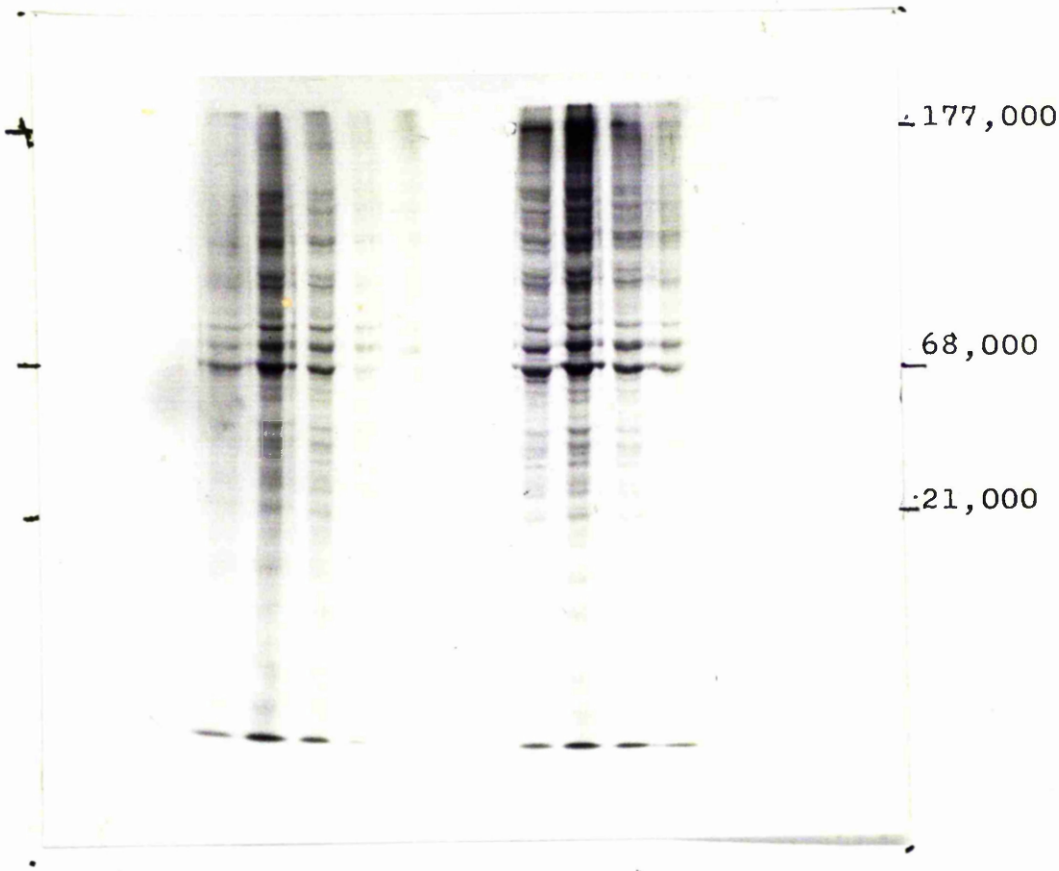

100
75
50
25
0
%
Protein
synthesis
50
100
150
200
300
mM NaCl added to the medium

Fig 7.3.1. Autoradiography of electrophoretically separated proteins synthesized in mock-infected and infected cells under hypertonic conditions.

Cells grown in 90mm petridishes were mock-infected or infected with PRV at 50 p.f.u./cell in the presence of Cycloheximide (50$\mu$g/ml). At 5 hrs post infection cells were washed to remove Cycloheximide and incubated for 5 min in normal medium. After an additional 10 min incubation in the presence of different concentrations of NaCl, cells were labelled with 25 $\mu$Ci $^{35}$S methionine per petridish for 30 min in methionine free medium again containing NaCl. The cells were washed with BSS, harvested and lysed. The cell lysates were processed for polyacrylamide gel electrophoresis, as described in Materials and Methods. The gel contained 10% (w/v) acrylamide and 0.3% (w/v) bis-acrylamide. The dried gel was exposed to X-ray films for autoradiography.

An equal amount of protein was applied onto each gel track.

Tracks 1 to 6 for mock-infected cell lysates and 7-12 for infected (IE) cell lysates. Tracks 1 and 7 with no added NaCl; 2 and 8 with 50mM NaCl; 3 and 9 with 100mM NaCl; 4 and 10 with 150mM NaCl; 5 and 11 with 200mM NaCl; 6 and 12 with 300mM NaCl.


177,000
68,000
21,000
50 100 150 200 300
mM NaCl
HeLa protein
50 100 150 200 300
mM NaCl
IE protein

protein and IE proteins from PRV infected cells are equally susceptible to high salt concentrations in the medium. Autoradiography shows that none of the infected cell proteins is resistant. Thus, PRV infected cell proteins behave differently from picornavirus infected cell protein and MCMV infected cell proteins in that the latter show some resistance to hypertonicity.

# Chapter 8. The effects of inactivated PRV on host macromolecule synthesis.

## 8.1. Introduction.

In studying the fact that infection of animal cells with viruses results in the inhibition of host macromolecule synthesis it has been shown, using metabolic inhibitors like actinomycin D and Cycloheximide and also u.v. irradiated virus, that in vaccinia virus infected cells a component(s) of the infectious particle can cause the inhibition (Moss 1968, Shatkin, 1963), and this effect requires no virus specific macromolecule synthesis.

Similarly, heat inactivated Frog virus can inhibit host macromolecule synthesis almost to the same extent as does the normal virus (Goorha & Granoff 1974; Raghow & Granoff, 1979), again indicating the involvement of a structural component(s) of the virion. In picornaviruses, mutants defective in the ability to inhibit host protein synthesis map in the regions of the genome coding for structural proteins (Steiner-Pryor & Cooper, 1973).

Within two hours of infection with herpes virus, host polyribosomes are dispersed, host protein synthesis declines and is superseeded by viral protein synthesis (Sydiskis & Roizman, 1966). With HSV-2 substantial inhibition of host protein synthesis occurs in whole cells after infection with u.v. irradiated virus and also in cytoplasts, the cells having been enucleated before infection (Fenwick & Walker, 1978). Thus

in many cases inhibition of host protein synthesis is caused, at least in part, by a structural component(s) of the virus. Inactivated PRV was used to investigate whether any structural component(s) could exert an inhibitory effect on host macromolecule synthesis.

## 8.2. Inactivation of PRV.

### 8.2.1. U.V. Irradiation.

A PRV preparation was diluted 15 times with PBS containing 1% (w/v) glucose and irradiated as required by a 15 w germicidal lamp, as described in Materials and Methods. The viability of the irradiated virus was checked by plaque assay. The inactivation is shown in Fig. 8.2.1.1. It was found that at 500 ergs/$mm^2$ input, virus inactivation was 90% and at 64,000 ergs/$mm^2$ input, inactivation was about 99.9% (Table 8.2.1.1.).

### 8.2.2. Heat treatment.

A 15 times diluted PRV preparation in PBS containing 1% (w/v) glucose was heated at 60°C for 60 min. The extent of heat inactivation was determined by plaque assay and was found to be more than 99.7% (Table 8.2.1.1).

To study the effects of inactivated PRV on host macromolecule synthesis, viruses irradiated at 64000 ergs/$mm^2$ and heat inactivated at 60°C for 60 min were used at a multiplicity of 50 original p.f.u./cell.

Fig. 8.2.1.1. Inactivation of PRV by u.v. irradiation

Virus stock was diluted 15 times with PBS containing 1% (w/v) glucose and irradiated at different energy levels, taking 2ml of diluted preparation per 50mm petridish, as described in Materials and Methods. The irradiated virus stock was tested for inactivation by plaque assay.

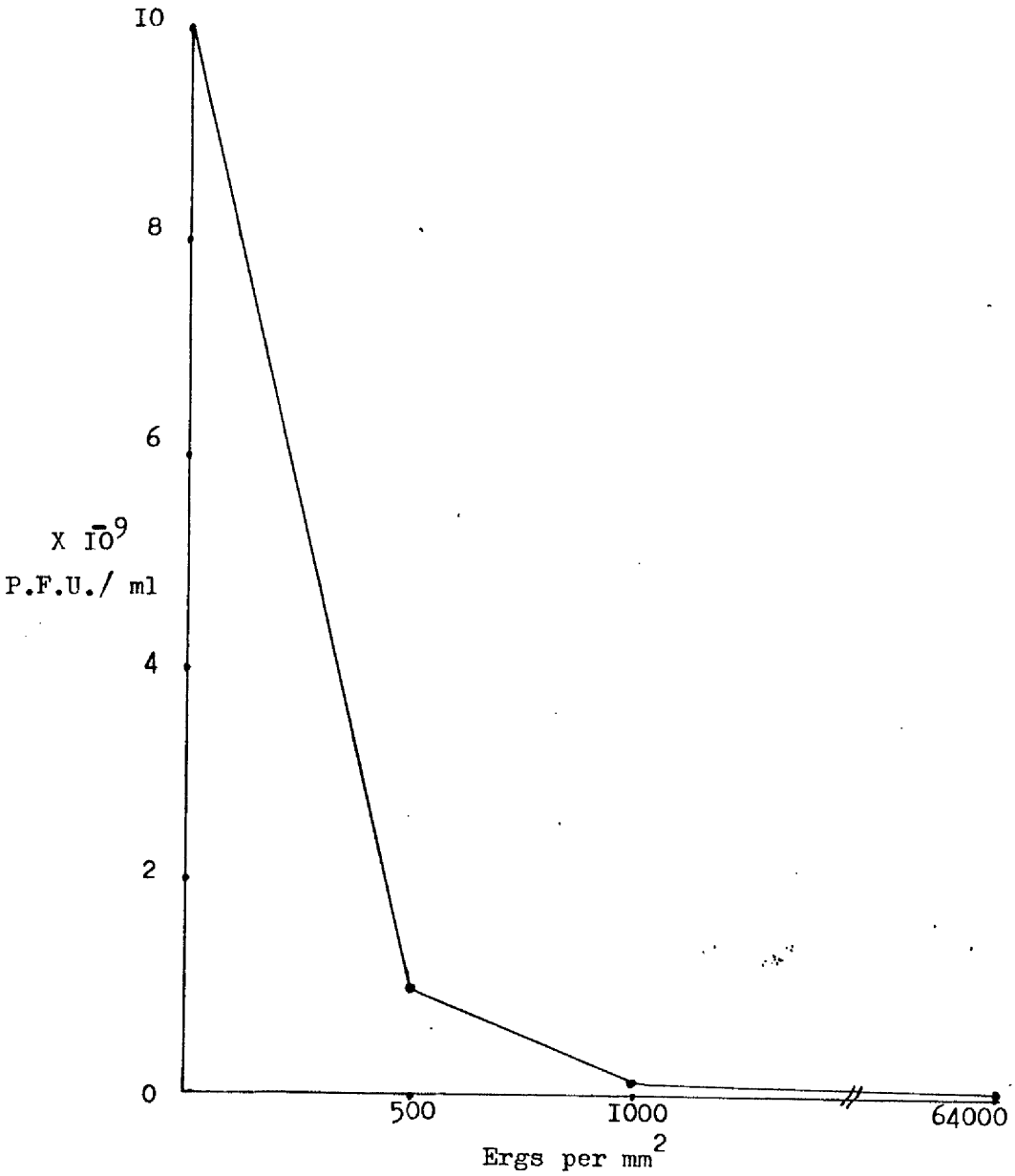

10
8
6
X $10^{9}$
P.F.U./ ml
4
2
0
500
1000
64000
Ergs per $mm^{2}$

Table 8.2.1.1.

Inactivation of PRV by u.v. irradiation and heat.

| Nature of treatment | Dose | % inhibition (a) |
|---|---|---|
| U.V. Irradiation | 500 ergs/mm$^2$ | 90.0 |
| | 1000 ergs/mm$^2$ | 98.5 |
| | 64000 ergs/mm$^2$ | 99.88 |
| Heat treatment | 60$^o$C. 60 min | 99.78 |

A virus stock diluted 15 times with PBS containing 1% (w/v) glucose was irradiated at different energy levels. Similarly, 10ml aliquots of dilute virus preparation were heated at 60$^o$C for 60 min. The extent of inactivation was followed by plaque assay, as described in Materials and Methods.

(a) Inhibition was determined by measuring the p.f.u./ml of the untreated PRV preparation and comparing the p.f.u./ml of the inactivated virus preparation with it. In the original PRV preparation the titre was 1 x $10^{10}$ p.f.u./ml.

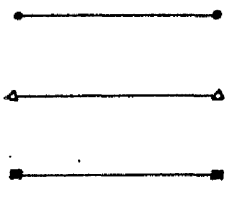
## 8.3. Protein synthesis in cells infected with inactivated PRV.

Incorporation of [$^{35}$S] methionine into TCA precipitable material in mock-infected cells as well as in cells infected with normal and inactivated PRV was used as a tool to study the effect of inactivated PRV on host protein synthesis. Confluent monolayers of HeLa cells grown on coverslips in petridishes were mock-infected or infected with normal and inactivated PRV at a multiplicity of 50 original p.f.u./cell. At different times after infection, cells were labelled for 30 min with 15 $\mu$Ci [$^{35}$S] methionine per petridish in methionine-free medium. Determination of radioactivity in TCA precipitable material was as described in Materials and Methods. The results of a typical experiment are plotted as a percentage relative to the incorporation into mock-infected cells (Fig 8.3.1). At 8 hr post infection, normal PRV caused almost 70% inhibition of host-protein synthesis. Heat inactivated and u.v. irradiated PRV also caused an almost equal extent of inhibition. Infection was continued up to 24 hrs in further experiments with inactivated PRV to determine whether recovery could occur in the absence of new virus formation. Here inhibition was 95% with normal virus compared to about 70% inhibition by inactivated virus at 24 hrs post infection (Fig 8.3.2). In this case early inhibition was less rapid. The reason is unknown. However, at 24 hrs post infection the differences between protein synthesis in cells infected with normal virus and inactivated virus are significant. The higher degree of inhibition by normal, untreated virus, at 24 hrs post infection compared to

Fig. 8.3.1. Protein synthesis in HeLa cells infected with inactivated PRV.

Cells grown on coverslips in 50mm dia. petridishes were mock-infected or infected with normal and inactivated PRV at a multiplicity of 50 original p.f.u./cell. At different times after infection cells were labelled with 15 $\mu$Ci [$^{35}$S]-methionine per petridish in methionine-free medium for 30 min. Then TCA precipitable radioactivity of the labelled cells was determined, as described in Materials and Methods.

100% ≡ $4 \times 10^5$ cpm (Measured in mock-infected cells).



Infected with normal virus

Infected with heat inactivated virus.

Infected with u.v. irradiated virus. (64K ergs/mm$^2$)

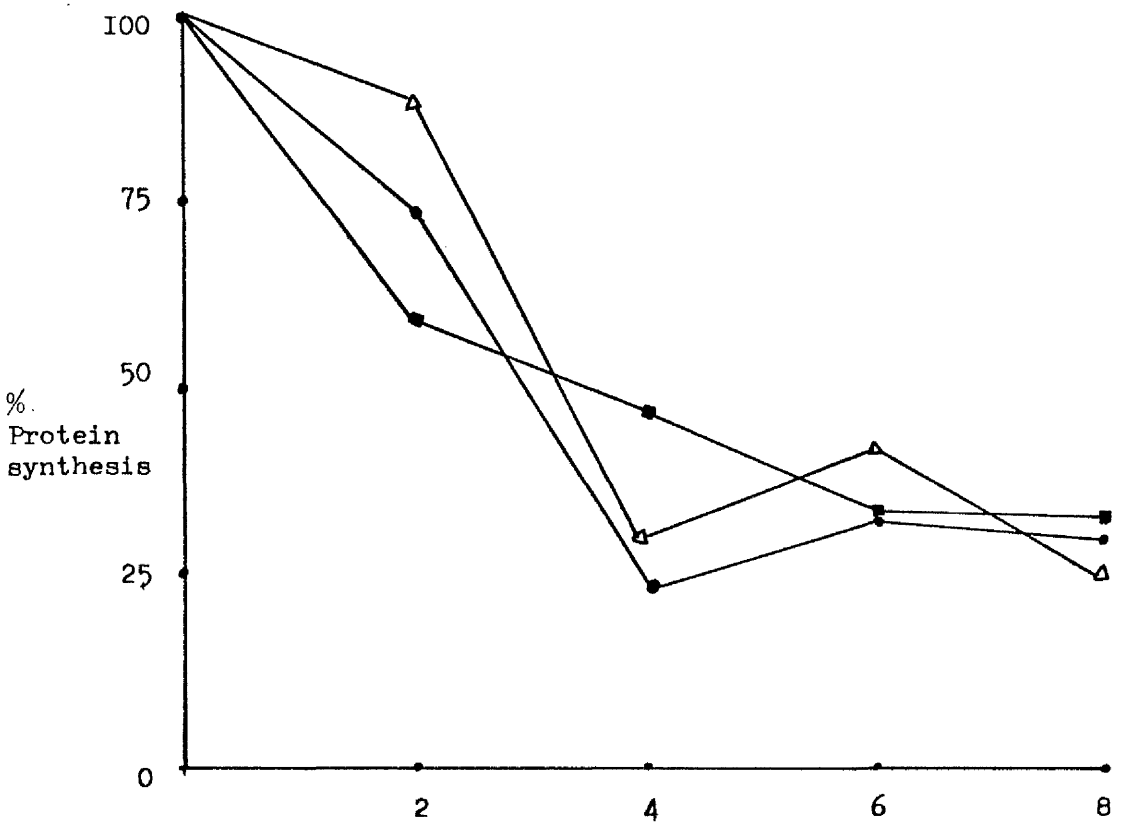
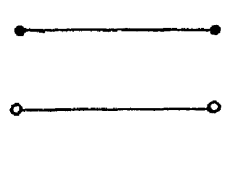

100
75
50
%
Protein
synthesis
25
0
2
4
6
8
Hours post-infection.

Fig. 8.3.2. Protein synthesis in HeLa cells infected with inactivated PRV.

Confluent monolayer of HeLa cells grown on coverslips in 50mm petridishes were mock-infected or infected with normal and inactivated PRV at a multiplicity of 50 original p.f.u./cell. At different times after infection cells were labelled with 15 $\mu$Ci [$^{35}$S] methionine in methionine-free medium for 30 min. The TCA precipitable radioactivity of the labelled cells was determined.

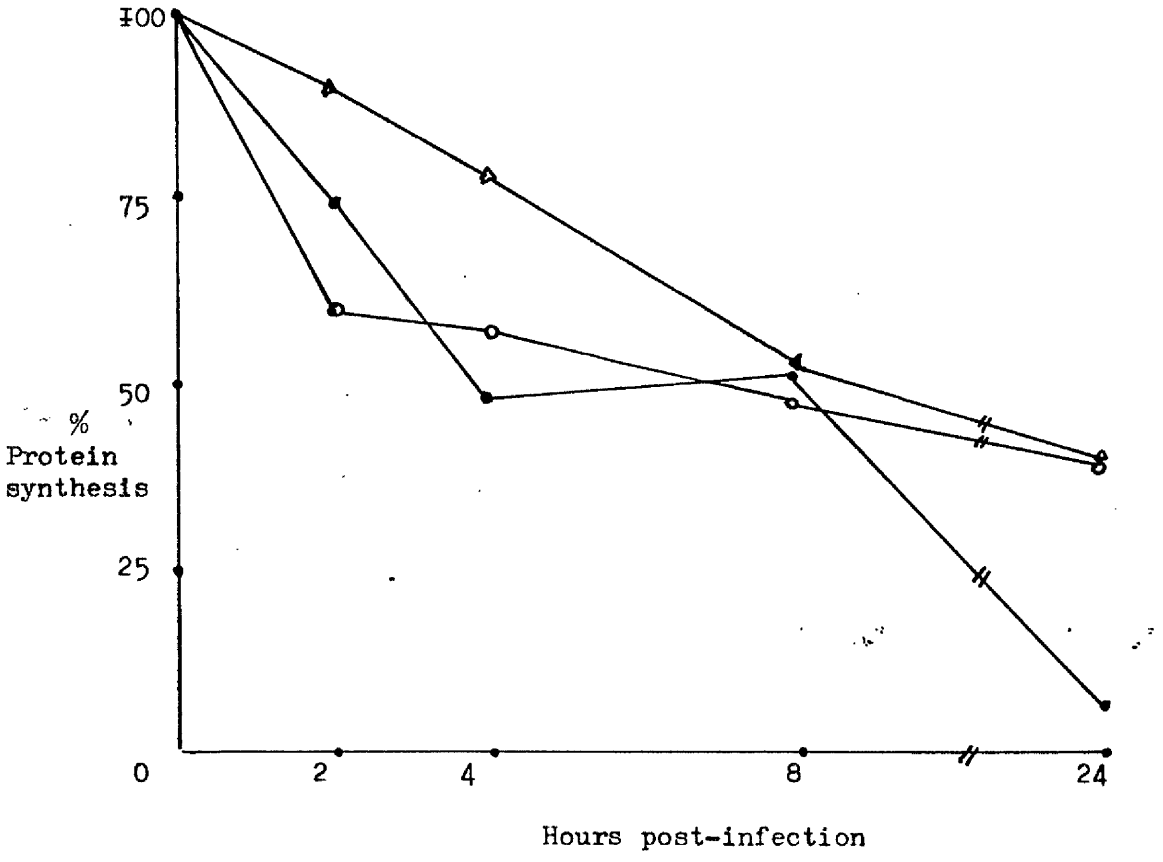
100% ≡ 2.08 x $10^4$ cpm (Measured in mock-infected cells).



Infected with normal PRV.

Infected with u.v. irradiated PRV (64 K ergs/mm$^2$)

Infected with heat inactivated PRV.


100
75
50
25
0
% Protein synthesis
2
4
8
24
Hours post-infection

that by the inactivated virus could be due to the cytopathic effect which is exerted by the normal virus but not by the inactivated virus. Thus it appears that inhibition of protein synthesis in HeLa cells after PRV infection does not necessarily require infective virus particles.

Inactivated PRV was also used to study the extent of inhibition of protein synthesis in C13 cells. Infection, labelling and determination of TCA precipitable radioactivity were carried out essentially in the same way as with HeLa cells. The results are plotted as percent cpm in infected cells compared to that in mock-infected cells (Fig 8.3.3). At 6 hr post infection normal PRV caused 75% inhibition and inactivated PRV inhibited in the range of 55 - 65%. Still the extent of inhibition by inactivated virus was substantial enough to argue that in C13 cells inactivated PRV could cause marked inhibition of protein synthesis.

## 8.4. RNA synthesis in cells infected with PRV.

Since it was found that inactivated PRV could inhibit host protein synthesis, it was of interest to study whether it could inhibit RNA synthesis as well. Infection and labelling of HeLa cells as well as determination of TCA precipitable radioactivity were essentially the same as for protein synthesis, except that $^{3}H$ - uridine was used to label the cells at 15 $\mu$Ci/petridish for 30 min. The results are shown as percent cpm in infected cells compared to that in mock-infected cells (Fig 8.4.1). It is found that 8 hrs post infection RNA synthesis was inhibited by

Fig. 8.3.3. Protein synthesis in C13 cells infected with inactivated PRV.

Confluent monolayer of C13 cells grown on coverslips in 50mm petridishes were mock-infected or infected with normal and inactivated PRV at a multiplicity of 50 original p.f.u./cell. At different times after infection cells were labelled with 15 $\mu$Ci [$^{35}$S]-methionine per petridish in methionine-free medium for 30 min. TCA precipitable radioactivity was then determined.

100% ≡ 15.5 x $10^5$ cpm (Measured in mock-infected cells)

●——● Infected with PRV.

○——○ Infected with u.v. irradiated PRV (64K ergs/mm²)

△——△ Infected with heat inactivated PRV.

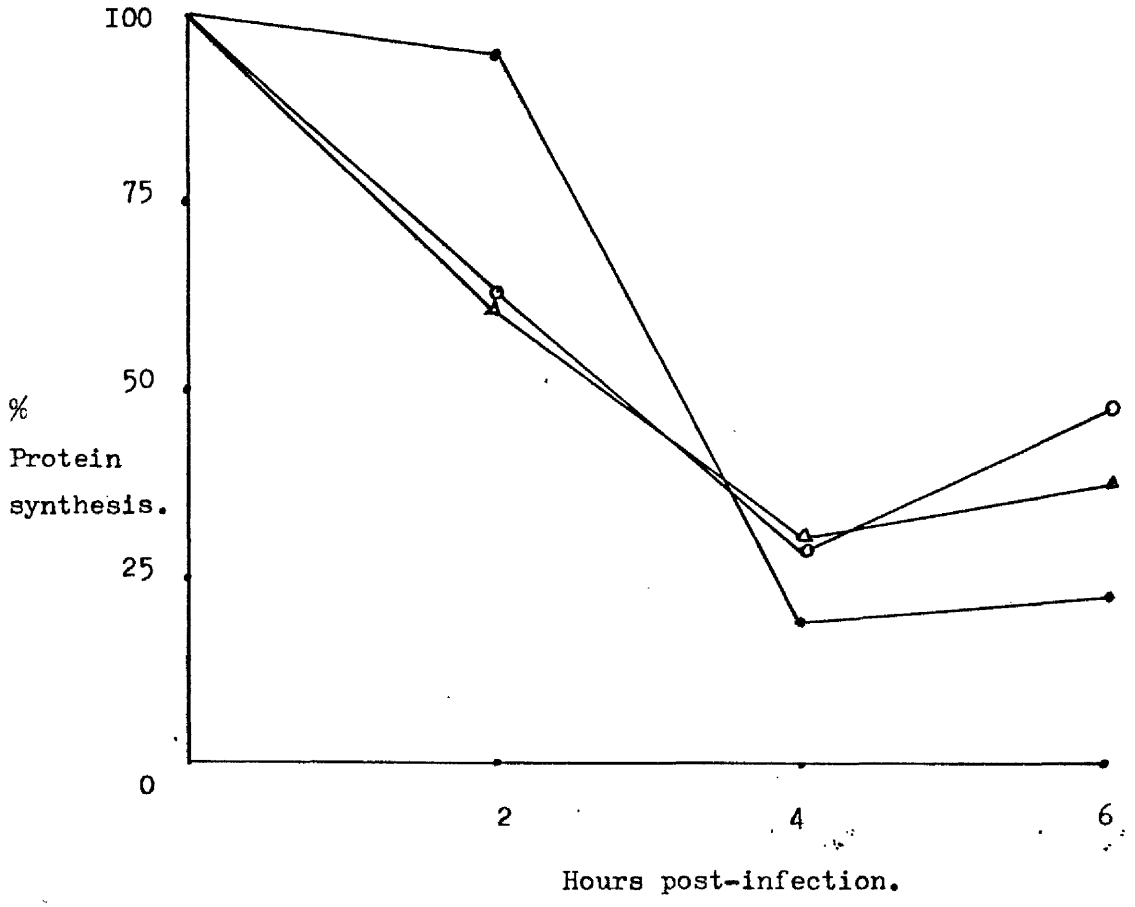
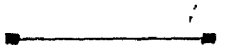

100
75
50
25
0
%
Protein
synthesis.
2
4
6
Hours post-infection.

Fig. 8.4.1. RNA synthesis in HeLa cells infected with inactivated PRV.

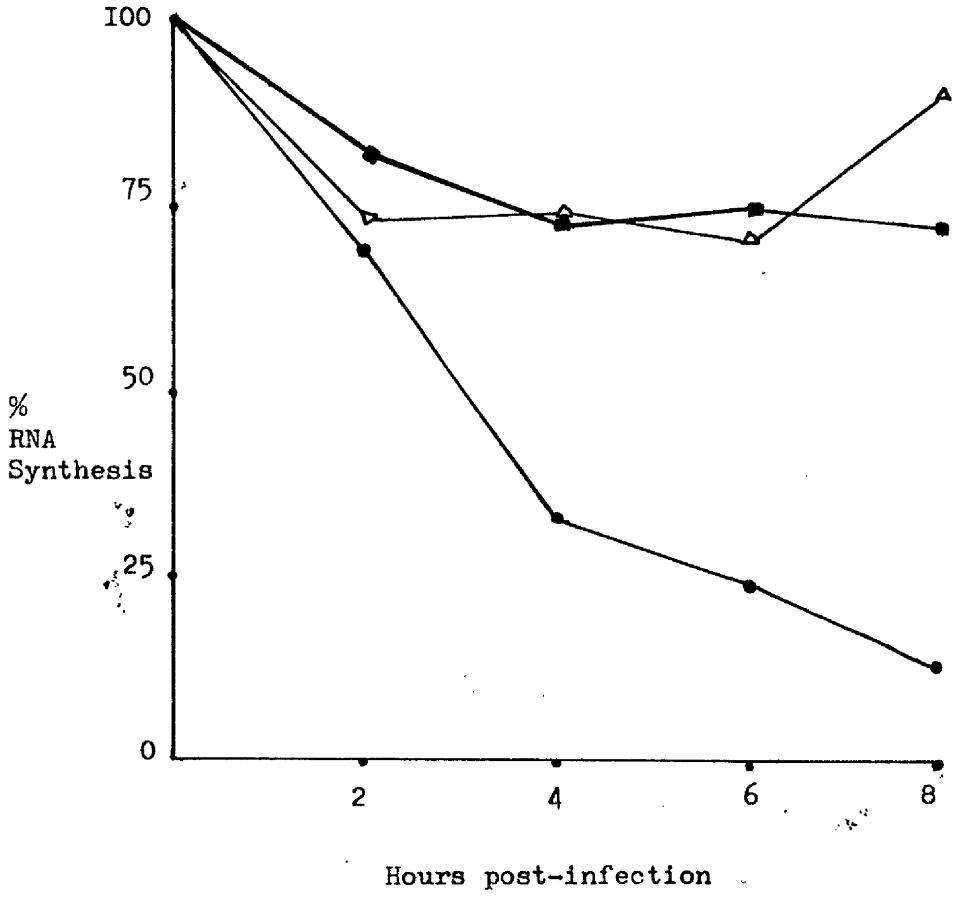
Confluent monolayer of HeLa cells grown on coverslips in 50mm petridishes were mock-infected or infected with normal and inactivated PRV at a multiplicity of 50 original p.f.u./cell. At different times after infection, cells were labelled with 15 $\mu$Ci $^{3}$H - uridine per petridish for 30 min. Then TCA precipitable radioactivity was determined.

100% ≡ $1.48 \times 10^{4}$ cpm (Measured in mock-infected cells)


Infected with normal virus.


Infected with u.v. irradiated virus.


Infected with heat inactivated virus.


100
75
50
25
0
%
RNA
Synthesis
2
4
6
8
Hours post-infection

normal PRV to the extent of 85% but to less than 25% by inactivated virus. So inactivated PRV is much less effective than normal virus in inhibiting at the level of transcription in infected cells.

## 8.5. Discussion.

The experiments show that inhibition of protein synthesis in the infected cells is caused not only by normal infective PRV but also by inactivated PRV. The extent of inhibition by inactivated PRV is almost to the same level as for normal virus. The effects of inactivated PRV are found both in HeLa and C13 cells. At 24 hr post infection normal PRV shows cytopathic effects but inactivated viruses do not. Studies of the effects of inactivated virus on host RNA synthesis show no marked inhibition. From the differential effects of inactivated PRV on translation and transcription, it appears that a component(s) of the virion can inhibit host protein synthesis by acting at some stages in the translation process, but no similar component acts on the RNA synthetic pathways though there is some inhibition of RNA synthesis by inactivated PRV. The significance of this inhibition is unknown. But if the extent of protein inhibition is taken into account, it appears unlikely that inhibition of protein synthesis is a consequence of inhibition of RNA synthesis. It could be a direct action of PRV on the translation process. In herpes virus infected cells, more than one mechanism is likely to be involved in causing inhibition of protein synthesis and one of them is due to the involvement of a virion component(s).

# Chapter 9. Effects of PRV on protein synthesis in the MDL system.

## 9.1. Introduction

Inhibition of translation of host mRNA has been observed in cell free extracts from poliovirus infected cells (Rose et al., 1978; Trachsel et al., 1980). It is also known that cytoplasm from poliovirus infected cells inhibit protein synthesis in reticulocyte lysates (Hunt & Ehrenfeld, 1971). The cytoplasm contains enough free virus protein to cause inhibition. Isolated nucleocapsids and empty capsids can also inhibit translation in cell free extracts (Levintow, 1974).

In vaccinia virus infected cells, a component(s) of the virion is responsible for inhibition (Moss 1968) and in rabbit reticulocyte lysates inhibition is caused by the core (Racevskis et al., 1976; Ben-Hamide & Beand 1978) and even heat disrupted virus can exert an inhibitory effect. In a coupled transcription-translation system, protein synthesis is again depressed by the cores (Cooper & Moss, 1978).

Very little is known about the regulation of herpes virus specific mRNA translation in cell-free system except that in mouse and rabbit reticulocyte lysates protein synthesis is not significantly enhanced by the addition of ribosomal salt wash fractions from HSV-1 infected cells whereas an equivalent fraction from mock-infected cells can stimulate translation of HSV specific mRNA, BHK mRNA and Globin mRNA (Preston, 1977). Since it has been shown that PRV particles can cause poly-

ribosome disaggregation and protein synthesis inhibition in the whole cell it is of interest to study the effect of the particles on cell-free translation.

## 9.2. Inhibition of protein synthesis in the MDL system

Details of the assay procedure for protein synthesis in the MDL system are described in Materials and Methods. Purified PRV was dialyzed overnight against 0.01M Tris-HCl (pH 7.4) at 4°C. Each assay of 11µl contained 20 µCi [$^{35}$S] methionine, a specified amount of mRNA and an appropriate amount of virus or its protein equivalent. At different time intervals during incubation 1µl aliquots were taken in duplicate onto filter discs to determine TCA precipitable radioactivity. There was a marked inhibition of protein synthesis in the presence of virus when an Immediate-early mRNA preparation (Fig 9.2.1), or a late mRNA (Fig. 9.2.2) or Globin mRNA (Fig 9.2.3) were being assayed. These results are also presented as percent inhibition of protein synthesis (Table 9.2.1). The extent of inhibition varied from 61 to 74% which is substantial. Normal and heat inactivated PRV did not show any significant difference in their inhibitory potential. As a control for the addition of exogenous protein to the MDL system, the effect of an equivalent amount of BSA was studied. The results (Table 9.2.2) show that added BSA caused only a small decrease in [$^{35}$S]-methionine incorporation compared to that caused by PRV. It was also found that the inhibition was dose dependent. The extent of inhibition increased with the addition of increasing amounts of virus (Fig. 9.2.4).

Fig. 9.2.1. Effects of PRV on protein synthesis, in vitro, in the MDL system specified by IE mRNA.

Details of the assay of protein synthesis in the MDL system are described in Materials and Methods. The 11$\mu$l assay mixture contained 0.25$\mu$g IE mRNA preparation, 20$\mu$Ci $^{35}$S-methionine and dialyzed PRV. After incubation at 30$^{\circ}$C, 1$\mu$l aliquots were taken in duplicates, at different time intervals, onto filter paper discs and TCA precipitable radioactivity was determined.

●——● IEmRNA + dialyzing buffer.

■——■ IEmRNA + 4$\mu$g protein equivalent of PRV

△——△ IEmRNA + 4$\mu$g protein equivalent of heat inactivated PRV.

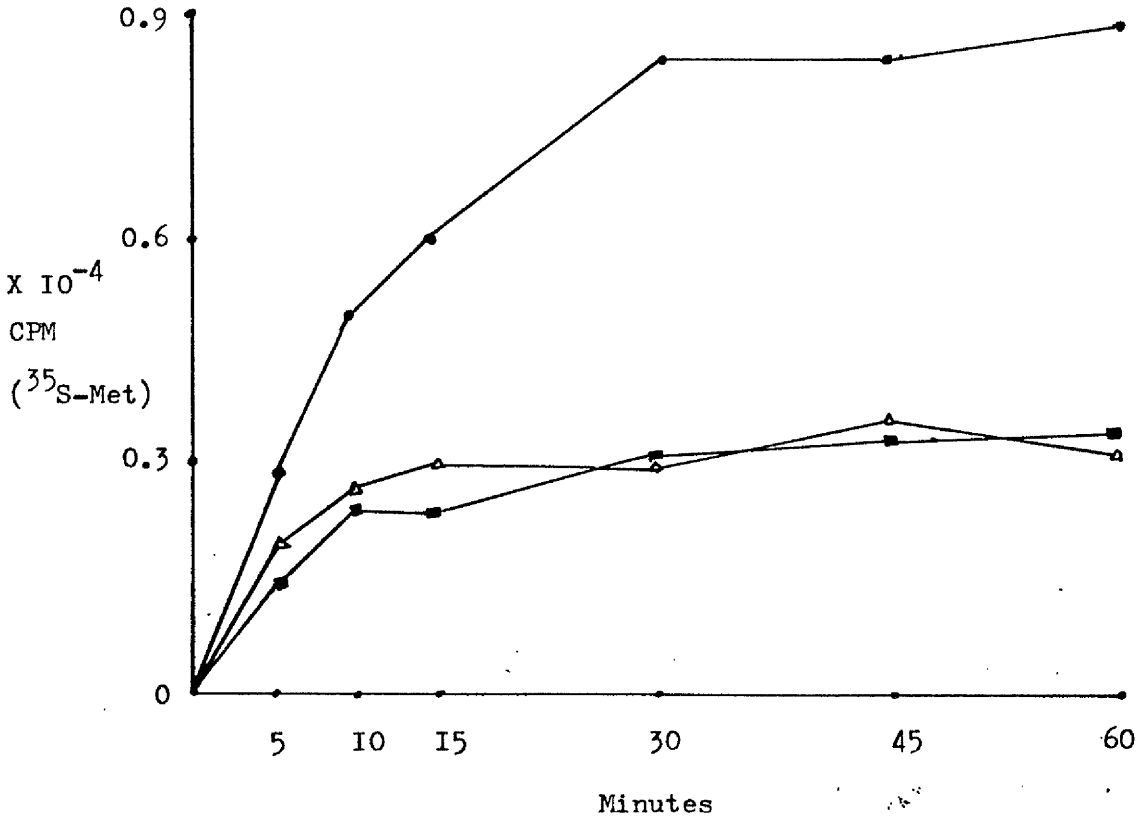

0.9
0.6
0.3
0
X IO$^{-4}$
CPM
($^{35}$S-Met)
5
IO
I5
30
45
60
Minutes

Fig. 9.2.2. Effects of PRV on protein synthesis in the MDL system specified by late viral mRNA.

The 11µl assay mixture contained 20 µCi $^{35}S$ methionine, 0.25µg late viral mRNA and 4µg protein equivalent of PRV. Incubation was at 30°C and at different times reactions were stopped by quick transfer onto ice. 1µl aliquots were taken in duplicate on filter paper discs to determine TCA precipitable radioactivity.

●——● Late mRNA + dialyzing buffer.

■——■ Late mRNA + 4µg protein equivalent of PRV

△——△ Late mRNA + 4µg protein equivalent of heat inactivated PRV.

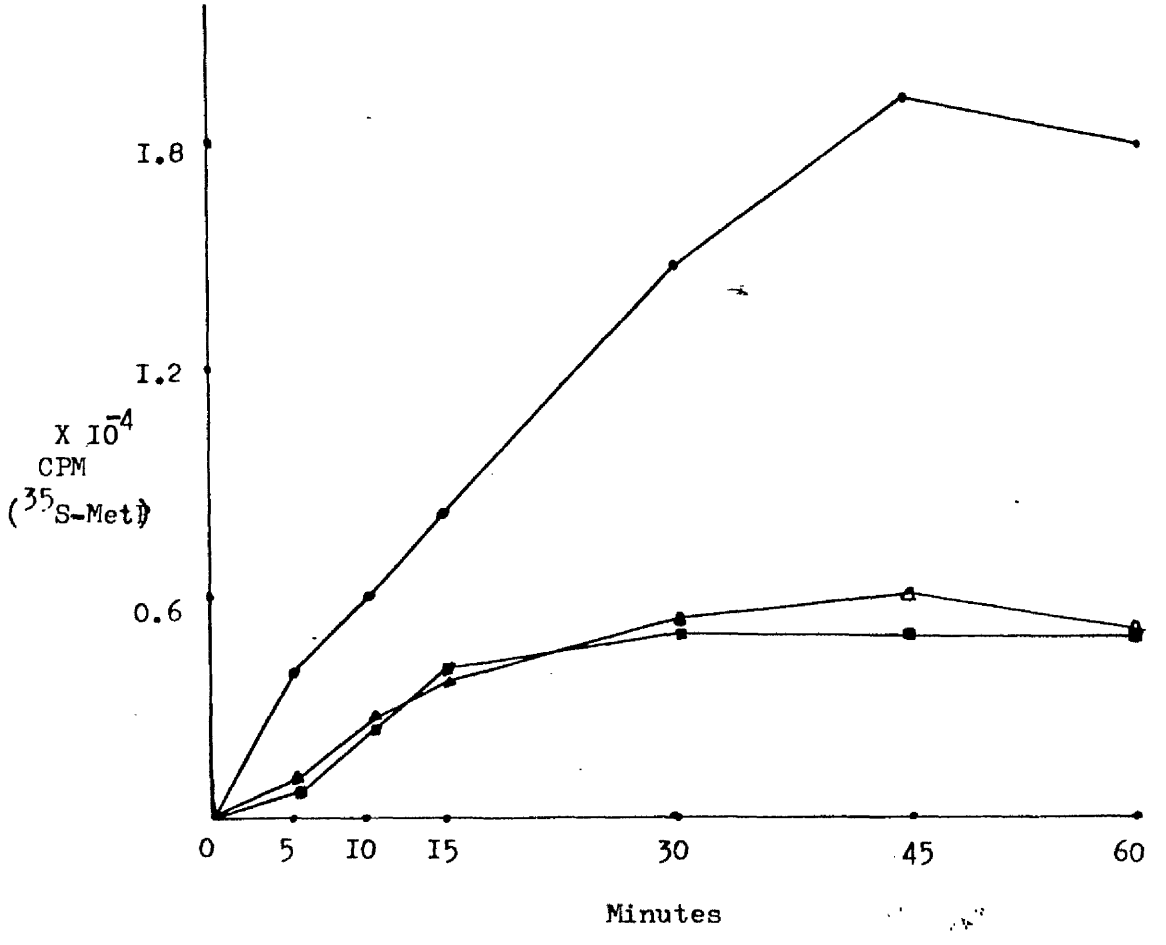

I.8
I.2
X $10^{-4}$
CPM
($^{35}$S-Met)
0.6
0
5
10
15
30
45
60
Minutes

Fig. 9.2.3. Effects of PRV on protein synthesis in the MDL system specified by Globin mRNA.

The 11$\mu$l assay mixture contained 20 $\mu$Ci [$^{35}$S]- methionine, 0.25$\mu$g Globin mRNA and 4 $\mu$g protein equivalent of PRV, normal and heat inactivated. After incubation at 30$^{\circ}$C the reaction was stopped at different time intervals by quick transfer onto ice. 1$\mu$l aliquots were taken in duplicate onto filter discs to determine TCA precipitable radioactivity.

●——● Globin mRNA + dialysing buffer.

■——■ Globin mRNA + 4$\mu$g protein equivalent of PRV.

△——△ Globin mRNA + 4$\mu$g protein equivalent of heat inactivated PRV.

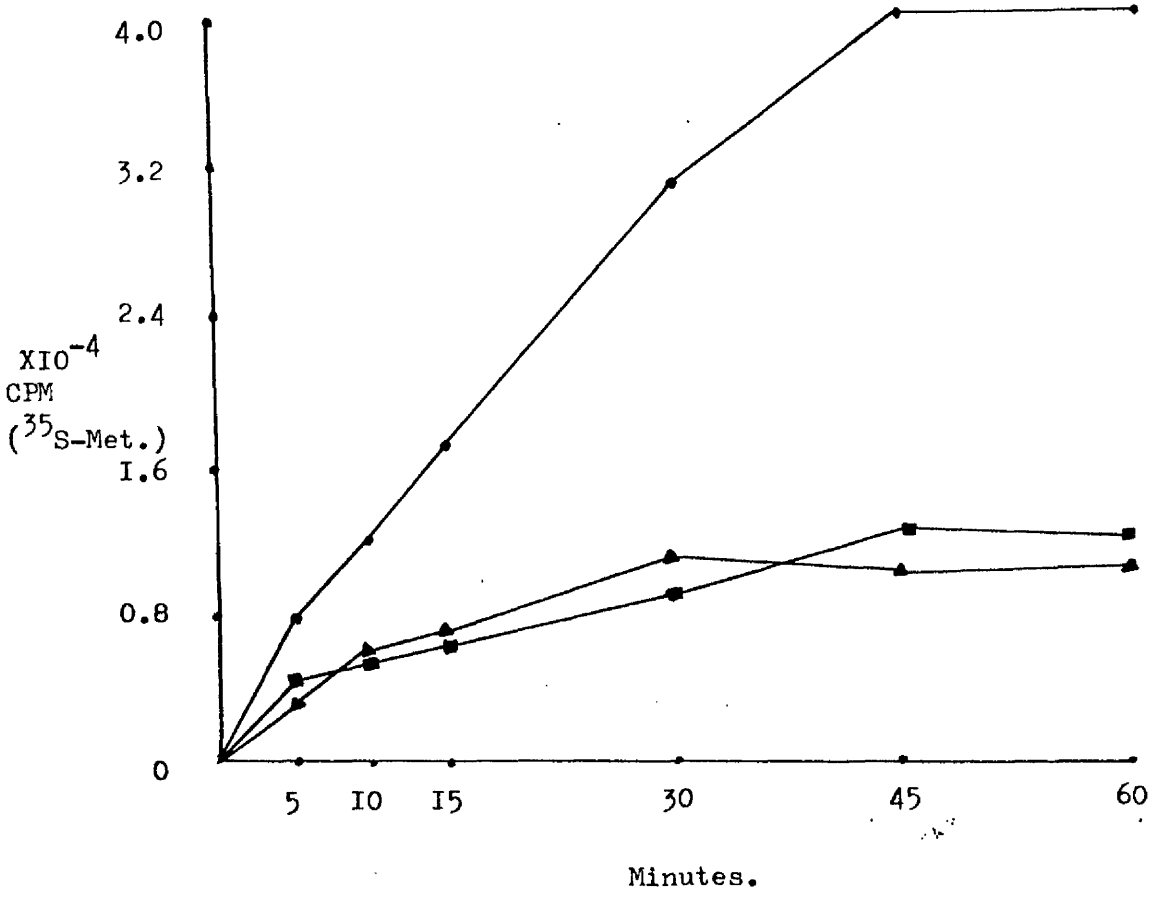

4.0
3.2
2.4
XIO$^{-4}$
CPM
($^{35}$S-Met.)
I.6
0.8
0
5
I0
I5
30
45
60
Minutes.

Table 9.2.1.

Inhibition of protein synthesis in the MDL system by PRV.

| Nature of mRNA | Virus, 4μg protein equivalent | % Inhibition |
|---|---|---|
| Immediate-early PRV mRNA | PRV | 61.0 |
| | ΔPRV | 62.5 |
| Late PRV mRNA | PRV | 72.2 |
| | ΔPRV | 71.0 |
| Globin mRNA | PRV | 70.0 |
| | ΔPRV | 74.0 |

A purified and dialyzed PRV preparation was added to 11 μl assay mixture containing 20 μCi [$^{35}$S]-methionine, 0.25μg IE mRNA or 0.25μg late mRNA or 0.25μg globin mRNA. After 60 min incubation at 30°C 1μl aliquots were taken on to filter paper discs to determine TCA precipitable radioactivity.

Table 9.2.2.

Effect of exogenous protein on protein synthesis, in vitro, in the MDL system

| Nature of mRNA | Nature of protein added | % Inhibition |
|---|---|---|
| | NO PROTEIN | 0 |
| G mRNA | BSA | 20 |
| | PRV | 75 |

The 11 $\mu$l assay mixture contained 20 $\mu$Ci [$^{35}$S]-methionine, 0.25$\mu$g G mRNA, 4$\mu$g BSA or 4$\mu$g protein equivalent of PRV. After 60 min incubation at 30$^{\circ}$C, 1$\mu$l aliquots were taken in duplicates on filter paper discs to determine TCA precipitaable radioactivity.

Fig. 9.2.4. Effects of different concentration of PRV on protein synthesis in the MDL system, specified by Globin mRNA

The 11 $\mu$l assay mixture contained 20 $\mu$Ci [$^{35}$S]-methionine, 0.25$\mu$g Globin mRNA and different concentrations of PRV. After incubation at 30°C the reaction was stopped at different time intervals by quick transfer onto ice. 1$\mu$l aliquots were taken in duplicates on to filter discs to determine the TCA precipitable radioactivity.

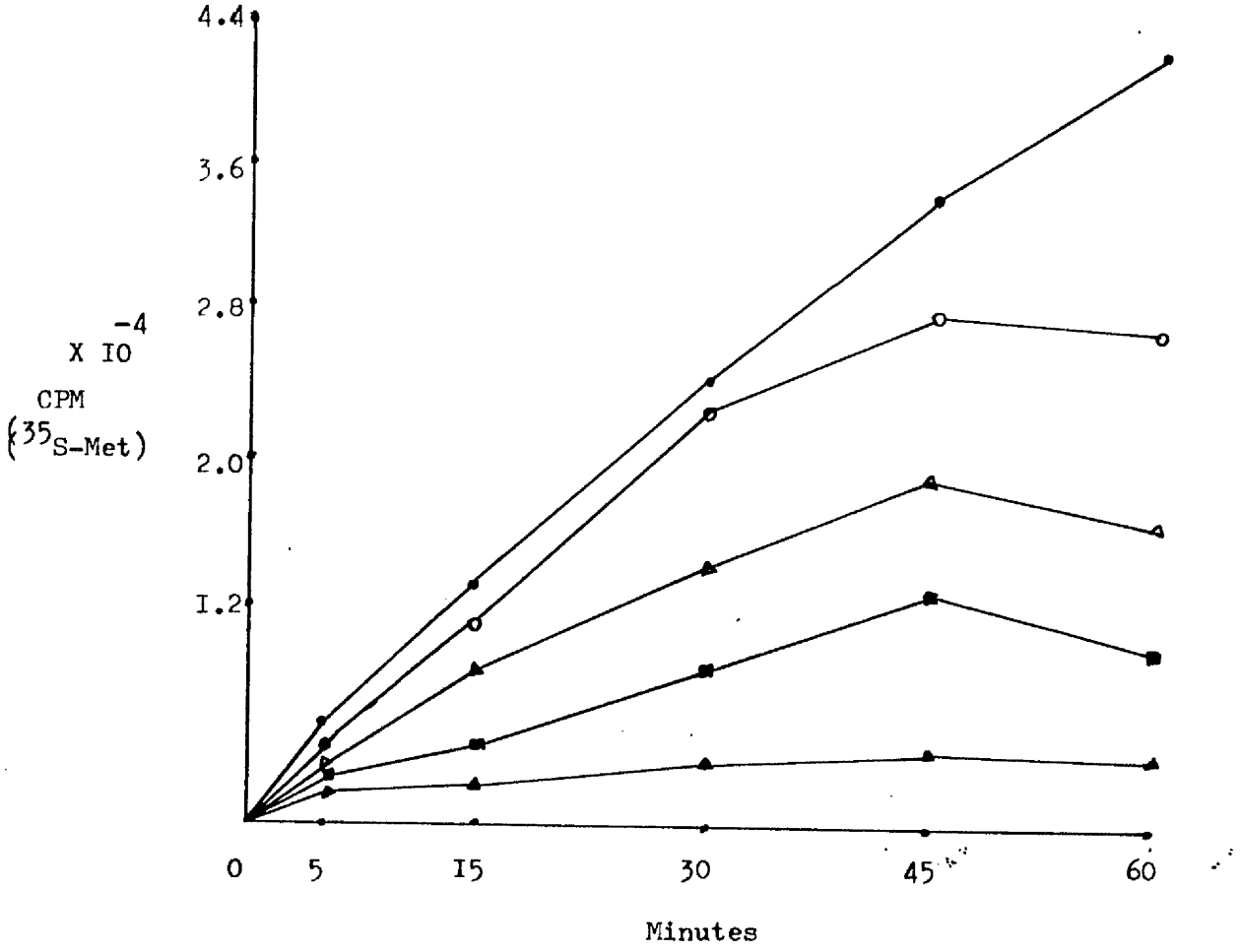
●——● Globin mRNA + dialysing buffer.

○——○ Globin mRNA + 1.6$\mu$g protein equivalent of PRV

■——■ Globin mRNA + 4.0$\mu$g protein equivalent of PRV

△——△ Globin mRNA + 1.6$\mu$g protein equivalent of heat inactivated PRV.

▲——▲ Globin mRNA + 4.0$\mu$g protein equivalent of heat inactivated PRV.


4.4
3.6
2.8
2.0
I.2
-4
X IO
CPM
(35S-Met)
0
5
I5
30
45
60
Minutes

Analysis of the translation products was carried out using SDS-polyacrylamide gel electrophoresis and autoradiography. This showed no increase in small molecular weight products, (Fig 9.2.5), which indicated that the inhibition was not apparently due either to premature termination or mRNase activity.

## 9.3. Discussion.

In these studies it has been found that PRV causes a marked inhibition of protein synthesis in the MDL system. Since in this system, there is absolutely no possibility of any virus specific macromolecule synthesis, inhibition appears to be due to some structural component(s) of the virus. This is supported by the dependence of inhibition on the concentration of virus added. The inhibition does not appear to be due to mRNA degradation or to premature termination. Since heat inactivated virus is equally active in causing inhibition, it is possible that some heat stable component(s) of the virus bind to items of the translation system causing inhibition.

In the intact cells virus causes selective inhibition of host protein synthesis whereas in the MDL systemthe virus particles cause a general inhibitionpresumably by binding to some component of the translation system.Probably in the cell a different mechanism is responsible for selectivity. In this experiment cent per cent purity of the virus preparation is not guaranteed so interference of any impurity cannot be ruled out.

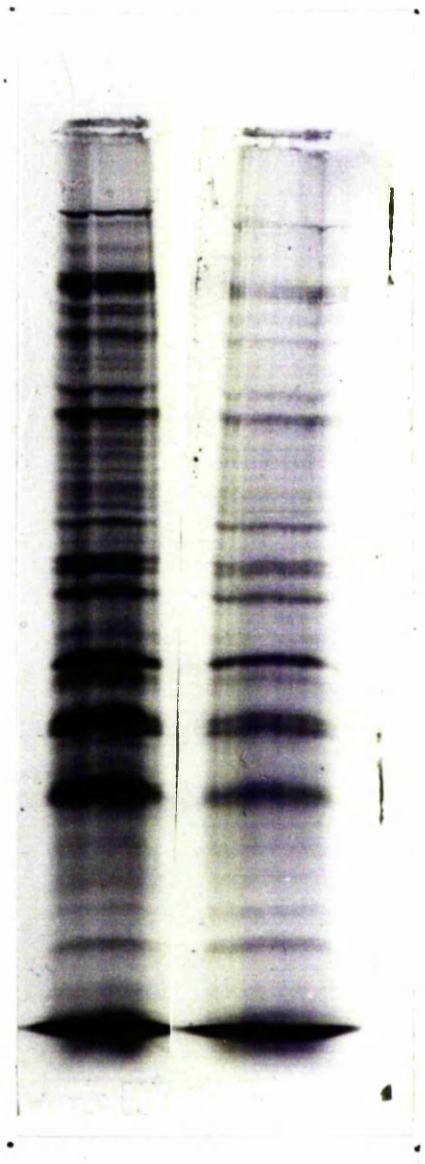
Fig. 9.2.5. Polypeptides synthesized in the MDL System in the presence of PRV particles.

The assay mixture of 11µl contained the MDL system plus 40µCi $^{35}$S-Methionine, 0.25µg late mRNA with or without 4µg protein equivalent of dialyzed PRV. After 60 min incubation at 30°C, the translation products were processed for polyacrylamide gel electrophoresis and autoradiography (Materials and Methods).

Equal amounts of protein were applied on to each gel track.

Track A Late mRNA + dialysing buffer

B Late mRNA + PRV particles.



A B

## Chapter 10. Effects of low concentration of Cycloheximide on protein synthesis and virus replication.

### 10.1. Introduction.

In EMC virus infected cells competition between host and viral mRNA's causes suppression of host protein synthesis (Lawrence & Thach, 1974). In the case of poliovirus, preferential synthesis of infected cell proteins at high salt concentration supports the speculation that there are differences in the efficiencies of initiation of host and viral messages (Nuss et al., 1975).

The high efficiency of many viral mRNA's in the initiation of protein synthesis (Lawrence & Thach, 1974; Nuss et al., 1975; Golini et al., 1976; Jen et al., 1978) might be used as a signal for discrimination between host and viral mRNA's. As such translation of these viral mRNA's is likely to be limited by the elongation rate whereas the translation of host mRNA's should be inhibited by the initiation rate. So any decrease in the elongation rate should inhibit elongation-limited viral mRNA's for more than initiation-limited host mRNA's.

The elongation inhibitor, cycloheximide, when present at low concentration during the infectious cycle, suppresses replication of VSV and EMC viruses (Ramabhadran & Thach, 1980) whereas cellular protein synthesis and synthesis of MLV proteins are inhibited to a much lesser extent (Yau et al., 1978). Attempts were made to study the effect of cycloheximide on PRV infected cell protein synthesis. Virus yield was monitored in these experiments to check that the concentration of

inhibitor used did not completely inhibit virus replication.

## 10.2. Inhibition of protein synthesis and virus replication.

Mock-infected and PRV infected cells (50 p.f.u./cell) were treated with different concentrations of Cycloheximide from the start of infection. At 4.5 hrs post infection, when viral protein synthesis is almost maximum cells were labelled for $\frac{1}{2}$ hr with [$^{35}$S]-methionine in the presence of the drug. Total cell protein synthesis was determined by counting the TCA precipitable radioactivity. It was found that at 1$\mu$M cycloheximide infected cell protein synthesis was inhibited by 80% compared to 65% inhibition of mock-infected cell protein synthesis (Fig 10.2.1) but Cycloheximide caused a substantial reduction in virus yield which was about 80% at 1$\mu$M Cycloheximide.

## 10.3. Discussion.

The data presented here suggest that the inhibition of infected cell protein synthesis is not significantly different from that of mock-infected cells. Inhibition of infected cell protein synthesis coincides with the reduction in virus yield at 1 $\mu$M concentration. Since the elongation rate of viral and host mRNA's are comparable (Silverstein & Engelhardt, 1979) this implies that initiation of viral protein synthesis is little different from that of host protein synthesis.

Fig. 10.2.1. Effect of Cycloheximide on Protein Synthesis and Virus Replication.

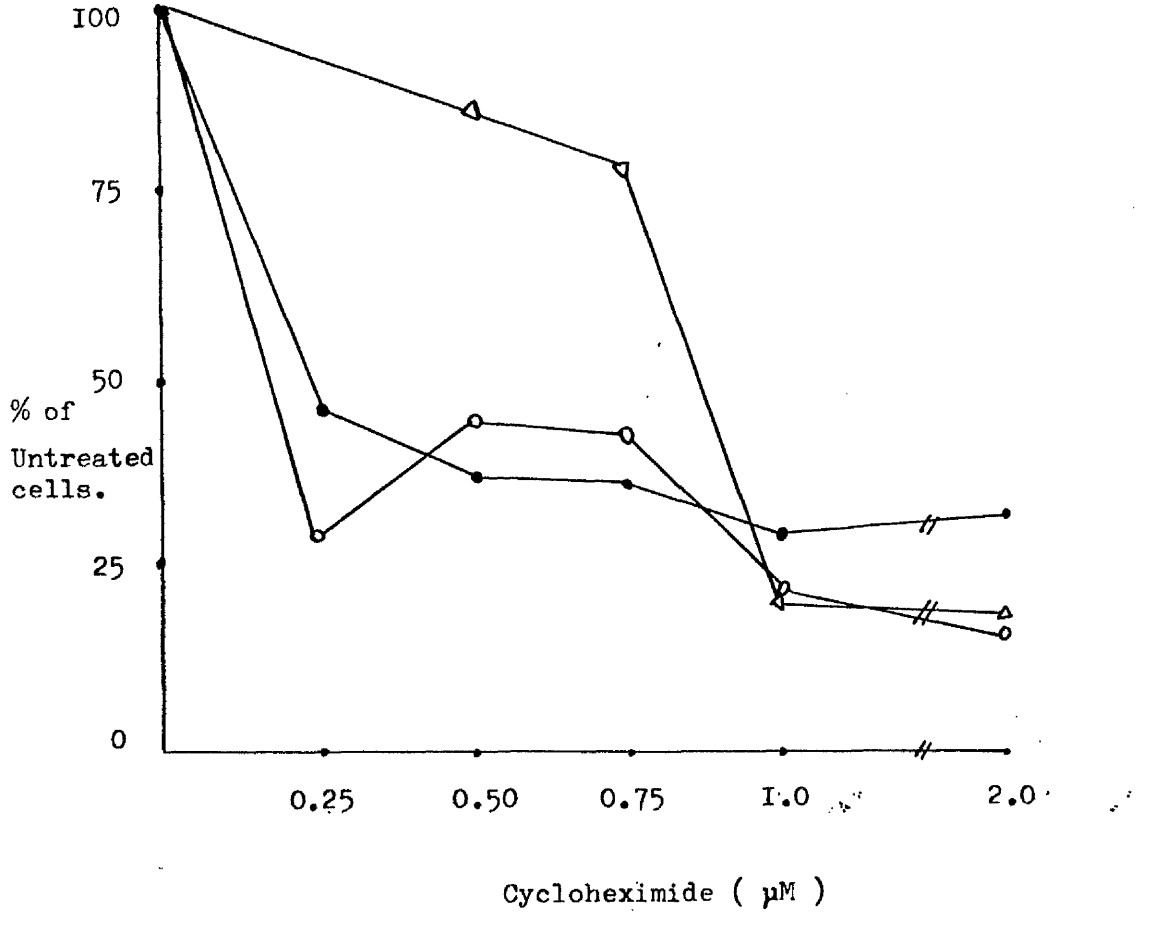
(a) HeLa cells grown on coverslips in petridishes were mock-infected or infected with PRV in the presence of different concentration of Cycloheximide. After 4.5 hrs infection, cells were labelled with 15 $\mu$Ci [$^{35}$S] methionine per petridish in methionine-free medium for 30 min again containing Cycloheximide. Protein synthesis was monitored by determining TCA precipitable radioactivity (Materials and Methods).

●——● Mock-infected, 100% = $8.9 \times 10^4$ cpm

o——o Infected, 100% = $8.0 \times 10^4$ cpm

(b) Confluent monolayers of HeLa cells in petridishes were infected with PRV for 12 hrs in the presence of different concentrations of Cycloheximide. Virus yield was determined by plaque assay as described in Materials and Methods.

Δ——Δ Virus yield, 100% = $0.25 \times 10^9$ p.f.u./ml


100
75
50
% of
Untreated
cells.
25
0
0.25
0.50
0.75
1.0
2.0
Cycloheximide ( μM )

# Chapter 11. RNase activities in cells infected with PRV.

## 11.1 Introduction

In picornavirus infected cells cellular mRNA's appear to be as stable as in mock-infected cells and as such inhibition of host protein synthesis is not due to nucleolytic degradation of host mRNA (Abreu & Lucas-Lenard 1976; Fernandez-Munoz & Darnell, 1976). Neither has host mRNA degradation been observed in cells infected with vaccinia virus (Rosemond-Hornbeck & Moss, 1975) or VSV (Nishioka & Silverstein 1978a). However, in Friend erythroleukemia cells infected with HSV-1 inhibition of host protein synthesis is accompanied by degradation of mRNA (Nishioka & Silverstein 1978a) and the cellular mRNA's are released from polyribosomes prior to degradation. Degradation does not occur in the presence of inhibitors of protein or RNA synthesis and thus expression of early virus function is found to be required for the degradation of cellular mRNA to occur (Nishioka & Silverstein, 1978b). In HSV-1 infected cells there is also an increase in the activity of RNase HI at times late in infection (Muller et al., 1980). However this increased RNase activity is likely to be required for the degradation of RNA primers in HSV DNA synthesis (Biswal et al., 1974, Muller et al., 1979). This enzyme hydrolyzes specifically RNA from DNA-RNA hybrids and is normally inactive against ds RNA or SS-RNA (Hausen & Stein 1970) and so does not appear to be a candidate for the mRNase activity which has been reported. Thus it is of interest to study the presence of

RNase activities in PRV infected cells, which could be responsible, in part, for the inhibition of protein synthesis.

## 11.2. Degradation of mRNA in PRV infected cells.

Mock-infected and 6 hrs infected cells were homogenized and centrifuged at 50,000 r.p.m. for 3 hrs at 4°C to prepare a post ribosomal supernatant, as described in Materials and Methods. This post ribosomal supernatant was used to study mRNase activities. An aliquot of PRV labelled mRNA was incubated for 30 min at 25°C with the post ribosomal supernatant to study the degradation of mRNA. The percent of mRNA recovered after incubation, in a typical experiment, is given in Table 11.2.1. Using a supernatant from mock-infected cells the percent recovery of HeLa mRNA was 94.3 whereas with supernatant from infected cells the percent recovery was 70.0. When late mRNA was used the recovery was close to 100% after incubation with either mock-infected or infected cell supernatants. The actual figure was 97% in both cases. From these results it appeared that the post ribosomal supernatant from infected cells possessed some RNase activities which degraded HeLa mRNA to an appreciable extent but did not degrade late mRNA from PRV infected cells.

## 11.3. RNase activities in PRV preparation.

PRV was purified and dialyzed overnight against 0.01M Tris HCl (pH 7.4), as described in Materials and Methods. Aliquots of labelled mRNA were incubated with the virus preparation. Degradation of mRNA was studied by determining

Table 11.2.1.

mRNA degradation by cell free extracts from PRV infected cells.

| Nature of the post ribosomal supernatant(a) | Nature of mRNA | Input | | Recovery | | % |
|---|---|---|---|---|---|---|
| | | cpm | µg | cpm | µg | |
| Mock-infected | $^3$H-HeLa | 1500 | 1.4 | 1420 | 1.32 | 94.3 |
| Infected | $^3$H-HeLa | 1500 | 1.4 | 1062 | 0.99 | 70.7 |
| Mock-infected | $^3$H-late | 5500 | 2.5 | 5335 | 2.42 | 97 |
| Infected | $^3$H-late | 5500 | 2.5 | 5324 | 2.42 | 96.8 |

Cells were mock infected or infected with PRV at 50 p.f.u./cell. At 6 hrs post infection cells were harvested and post ribosomal supernatant was prepared as described in Materials and Methods. The assay mixtures contained 30 µl and 20 µl of post ribosomal supernatants from mock-infected and infected cells respectively and a specified amount of labelled mRNA. After 30 min incubation at 25°C, 10µl aliquots were collected on Whatman No. 1 filter discs, washed with TCA and then ethanol and then dried. The radioactivity of TCA precipitable material was determined by liquid scintillation counting.

(a) Protein content of the post ribosomal supernatants :

from mock-infected cells = 3.0mg/ml.

from infected cells = 4.4mg/ml.

the radioactivity of the TCA precipitable material in the assay mixture after incubation. The results of a typical experiment are shown in Table 11.3.1. The PRV preparation did not cause any degradation of host or virus specific mRNA's. This was true both for the normal and the heat inactivated PRV preparations.

## 11.4. Discussion.

The results show that in the infected cells there is an RNase activity which selectively degrades host mRNA. But no RNase activity is associated with either normal or heat inactivated PRV preparations and therefore the component(s) of the infecting virions which causes inhibition of protein synthesis cannot itself be an mRNase.

Table 11.3.1.

mRNA degradation by PRV preparation.

| Nature of the virus | Nature of the mRNA | Input cpm | Input μg | Recovery cpm | Recovery μg | Recovery % |
|---|---|---|---|---|---|---|
| Normal PRV | $^{3}$H-HeLa | 1500 | 1.4 | 1390 | 1.29 | 93.0 |
| Normal PRV | $^{3}$H-Late | 11000 | 5.0 | 10540 | 4.78 | 96.0 |
| Heat-inactivated PRV | $^{3}$H-HeLa | 1500 | 1.4 | 1462 | 1.36 | 97.0 |
| Heat-inactivated PRV | $^{3}$H-Late | 11000 | 5.0 | 10780 | 4.91 | 98.0 |

A purified PRV preparation was dialyzed against 0.01M Tris HCl (pH 7.4) and some of the preparation was inactivated by heating at 60°C for 60 min (Materials and Methods). The assay mixture contained 20 μl of the PRV preparation and a specified amount of labelled mRNA. After 30 min incubation at 25°C aliquots were collected on filter discs to determine TCA precipitable radioactivity.

## Chapter 12. Discussion and Conclusions

The aim of this project was to examine a number of aspects of PRV infection in order to explain the mechanism(s) by which the virus causes inhibition of host protein synthesis. This problem was approached by undertaking experiments both *in vivo* and *in vitro*.

In general, virus induced inhibition of host protein synthesis could involve any one or more of a number of effects, for example, degradation of host mRNA, or inhibition of initiation or elongation of host peptide chain formation. However, in this project the aim was, first, to answer the question whether inhibition of host protein synthesis is selective in PRV infected cells and second, what effects do contribute to the overall inhibitory mechanism.

A number of experiments were carried out to study selectivity of inhibition, of which the first ones discussed involved the use of different cell lines to examine protein synthesis following infection to investigate, therefore, whether selectivity resides in the host cell. Initiation of protein synthesis in eukaryotes is a complex phenomenon, involving at least eight initiation factors in addition to mRNA, Met-tRNA$_f$, 40 S and 60 S ribosomal subunits, ATP and GTP (Weissbach & Ochoa, 1976). Different cell lines might have different capabilities of translating the same mRNA. It was found that both PRV and HSV-1 (KOS) caused a rapid and marked inhibition of protein synthesis in HeLa cells. But in PRV infected C13 cells though inhibition was rapid and substantial, there was an initial stimulation at 1 hr post-infection. A similar stimulation was found in PRV infected

BS-C-1 cells, in which case the subsequent inhibition was gradual. It is possible that the initial stimulation is not a real increase in protein synthesis but a change in the methionine pool in BS-C-1 and C13 cells early after infection. Alternatively, initial stimulation may be due to a sensitization of the translation machinery resulting in rapid initiation. Modification of ribosomal protein(s) or initiation factor(s) soon after infection might contribute to this phenomenon. Phosphorylation of ribosomal proteins has been observed in vaccinia virus infected cells (Kaerlin & Horak, 1976). Similar phosphorylation has been observed in HSV-infected cells (Fenwick & Walker, 1979) and in PRV infected C13 cells (Kennedy, personal communication), but no connection between phosphorylation and alterations in protein synthetic ability has yet been established. It was found that early after infection almost all the host mRNA's are active in each of these cell lines as shown in the autoradiographs in Chapter 5, which indicates that the cellular translation machinery probably utilizes all the mRNA's available. Late in infection it is likely that translatable host mRNA's are scanty and the translation machinery then synthesizes mainly viral proteins. In HeLa cells the kinetics of inhibition seemed to be most simple and so this cell line was used in most subsequent experiments. However, these results indicate that selectivity does not reside in the host cell.

Selective effects on the inhibition of host protein synthesis can also be studied in cell-free systems, in which case it is necessary to isolate the mRNA's present in the cell and to translate them in an efficient cell-free translation system.

In PRV infected cells inhibition of protein synthesis occurs early after infection and so most of the studies were concentrated on events immediately after infection. Following the published results of Ben-Porat et al., (1971) and Rakusanova et al., (1971), Immediate-early and Late PRV mRNA's were isolated from HeLa cells infected for 5 hrs in the presence and in the absence of Cycloheximide respectively. Filter disc hybridisation studies showed the presence of at least 5% viral transcripts in the IE mRNA preparation. Since hybridisation did not reach the saturation level, the proportion of the genome represented in the transcripts was not estimated. Translation studies in vitro showed the presence of viral transcripts in the preparation (see 3.3). Rakusanova et al., (1972) have shown that most of the polysome bound mRNA's late in infection are viral and McGrath (1978) has found that at least 50% of the poly(A) RNA isolated from the polysomes of PRV infected cells are viral transcripts.

The mRNA activity of a transcript is judged by its ability to stimulate incorporation of radioactive amino acids into TCA precipitable material in a cell-free translation system. The cell-free translation system mostly used in this project was the unfractionated Messenger Dependent Lysate (MDL) system which is capable of efficient and reproducible translation of exogenous mRNA's (3.1). Wheat germ extracts which had been used previously in this lab (McGrath, 1978) suffer from premature termination of polypeptide synthesis. Moreover, Shih et al., (1980) have shown that EMC coat protein is not synthesized completely under standard conditions in the wheat germ extract

unless it is supplemented with a reticulocyte ribosomal salt wash. So proteins synthesized in the MDL system appear to be more representative of the mRNA species. Two MDL systems used - one prepared in the lab and the other commercially prepared - showed good agreement in their ability to translate exogenous mRNA's. Analysis of the translation products specified by IE mRNA preparation showed the presence of a large number of host proteins in addition to a few viral proteins. This is in agreement with some studies on HSV-1 (Preston, 1979) but contrast to previous studies (Roizman et al., 1974) which claimed that in Cycloheximide treated HSV-1 infected HEp-2 cells IE proteins are mainly synthesized when the drug is removed. Our results showed that host protein synthesis was not inhibited completely in PRV infected HeLa cells when IE proteins were made. The hybridisation data also showed the presence of a large number of host mRNA's in the IE mRNA preparation. Thus it appears that active host mRNA's are present in abundance in the infected cells early after infection. Probably differences in the nature of the host and in the nature of the virus result in differences in the extent of inhibition described in the literature. But our experimental results confirm our observation that no selective inhibition of host protein synthesis occurs at least for the first few hours of infection.

In HSV-1 infected cells viral mRNA's are said to be selectively translated even though host specific mRNA's are present on the polysomes at times late in infection (Stringer et al., 1977). Certainly, this may be true for other viruses. For example, in a cell-free translation system, EMC viral mRNA

is preferentially translated when both host and viral mRNA's are present in saturating amounts (Lawrence & Thach, 1974). Again in a cell-free system prepared from mengovirus infected EAT cells viral mRNA is selectively translated (Egberts et al., 1978). 70% of the selectivity is due to the initiation factors and 30% due to pH 5.0 enzyme fractions. Globin mRNA can compete with calf lens mRNA and TYMV mRNA in the MDL system (Asselbergs et al., 1980). Competition of mRNA's for initiation factor and/ribosomes might be part of a cellular mechanism to regulate the relative ratio of synthesis of different proteins (Lodish, 1974). Conversely, selectivity may be promoted by the particular ratio of various initiation factors (Revel & Groner, 1978). Since globin mRNA and TYMV mRNA have the same nucleotide sequence at the 5' side of AUG (see Asselbergs et al., 1980), it is possible that the binding affinities of initiation factors and ribosomes and different mRNA's vary due to differences in the nucleotide sequence of points other than the initiation regions of the mRNA's.

Studies in the MDL system using equal amounts of host and viral mRNA's showed no preferential translation of viral mRNA. Though we do not know the structure of either HeLa or PRV mRNA's, at least based on this criterion it could be argued that any selectivity of translation does not reside in the mRNA. Again it was found that cytoplasmic and polysomal poly(A) mRNA's from PRV infected cells did not differ in the pattern of the proteins specified in the MDL system. Cumulatively these results make it clear that host protein synthesis is not inhibited substantially early after infection as translatable host mRNA's are found on the polysomes when IE proteins are

made. Viral mRNA is not selectively translated in the cell-free system when present with host mRNA's. And since both cytoplasmic and polysomal poly(A) mRNA's gave the same translation products it appears that viral mRNA's are not preferentially localized on the polysomes.

Another explanation for the selective inhibition of host protein synthesis in virus infected cells comes from the model proposed by Carrasco (1977). He proposes that a viral structural protein binds to the cell membrane and thus, causes a change in the permeability of the membrane. Under such conditions the intracellular ion distribution changes (high $Na^+$, low $k^+$) and is favourable for the translation of viral mRNA's but inhibits host protein synthesis.

The apparent loss of the permeability barrier in animal cells after virus infection has been reviewed by Kohn (1979). Binding of a ligand to a macromolecule in the plasma membrane causes lateral displacement of the protein-lipid complex (Nicolson, 1976) and this is also found in the binding of mengovirus capsid protein to membrane receptor (Gschwender & Traub, 1979). Thus adsorption of normal or even heat or u.v. inactivated virus to the membrane receptor may result in a distortion of membrane permeability with the consequent distortion of ion gradients across the membrane. Carrasco (1978) suggests that changes in the permeability barrier are a general phenomenon for virus infected cells and this involves disturbance in the distribution of not only $Na^+$ ions (Carrasco, 1978) but also $Mg^{++}$ and $Ca^{++}$ ions as well (Durham, 1977).

In support of this model it has been found that Picornavirus, Papovavirus, Rhabdovirus, Togavirus and Myxovirus mRNA,s are translated in cell free systems at $K^+$ optima higher than that of corresponding host mRNA's (Carrasco & Smith, 1976; Carrasco et al., 1979) and that the EMC virus infected cell membrane becomes leaky to monovalent ions when "shut-off" occurs (Carrasco, 1977, 1978, 1979). Picorna and Papovavirus infected cell membranes become leaky to low molecular weight translation inhibitors when viral proteins are being made (Contreras & Carrasco, 1979). Again membrane leakiness is detectable in VSV infected L-cells and in HSV-1 and Sendai virus infected 37RC cells at times late in infection (Bendetto et al., 1980).

In the Krebs II ascites cell-free translation system an elevated $K^+$ concentration is required for complete translation of EMC viral mRNA (Mathews & Osborn, 1974). However, this phenomenon is not universal. In VSV infected cells the intracellular $K^+$ concentrations do not change early after infection but changes are observed late in infection when the cells are already showing obvious signs of deterioration (Francoeur & Stanners, 1978). Again in mengovirus infected EAT cells, an initial $K^+$ influx is balanced by $Na^+$ efflux, leaving the total ion concentration unchanged (Egberts et al., 1977). Whether such changes in $Na^+/K^+$ ratio early in infection have any effect on the regulation of host protein synthesis is not yet known. Here also the $Na^+$ concentration increases late in infection.

Studies were carried out to investigate $K^+$ optima for host

and viral mRNA translation in the cell-free systems on the assumption that if variation in the ionic concentrations in the infected cells is responsible for selective inhibition of host protein synthesis, PRV mRNA's would have a higher $K^+$ optima. In a wheat germ system late PRV mRNA has $K^+$ optima identical to that of host mRNA (McGrath & Stevely, 1980). In Carrasco's model, it is the virus structural protein(s) whose binding to the membrane causes permeability changes on infection and so it was important to examine early events. IE, Late and HeLa mRNA's all showed an identical $K^+$ optima for translation in the MDL system. Since the IE mRNA preparation contained 5% of the viral transcripts, a major change in the $K^+$ optima curve, if there is any, could not be expected. However, analysis of the translation products did not show any major difference though at least one IE protein and one host protein showed weak resistance at elevated $K^+$ concentrations. A similar result was found in the wheat germ extract system. So the idea of preferential translation of viral mRNA's early and late in infection in the presence of high salt concentrations, in other words, Carrasco's model, is not applicable to this PRV infected cell system, though it could be relevant to the general inhibition.

The model was tested <u>in vivo</u> also since it is possible that the cell-free system is unable to respond faithfully to the elevated salt concentration. In the light of Lodish's Model (1974) the effect of hypertonic initiation block (HIB), observed at high salt concentrations, implies that under conditions when the overall rate of protein synthesis is decreased the mRNA's having higher affinities for ribosomes or initiation

factors e.g. viral mRNA's would be translated preferentially whereas host mRNA's having lower affinities or rate constants would not be translated.

In MCMV infected cells the synthesis of both host and a few viral proteins is inhibited at high salt concentrations (Chantler et al., 1978). On the other hand, synthesis of HSV-2 proteins (Fenwick & Walker, 1978) and Late PRV proteins (Stevely & McGrath, 1978) are susceptible to high salt concentrations. Again in order to study the effects on early events IE protein synthesis was studied at elevated NaCl concentrations and it was found that IE protein synthesis was inhibited to the same degree as the host protein synthesis when the salt concentration of the medium was increased. So it seems unlikely that hypertonic initiation block has anything to do with preferential inhibition of host protein synthesis in PRV infected cells.

The high efficiency of many mRNA's in the initiation of protein synthesis (Lawrence & Thach, 1974; Nuss et al., 1975; Jen et al., 1978; Ramabhadran & Thach, 1980) implies that translation of these mRNA's is limited by the elongation rate. So any decrease in the elongation rate would preferentially inhibit viral mRNA translation and any decrease in the initiation rate would inhibit slow initiating host mRNA translation. In this project PRV infected cell protein synthesis was studied in the presence of an inhibitor of elongation rate. Studies were carried out at 5 hrs post infection when viral protein synthesis is maximum. PRV protein synthesis was inhibited almost to the same extent as host protein synthesis. It is known that in herpesvirus infected cells the elongation rates of host and

viral protein synthesis are comparable (Silverstein & Engelhardt, 1979). So it appears that initiation of PRV protein synthesis is little different from that of host protein synthesis which indicates that apparently there is not selective initiation of viral protein synthesis.

Nishioka & Silverstein (1978b) have suggested that an early viral protein, possibly an IE polypeptide, is required for the degradation of host mRNA in the infected cells and host mRNA is released from the polysomes before being degraded. Such a degradation of host mRNA in HSV-1 infected cells is thought by them to be responsible for selective inhibition of host protein synthesis. An mRNase activity was observed in PRV infected cells. Cell-free extracts from 6 hrs infected cells caused about 30% degradation of host mRNA and apparently no degradation of late viral mRNA. Probably this extent of degradation of host mRNA is sufficient to cause the marked inhibition of protein synthesis observed in the intact cells. Selective degradation of mRNA by RNase is not unique. The antiviral state in interferon treated infected cells is thought to be the result of an induction of an mRNase activity and degradation of viral mRNA (see 1.8). The PRV genome has higher G + C content than the host (1.3.3) and so the viral mRNA is likely to contain a higher proportion of G + C. This difference in the nucleotide composition may contribute to selective degradation of host mRNA since ribosomal RNA, which also has a high G + C content is apparently not cleaved by this RNase. Further experiments to characterize this RNase are necessary to determine its properties more fully.

All these studies suggest that in PRV infected cells except for the role of the RNase activities mechanisms for the selective inhibition of host protein synthesis has not been found

In examining the general inhibition of protein synthesis in infected cells we first examined the phenomenon of polysome disaggregation which is known to occur in the infected cells. Disaggregation of polysomes within 2-3 hrs of herpesvirus infection is considered to be an index of the inhibition of protein synthesis (Sydiskis & Roizman, 1966). Virus induced polysome disaggregation has a general effect on host protein synthesis (6.1). Of the various elements responsible for polysome disaggregation a viral structural component(s) is considered as an active candidate in the case of many animal viruses (see 1.7.5). Raghow & Granoff (1979) have observed that inactivated Frog Virus 3 can disaggregate both free and membrane bound polysomes in the absence of viral protein synthesis and this effect is not due to host mRNA degradation. U.V. inactivated HSV-1 can also disaggregate FL cell polysomes (Nishioka & Silverstein, 1978b). Fenwick & Walker (1978) have observed that in HSV-2 infected cells polysome disaggregation is caused by a component(s) of the virus.

To study the particle effect on polysome disaggregation in PRV infected cells both normal and inactivated viruses were used. Ross et al., (1972) have found that a high dose of u.v. irradiation does not affect adsorption and penetration of cells by herpesviruses. Similarly heat inactivated HSV can adsorb to mammalian C6 cells (Ianez, 1980). Experiments described in this thesis showed that both PRV and HSV-1 (MP17) could disaggregate host

polysomes in the absence of viral protein synthesis. This is supported by the observation of increased disaggregation when the number of infecting PRV particles was increased in the presence of Cycloheximide and thereby in the absence of protein synthesis. Moreover the use of both heat and u.v. inactivated PRV showed sufficient disaggregation of host polysomes to make it clear that polysome disaggregation is due to a structural component(s) of the virus.

It is also important to interpret the possible effects of inactivation processes employed on the virus particles themselves. The replicating capacity of VSV is destroyed upon irradiation whereas it has little effect on the inhibitory potential of the virus (Yaoi et al., 1970). U.V. irradiation at high doses or heat inactivation causes an alteration in the polio virus structure as RNA becomes sensitive to RNase treatment and empty capsids are formed (Katagiri et al., 1967; Briendl, 1971) but the inactive virus can still inhibit host macromolecule synthesis (Helentjaris & Ehrenfeld, 1977). U.V. irradiation of mengovirus makes the capsid fragile and some virion proteins become covalently linked to viral mRNA, but high doses of irradiation cause disaggregation of the virus (Miller & Plagemann, 1974). At high u.v. doses reverse transcriptase activity of Rauscher Leukemia Virus is inhibited completely (Lovinger et al., 1974). Heat inactivation of HSV results in the destruction of the replicating capability of the virus while exerting minimal effects on the gross physical integrity of the virus particles as well as their adsorbing capacity (Lancz, 1980). Inactivation of PRV by heat and u.v. irradiation might involve any of these

effects on the virus particles. Though u.v. irradiation is normally found to act on the nucleic acid causing thymidine dimer formation or even destruction of part of the genome, it might cause binding of certain viral proteins to DNA rendering it inexpressable. An effect on regulatory viral proteins can not be ruled out. Similarly, heat inactivation might involve denaturation of an active component(s) required for proper functioning of the viral genome or binding of viral proteins to DNA rendering it inactive. Even some sort of modification of the viral DNA which does contain a number of nicks (1.3.3) can not be ruled out after heat treatment.

The effects of inactivated virus on host protein synthesis have been discussed (1.7.5). Experiments carried out in this project showed that both normal and inactivated PRV caused an almost equal extent of inhibition of total protein synthesis in HeLa and C13 cells at 8 hrs post infection. When infection was studied up to 24 hrs, infective virus was found to exert cytopathic effects whereas inactivated viruses did not. The inhibitory effect of inactivated virus was almost the same at 8 hrs and 24 hrs post infection. Since no new viral structural proteins are likely to be made in cells infected with inactivated virus it is not surprising that no further increase in the inhibition was observed. Inactivated viruses caused only 25% inhibition of RNA synthesis whereas infective viruses caused more than 80% inhibition. The significance of this inhibition is not clear. It is not known whether this 25% inhibition of RNA synthesis is sufficient to cause the greater inhibition of protein synthesis (70% approx.) which is observed. Furlong (personal communication) has found that u.v. inactivated PRV

caused about 20% inhibition of rRNA synthesis whereas heat inactivated PRV had little effect. The significance of these data with respect to RNA and protein synthesis in PRV infected cells is not clear. However, the marked inhibition of protein synthesis and little effect on RNA synthesis indicate that inactivated viruses appear to exert selective effect on the translation process directly. Some heat and u.v. resistant component(s) of the virus must play this role.

The inhibitory effects of the virion component(s) can be understood more clearly if studied in cell-free systems. The effects of various viruses and their components on protein synthesis have been discussed (9.1). These studies reveal either the involvement of the virus particles themselves in exerting inhibition or indicate the effects of infection on some property of a component essential for protein synthesis. A viral protein solubilized from Frog Virus 3 suppresses protein synthesis in cell free systems (Aubertin et al., 1978) and a similar inhibitory effect of poliovirus core is observed in reticulocyte lysates (Racervskis et al., 1976).

In the MDL system addition of both normal and heat inactivate PRV caused substantial inhibition of protein synthesis. Since in this system virus macromolecule synthesis is not possible, it indicates that virus structural component(s) are responsible for the inhibition. This is substantiated by the finding that the inhibition increased with the addition of increasing number of particles. Analysis of the translation products showed that inhibition did not appear to be due to mRNA degradation.

In the MDL system substantial inhibition was observed when the viral protein was about 1.8% of the protein content of the system, whereas in HeLa cells infected with PRV at 50 p.f.u./cell a similar inhibition was observed when viral protein was about 0.2% of the total cell protein. The relatively high quantity of viral proteins needed to cause inhibition in the MDL system does not necessarily reflect the in vivo situation where compartmentalization and membrane effects might drastically alter the mechanism of action and the magnitude of the effects. Since inhibition has normally been found to occur at the level of initiation, the large number of nuclear proteins in HeLa cells might not be involved and so the calculation should be modified on the basis of cytoplasmic protein content. In the MDL system during the inhibition studies total protein content of the virus was considered, but during infection of HeLa cells the envelope of the virus is left on the membrane, not taken into the cells. So the relative variation in the percent of viral proteins during inhibition in the MDL system and in theHeLa cells may not be absolute. However, these experiments again support the idea of involvement of viral structural component(s) in inhibition.

A further possibility for virus induced inhibition of protein synthesis which was studied is the degradation of mRNA. About 30% degradation of host mRNA was observed when HeLa mRNA was incubated with postribosomal supernatant from the cells infected for 6 hrs. This could be a contributing factor in addition to the particle effects observed. However, neither

normal nor heat inactivated PRV showed any RNase activities associated with them. So it seems clear that the particle effect on the inhibition is not due to mRNA degradation but is a separate feature of infection.

Final conclusions on the mechanism of inhibition or protein synthesis in herpesvirus infected cells include a few suggestions and rule out a few possibilities which are applicable in other virus infected cell systems. Inhibition of IE protein synthesis at high $Na^+$ concentrations and susceptibility of Immediately early mRNA translation at elevated $k^+$ concentrations in the cell free systems rule out a simple application of Carrasco's model to explain inhibition in PRV infected HeLa cells. Viral mRNA does not show any high efficiency of initiation of protein synthesis when elongation rate is inhibited. Neither does selective translation of viral mRNA's occurs when both host and viral mRNA's are present in equal amounts in the cell-free system. Except for selective degradation of host mRNA by an RNase induced in cells at 6 hr post infection no other selectivity was observed which could lead to the preferential translation of viral mRNA's after infection.

Though Carrasco's model is not applicable in terms of selectivity it could contribute towards an understanding of inhibition in general. Both in the intact cell and in the cell free systems synthesis of both host and viral proteins was inhibited and no selective translation of viral mRNA was observed. If we consider the hypothesis put forward by Lodish (1974) that when overall protein synthesis is decreased

an mRNA having a high efficiency of initiation will be translated, we found no such mRNA's. But our translation studies showed the translation of all available mRNA's present in the preparation. This is in agreement with Preston (personal communication) who claims that in HSV-1 infected cells the spectrum of proteins synthesized is a true reflection of the mRNA's available in the cells at that time. Nonetheless, it is possible that changes in the ionic strength may contribute to the overall inhibition which occurs.

Particle effects on protein synthesis have been observed both in intact cells and in cell free systems. It is known that the average size of the polysome is proportional to the rate of initiation and inversely proportional to the rate of elongation (Lodish, 1976). Since in HSV infected cells the elongation rates of host and viral protein synthesis are comparable (Silverstein & Engelhardt, 1979), the particle effects on polyribosome disaggregation may occur at the level of initiation and do not appear to be due to mRNA degradation.

Induction of an RNase activity indicates that of the possible causes of inhibition host mRNA degradation must be taken seriously.

The project gives some understanding of the mechanism of inhibition of protein synthesis in PRV infected cells. Any further work should include.

(a) a study of the effect of virus particles on the polysomes in a cell-free system, e.g. the MDL System,

(b) studies on the specificity of the mRNase.

# MATERIALS AND METHODS

## Materials

Appendices A :

A.1. Biological Materials

A.1.1. HeLa cells : A human cell line derived from cervical carcinoma (Gey et al., 1952)

A.1.2. C13 cells (BHK-21) : A kidney cell line derived from 1-day-old baby hamster. The C13 clone was first isolated by Stoker & Macpherson (1962).

A.1.3. BS-C-1 cells : A continuous cell line derived from primary cultures, by Hopp s et al., (1963) from African Green Monkey Kidney.

A.1.4. Pseudorabies Virus (PRV) : PRV (Pig Herpes virus 1) was originally derived from a stock preparation (Kaplan & Vatter, 1959) and has subsequently been plaque purified several times. Virus stocks were prepared from infected monolayers of C13 cells.

A.1.5. HSV-1, MP17 : This strain of Herpes Simplex virus, type 1 was isolated following infection of Friend erythroleukemia cells with HSV-1mp (Hoggan & Roizman, 1959). Our stocks were obtained from Dr. J. Morrison of the Biochemistry Department, Glasgow University.

A.1.6. HSV-1,KOS : Stocks of this strain were obtained from Prof. Y. Becker of Hebrew University, Jerusalem.

A.2. Chemicals

A.2.1. Materials for mRNA isolation :

| | | |
|---|---|---|
| Cycloheximide | : | Sigma Chemical Co., |
| Heparin (159 u/mg) freeze dried. | : | Evans Medical Ltd., Speke, Liverpool. |
| Oligo-[dT]-cellulose | : | Collaborative Research, Waltham, Mass. |

A.2.2. Materials for DNA preparation :

| | | |
|---|---|---|
| pronase | : | Calbiochem. La Jolla, Ca. |
| CsCl | : | FISONS. |

A.2.3. Materials for <u>in vitro</u> translation system.

| | | |
|---|---|---|
| Adenosine 5'-triphosphate (ATP) | : | P-L Biochemicals Inc., Milwaukee, Wis. |
| L-Amino acids | : | Calbiochem Ltd., La Jolla, Ca |
| Creatine Kinase | : | Boehringer Mannheim, GnbH, Mannheim, West Germany. |
| Creatine phosphate | : | " " " |
| Dithiothreitol (DTT) | : | Sigma Chemical Co., |
| N'-2-ethanesulfonic acid (HEPES) | : | " " " |
| Ethylene glycol-bis (2-amino ethylether)-NN'-tetraacetic acid (EGTA) | | " " " |
| Glutathione, reduced from Guanosine 5'-triphosphate (GTP) | : | P-L Biochemicals Inc., Milwaukee, Wis. |
| Haemin, Bovine | : | Sigma Chemical Co., |
| Micrococcal nuclease (800 u/mg) | : | Boehringer Mannheim, West Germany. |
| Phenylhydrazine hydrochloride | : | Sigma Chemical Co., |
| Spermidine trihydrochloride | : | " " " |

| | | |
|---|---|---|
| Spermine tetrahydrochloride | : | Sigma Chemical Co., |
| Sephadex G 25 (coarse) | : | Pharmacia Ltd., Uppsala, Sweden. |
| Sodium phenobarbitone (Nembutal) | : | May & Baker Ltd., Dagenham. |
| Rabbit Reticulocyte lysate | : | New England Nuclear, Mass. Amersham, Bucks. |
| Wheat germ extract | : | Bathesda Research Laboratories, N.Y. |

A.2.4. Chemicals for Liquid Scintillation Spectrophotometry

| | | |
|---|---|---|
| 2.5-diphenyloxazole (PPO) | : | Koch-Light Laboratories, Colnbrook, Bucks. |
| Toluene, Analar | : | " " " |
| p-bis-(O-methylstyryl) benzene (bis MSB) | : | Eastman Biochemicals Kodak Co. |
| Triton X-100 | : | Rohn & Haas (U.K.) Ltd., Croydon. |

A.2.5. Materials for polyacrylamide gel electrophoresis

| | | |
|---|---|---|
| Acrylamide (specifically purified for electrophoresis) | : | B.D.H. Ltd. |
| NN'-Methylene-bis-acrylamide (specifically purified for electrophoresis) | : | B.D.H. Ltd. |
| Ammonium persulfate | : | B.D.H. Ltd. |
| NNN'N'-tetramethylene-diamine (TEMED) | : | Koch-Light Laboratories Ltd. |

A.2.6. Photographic materials

| | | |
|---|---|---|
| Kodak X-omat H1 X-ray films | : | Kodak Ltd., London. |
| Kodak DX-80 developer | : | " " " |
| Kodak FX-40 Liquid fixer | : | " " " |

A.2.7. Radiochemicals

These were obtained from the Radiochemical Centre, Amersham, Bucks.

L- [ 4.5-$^{3}$H ] leucine 50 - 60 Ci/mmol.

[5,6-$^{3}$H] uridine 40 - 60 Ci/mmol.

L- [$^{35}$S] Methionine 800 Ci/mmol.

$^{32}$P-orthophosphate (carrier free)

A.2.8. Other Materials

| | | |
|---|---|---|
| Actinomycin D | : | Sigma Chemical Co., |
| Absolute Alcohol, Analar | : | James Burroughs Ltd., London. |
| Dextran 10 | : | Pharmacia Fine Chemicals, Uppsala, Sweden. |
| Glacial Acetic acid<br>Dimethyl sulfoxide | : | Sigma Chemical Co., |
| β-mercaptoethanol | : | Koch-Light Laboratories Limited. |
| 3-Methyl-1-butanol<br>(Isoamylalcohol) | : | " " |
| Molecular weight standards<br>(BSA, Trypsin inhibitor, RNA polymerase) | : | Boehringer Mannheim |
| Trichloroacetic acid | : | Koch-Light Laboratories Limited. |
| Whatman No. 1 filter paper discs (2.5 cm diameter) | : | Whatman Ltd., Maidstone, Kent. |

| | | |
|---|---|---|
| Newborn calf serum, foetal calf serum. | : | Gibco Biocult. |
| Phenyl hydrazine hydrochloride | : | Sigma Chemical Co. |
| Tryptose phosphate broth | : | Gibco Biocult, Difco-bacto. |

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

## A.3. Standard Solutions

### A.3.1. Cell culture media

I. 1F

This is the Glasgow modification of Eagle's Minimum Essential Medium (MEM), which is supplied by Flow Laboratories as a 10X concentrate (Busby et al., 1964).

| Components | mg/litre | Components | mg/litre |
|---|---|---|---|
| D-glucose | 4500 | L-leucine | 52.46 |
| Magnesium sulfate $7H_2O$ | 200 | L-lysine hydrochloride | 73.06 |
| Potassium chloride | 400 | L-methionine | 14.92 |
| Sodium chloride | 6400 | L-phenylalanine | 33.02 |
| Sodium dihydrogen phosphate, $2H_2O$ | 140 | L-threonine | 47.64 |
| Calcium chloride, $2H_2O$ | 264.9 | L-Tryptophan | 8.16 |
| L-Arginine hydrochloride | 126.4 | L-Tyrosine | 36.22 |
| L-Cystine, disodium | 28.42 | L-valine | 46.86 |
| L-glutamine | 584.6 | D-Ca-pantothenate | 2.00 |
| L-Histidine hydrochloride $H_2O$ | 21.0 | Choline chloride | 2.00 |
| L-Isoleucine | 52.46 | Folic acid | 2.00 |

| Components | mg/litre |
|---|---|
| I-Inositol | 4.00 |
| Nicotinamide | 2.00 |
| Pyridoxal hydrochloride | 2.00 |
| Riboflavin | 0.2 |
| Thiamin hydrochloride | 2.0 |
| Phenol Red, sodium | 17.0 |

II. Earle's Balanced Salt Solution, BSS.

| | | | |
|---|---|---|---|
| NaCl | 0.13M | Phenol Red | 0.015% (w/v) |
| KCl | 0.006M | chloroform | to a final conc. of 0.1% (v/v) |
| $MgSO_4$ | 0.001M | | |
| $NaH_2.PO_4$ | 0.001M | sterilized by 15 $lb/in^2$ for 20 min. | |
| $CaCl_2$ | 0.002M | | |

III. Eagle's Stock, 10X concentrated (ES10-Met)

| | |
|---|---|
| MEM amino acids (-Met) X100 | 20ml |
| MEM vitamins X100 | 20ml |
| glucose | 4 g |
| glutamine | 0.6375 g |

pH to 7.1 with 5M NaOH, Final volume 220ml. Sterile filtration through 0.22 micron millipore membrane.

IV. Sodium bicarbonate

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

V. Penicillin and Streptomycin

| | |
|---|---|
| Penicillin | $10^5$ units/litre |
| Streptomycin | 10 g/litre |

Sterilized by millipore filtration through 0.22 micron membrane.

VI. Tryptose phosphate Broth

Tryptose phosphate broth (Difco bacto.) 29.5 g/litre
Sterilized by 15 $lb/in^2$ for 15 min.

VII. EC, Medium for HeLa Cells

| | |
|---|---|
| Dist. $H_2O$ | 450 ml |
| IF | 50 ml |
| $NaHCO_3$ | 20 ml |
| Penicillin/Streptomycin | 5 ml |
| Newborn calf serum | 50 ml |

VIII. ETC, Medium for BHK-21 (C13) cells

| | |
|---|---|
| Dist. $H_2O$ | 400 ml |
| IF | 50 ml |
| Tryptose phosphate broth | 50 ml |
| Calf serum | 50 ml |
| $NaHCO_3$ | 20 ml |
| Penicillin/Streptomycin | 5 ml |

IX. EFC, Medium for BSC-1 cells

| | |
|---|---|
| Dist. $H_2O$ | 450 ml |
| IF | 50 ml |
| Foetal calf serum | 50 ml |
| $NaHCO_3$ | 20 ml |
| Penicillin/Streptomycin | 5 ml |

X. EC -Met, Methionine free medium

| | |
|---|---|
| BSS | 450 ml |
| ES10 (-Met) | 50 ml |
| Calf serum | 50 ml |
| $NaHCO_3$ | 20 ml |
| Penicillin/Streptomycin | 5 ml |

XI. Trypsin/Versene

| Trypsin | | Versene | |
|---|---|---|---|
| Trypsin | 0.25% (w/v) | Sodium chloride | 0.8% (w/v) |
| Trisodium citrate | 0.29% (w/v) | Potassium chloride | 0.02% (w/v) |
| Sodium chloride | 0.61% (w/v) | Sodium dihydrogen phosphate | 0.115% (w/v) |
| Phenol red | 0.15% (w/v) | Potassin dihydrogen phosphate | 0.02% (w/v) |
| pH 7.8 with NaOH | | Versene (Ethylene diamine tetra-acetate disodium salt) | 0.02% (w/v) |
| Sterilize by membrane filtration through 0.22 micron membrane. | | Phenol red | 0.15% (w/v) |
| | | Sterilized by 15 $lb/in^2$ for 15 min. | |

1 volume Trypsin + 4 volumes Versene.

XII. BSS + Bicarbonate

| | |
|---|---|
| BSS | 450 ml |
| $NaHCO_3$ | 20 ml |

XIII. Formol Saline

| | |
|---|---|
| NaCl | 0.077M |
| $Na_2SO_4$ | 0.1M |
| Formaldehyde | 3.6% (v/v) |

XIV. Giemsa Stain

0.75% (w/v) Giemsa in Glycerol:Methanol
1:1 (v/v)

XV. Phosphate buffered saline, PBS.

| | | | | |
|---|---|---|---|---|
| NaCl | 0.17M | $KH_2PO_4$ | 1.8mM | |
| KCl | 3.4mM | $CaCl_2$ | 0.7mM | pH 7.2 |
| $NaH_2PO_4$ | 10.0mM | $MgCl_2$ | 0.5mM | |

A.3.2. Solutions for mRNA Preparation

I. Reticulocyte Standard Buffer, RSB.

| | | |
|---|---|---|
| NaCl | 0.01M | |
| $MgCl_2$ | 0.003M | pH 7.4 with HCl |
| Tris-HCl | 0.01M | |

(a) 15% (w/v) Sucrose in RSB

(b) 30% (w/v) Sucrose in RSB

II. Extraction buffer.

NaCl 0.1M ; EDTA 0.001M ; SDS 2.0% (w/v)

Tris-HCl 0.1M. pH 9.0

III. Phenol

Redistilled phenol saturated with extraction buffer.

IV. Buffers for oligo [dT] cellulose chromatography

| | | | |
|---|---|---|---|
| (a) Loading buffer : | LiCl | 0.5M | |
| | EDTA | 0.001M | |
| | SDS | 0.1% (w/v) | pH 7.5 |
| | Tris-HCl | 0.01M | |

| | | | | |
|---|---|---|---|---|
| (b) Intermediate buffer | : | LiCl | 0.1M | |
| | | EDTA | 0.001M | |
| | | SDS | 0.1% (w/v) | pH 7.5 |
| | | Tris-HCl | 0.01M | |
| (c) Elution buffer | : | EDTA | 0.001M | |
| | | Tris-HCl | 0.01M | pH 7.5 |

A.3.3. Hybridisation Solutions

I. Standard saline citrate, SSC

NaCl 0.15M, $Na_3$-citrate 0.015M.

II. Hybridisation buffer :

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

A.3.4. Solutions for Reticulocyte Lysate Translation System.

I. Glutathione

0.5M Glutathione in $H_2O$, pH 6.0 with 1M NaOH.

II. Phenylhydrazine

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

III. Haemin

Haemin 10mM; Tris-HCl 0.2M pH 8.2

KCl 0.5M;

The prep was in 95% (v/v) ethylene glycol.

IV. Creatine Kinase

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

V. $K^+/Mg^{++}$ solution

$MgCl_2$ 0.01M, KCl 2.0M, Autoclaved.

$K^+$ concentration made 0.8M when the prep was used in $K^+$ dependence studies.

VI. Creatine phosphate

Creatine phosphate 0.2M in sterile water

VII. Amino acids

Approximately 1.4mM solution was made in sterile deionized water with mild heating to dissolve and sterile filtration through 0.45 micron membrane.

glycine, alanine, arginine, aspertate, histidine, isoleucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, cysteine, glutamine, glutamate, aspargine, methionine or leucine.

VIII. Master mix

$K^+/Mg^{++}$ solution : Creatine phosphate : Amino acids

1 : 1 : 1 (v/v/v)

IX. $CaCl_2$

0.1M $CaCl_2$. Autoclaved.

X. Micrococcal nuclease

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

XI. EGTA

0.1M EGTA, pH 7.4 with KOH, stored at 4°C.

XII. Assay mixture

Messenger dependent lysate, MDL 50 µl

$H_2O$/mRNA and isotope 7.5 µl

A.3.5. Solutions for Wheat Germ Extract Preparation and Translation Studies.

I. Extraction buffer.

Hepes 20mM; KCl 100mM; $Mg(oAc)_2$ 1mM; $CaCl_2$ 2mM; Mercaptoethanol 6mM. Solutions were made in deionized water, pH 7.6 with KOH and autoclaved before addition of Mercaptoethanol.

II. Column buffer

Hepes 20mM; KCl 120mM, $Mg(oAc)_2$ 5mM. Mercaptoethanol 6mM, as in extraction buffer.

III. Energy mix

ATP 1mM; GTP 0.02mM; Creatine phosphate 5.5mM; pH 7.6 with KOH ; solution in sterile deionized water. Stored at -70°C.

IV. DTT

0.12M DTT in sterile deionized water, $N_2$ gas bubbled through for 10 min. Stored at -70°C.

V. Creatine Kinase

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

VI. Salt Solutions

1M Hepes, pH 7.6. Autoclaved.

0.9M KCl and some other concentrations when $K^+$ dependence studied.

0.05M $Mg(oAc)_2$

VII. Spermidine

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

VIII. ATP mix, (prepared fresh for each set of assays)

| | |
|---|---|
| Energy mix | 25 µl |
| Hepes | 10 µl |
| KCl | 10 µl |
| $Mg(oAc)_2$ | 10 µl |
| DTT | 10 µl |
| Spermidine | 10 µl |
| Creatine Kinase | 5 µl |
| Sterile deionized water | 20 µl |

IX. Assay mixture

| | |
|---|---|
| ATP mix | 10 $\mu$l |
| Wheat germ extract | 15 $\mu$l |
| $H_2O$/mRNA and isotope | 25 $\mu$l |

A.3.6. Solutions for Gel Electrophoresis.

I. Electrode buffer

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

II. Main gel solution

SDS 0.1% (w/v); TEMED 0.1% (v/v); Ammonium persulfate 0.14% (w/v); Tris-HCl 0.375M, pH 8.8, with acrylamide and bis-acrylamide in the ratio of 1: 0.033.

III. Stacking gel solution

SDS 0.1% (w/v), TEMED 0.1% (v/v); ammonium persulfate 0.09% (w/v); agarose 1.0% (w/v), Tris-HCl 0.125M, pH 7.0 with acrylamide 3% (w/v) and bis-acrylamide 0.1% (w/v).

IV. Staining solution

Coomassie brilliant blue 0.25% in 45% (v/v) Methanol and 5% (v/v) glacial acetic acid.

V. Destaining solution

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

A.3.7. Solutions for PRV purification.

I. Phosphate buffer

1mM $KH_2PO_4$

1mM $K_2HPO_4$ pH 7.4 Autoclaved.

II. Tris-HCl solution

Tris-HCl 0.01M, pH 7.4 Autoclaved

III. Dextran 10

12% (w/v) and 32% (w/v) in 1mM phosphate buffer, pH 7.4. Mild heat treatment for dissolving and then sterilized by filtration through 0.45 micron membrane filter.

A.3.8. Scintillation solutions

I. Toluene- P.P.O.

5% (w/v) P.P.O. in Toluene.

II. Triton-Toluene-P.P.O.

5% P.P.O., 0.05% (w/v) bis-MSB, 35% Triton X-100 in Toluene.

## Methods

Appendices B :

B.1. Cell culture technique.

B.1.1. Propagation of cells.

HeLa, C13 and BS-C-1 cells were cultured as monolayers in 80 oz Winchester bottles (burlers) according to the procedure of House and Wildy (1965). Cells were normally seeded in sterile burler containing 180ml media with 16-22 x $10^6$ cells per burler in an atmosphere of 95% air and 5% $CO_2$. Cells grew to a confluent monolayer in 72 hrs incubation at 37°C.

For serial passage cells were dislodged from the burler surface by Trypsin-Versene (A.3.1.XI) treatment, suspended in a small volume of the medium, counted and then seeded as before.

Maintenance of cell culture, contamination check of the medium and cells for bacterial, fungal and PPLO were carried out by the staff of the Wellcome Cell Culture Unit.

B.1.2. Propagation of virus.

Pseudorabiesvirus (PRV) stocks were prepared from infected C13 cell culture according to the procedure of Chantler & Stevely (1973).

Monolayers of C13 cells were infected at a multiplicity of 5 p.f.u./cell in 25 ml medium. After 1 h adsorption, the medium was poured off and fresh medium was added. At 36 hrs post-infection, cells were shaken off from the burler surface and transferred aseptically to centrifuge bottles. Cells were pelleted by centrifugation at 600 g for 10 min at 4°C. The supernatant was recentrifuged at 15,000 g for 2 hrs at 4°C.

The pelleted virus was suspended in a small volume of EC10 (A.3.1.VII), dispersed and aliquoted at -70°C.

Cell associated virus prepared by resuspending the cell pellets and sonicating the suspension, normally gave a low titre and was rarely used for infection studies, but mainly used for production of virus stocks.

The infectivity of the virus preparation was determined by plaque assay. What titre ?

B.1.3. Plaque assay for PRV.

Confluent monolayers C13 cells grown in 50mm petridish, seeded at 8 x $10^6$ cells/petridish, were infected with serial dilutions of the PRV preparations in 0.2ml medium. After 1 hr adsorption, the medium was poured off, 2ml medium added and incubation continued. After another 1.5 hrs incubation, 250µg heparin was added to the contents in each dish to restrict the vertical transmission of the virus. At 30 hrs post-infection, cells were fixed by removing the medium and adding enough formol saline (A.3.1.XIII) to cover the cell sheet. After 30 min at room temperature, the formol saline was poured off and cells were stained with Giemsa Stain (A.3.1.XIV) for 30 min. Excess stain was removed very gently with water and plaques were counted under a low power microscope.

B.1.4. Purification of PRV.

Purified PRV was made by following the procedure of Spear & Roizman (1972).

Confluent monolayers of Cl3 cells were infected at a multiplicity of approximately 20 p.f.u./cell and harvested as before after 18 hrs infection. Cell pellets were swollen in 2 volumes of 1mM phosphate buffer (pH 7.4) (A.3.7.1) for 10 min on ice and then disrupted with six strokes of Dounce homogenizer. Immediately afterwards, a concentrated sucrose solution was added to give a final concentration of 0.25M. Cytoplasmic extracts were separated by centrifugation at 1500 r.p.m. for 10 min in the MSE Major Centrifuge. 2ml of the extract was layered on 34ml dextran 10 gradients (12-32% w/v) (A.3.7.III) made up in 1mM phosphate buffer, pH 7.4 and filtered through a 0.45 micron membrane filter prior to use. Centrifugation was at 20,000 r.p.m. for 1 hr in the SW27 Beckman Ultracentrifuge rotor. Virions were found in a diffuse light scattering band just above the middle of the gradient. The band was aspirated with a needle and a syringe, diluted four fold with 0.01M Tris-HCl (pH 7.4) and virions were pelleted by centrifugation at 25,000 r.p.m. for 2 hrs in SW 27 rotor. The virus pellets were suspended in a small volume of 0.01M Tris-HCl (pH 7.4) (A.3.7.II) and dialyzed overnight against 500 volumes of 0.01M Tris-HCl (pH 7.4) at 4°C. The preparation was aliquoted and stored at -70°C.

B.1.5. Inactivation of PRV.

B.1.5.1. U.V. Light Irradiation

A PRV stock was diluted 10 fold with PBS (A.3.1.XIV) containing 1% (w/v) glucose. 2.5ml of this dilute preparation was put in each 50mm petridish and put under a 15 w germicidal lamp (calibrated by Dr. J. Pitts, Biochemistry Dept., University of Glasgow). The preparation was irradiated at appropriate energy level. Plaque assay was carried out to determine the extent of inactivation.

B.1.5.2. Heat

A dilute PRV preparation was heated at 60°C for 60 min and the infectivity was checked by plaque assay.

B.1.6. Isolation of PRV-DNA.

PRV DNA was isolated according to the procedure of Stevely (1977).

Viral pellets, obtained as in (B.1.2.), were suspended in a small volume of 1 x SSC. Pronase (50µl/ml) was added to the suspension, to which a few drops SDS (5% w/v) were also added until the suspension became clear. It was then incubated undisturbed in a water bath at 37°C for 30 hours. CsCl solution as well as powdered CsCl was added to give a density of 1.4002g/cm$^3$. Mild centrifugation removed the SDS precipitate. The preparation was then centrifuged at 30,000 r.p.m. for 72 hrs at 17°C in the 50 Ti Beckman rotor. 0.5ml fractions were collected by needle and syringe and their refractive indices were measured. Fractions containing PRV DNA were pooled and dialyzed overnight against 500 volumes of distilled water at 4°C. The DNA concen-

tration was determined by spectrophotometry and samples were aliquoted and stored at -20°C.

B.2. Preparation of polysomes

B.2.1. Infection

The procedure for isolating Immediate-early (IE) mRNA was basically that of Rakusanova et al. (1971).

HeLa cells seeded at 20 x $10^6$ per burler formed confluent monolayers on the third day at about 80-100 x $10^6$ cells per burler. Cells were infected in the presence of cycloheximide (50µl/ml) in 25ml medium at a multiplicity of 50 p.f.u./cell. After 1 hr adsorption, the excess was decanted and fresh medium containing cycloheximide was added. At five hours post-infection cells were washed six times with normal medium to remove cycloheximide and then incubated in normal medium for 15 min. The cells were then harvested for IE mRNA preparation.

When late PRV mRNA's were to be prepared, cells were infected in normal medium and harvested after five hours. For labelling the mRNA's with $^3$H - uridine, radioactive isotope was added at appropriate times after infection and before harvesting. Normally more than twelve burlers were used for preparing each batch of poly(A) containing mRNA.
For labelled mRNA prepara tion infected cells are treated for I hr. with $^3$H-uridine at 4 hrs. post infection and mock infected cells are labelled for I hr.

B.2.2. Preparation of polysomes

After mock infection or infection, medium was poured off and 10 ml BSS-bicarbonate mixture (A.3.1.XII) was added. Cells were harvested from the burler surface with a rubber scraper

while rotating the burler on ice. Cells were then pelleted by centrifugation at 1500 r.p.m. for 10 min at 4°C. After swelling the cells in small volumes of RSB (A.3.2.1) (at about 2 x $10^8$ cells per ml), they were disrupted by 4-6 strokes in a Teflon glass homogeniser, at low speed. Nuclei and cell debris were removed by centrifugation at 2,500 r.p.m. for 10 min at 4°C. 2ml of this cytoplasmic extract was layered onto 34ml 15-30% (w/v) sucrose gradients in RSB containing 1mg/ml heparin. Gradients were centrifuged at 27,000 r.p.m. for 110 min at 4°C in the Beckman SW27 rotor and then harvested by pumping through a Gilford 2000 recording spectrophotometer set at 260nm. From the gradients polysomal fractions were separated and pelleted by centrifugation at 20,000 r.p.m. for 16 hours.

In order to study the effects of infecting virus particles on host polysome disaggregation, cells were infected or mock-infected in the presence of cycloheximide (50μg/ml). At 2 hrs post-infection cells were harvested and polysome profiles were recorded as before. The percentage of polysomes was determined by cutting and weighing appropriate portions of the recorder tracing.

B.3. Extraction of polysomal RNA.

RNA extraction was carried out according to the procedure of Mendecki et al., (1972). Polysomal pellets were rinsed with sterile 2M sucrose and then suspended in extraction buffer (A.3.2.II) at a concentration of 10 $A_{260}$ units per ml. The suspension was diluted with an equal volume of phenol:chloroform: isoamylalcohol (50:50:1, v/v/v), mixed vigorously for 10 min and centrifuged for 10 min at 2,000 r.p.m. at 10°C. The aqueous layer was removed and the organic layer reextracted with an equal volume of extraction buffer. The two aqueous phases were pooled together, then extracted with phenol,chloroform solution. From the final aqueous phase, RNA was precipitated by the addition of 2 volumes of ethanol. Precipitates were allowed to form overnight at -20°C.

B.4. Isolation of poly(A) containing RNA.

A modification of the technique followed by Aviv & Leder (1972) was used.

Approximately 0.1g oligo-[dT] -cellulose powder was used for isolating RNA from 120 $A_{260nm}$ units. The oligo-[dT]-cellulose was suspended in a small volume of loading buffer (A.3.2.IVa) for 5 min to make a paste and poured into a mini-column made from a pasteur pipette plugged with glass wool and baked at 250°C for 4 hours. Polysomal RNA, pelleted by centrifugation at 11,000 r.p.m. for 10 min at 4°C, was dissolved in loading buffer and cycled thrice through the column at a rate of 0.5ml per min. The column was washed with the intermediate buffer

(A.3.2.IVb) until the washings had $A_{260nm}$ of less than 0.01. Bound RNA was then eluted with 1.5ml of elution buffer (A.3.2.IVc) made 0.4M with 4M NaCl and the RNA was precipitated with 2 volumes of ethanol, by standing overnight at -20°C. The RNA, pelleted by centrifugation at 11,000 r.p.m. for 10 min at 4°C, was dissolved in 0.4M NaCl and reprecipitated with 2 volumes of ethanol at -20°C. Finally the RNA was dissolved in a small volume of sterile deionized water and stored at -20°C.

B.5. Filter disc hybridisation.

Filter disc hybridisation consisted of denaturation of DNA, fixation to millipore filters, hybridisation with labelled RNA and determination of the amount of labelled mRNA bound to the DNA.

DNA purified by overnight dialysis was made 0.1 x SSC (A.3.3.1) and sheared by passing 12 times through a 25g needle. It was then denatured by addition of anequal volume of 1M NaOH, which was neutralized 20 min later with 3 volumes of a solution containing 3M NaCl, 1M Tris-HCl (pH 8.0), 1M HCl in the ratio of 2:1:1, v/v/v. DNA was immobilized on 13mm HAWp millipore filters, presoaked for 2 hrs in 2 x SSC, by slow filtration (Gillespie & Spiegelman 1965). Filters were washed with 2 x SSC, dried 1 hr in air, 2 hrs at 80°C and then stored at -20°C.

When required, filters were soaked for 2 hrs in 2 x SSC prior to use in hybridisation studies. Labelled mRNA in 0.1 x SSC was denatured by heating at 115°C for 5 min. Aliquots of RNA was then added to the filter discs placed at the bottom of the baked glass scintillation vials with the DNA side up and

approximately 400µl hybridisation buffer (A.3.3.II) was added to allow the discs to float freely. Hybridisation was at 66°C for 20 hrs. The filters were washed thrice in 500 ml 2 x SSC for 30 min each time at room temperature. These were then incubated in 5ml 2 x SSC at room temperature with pancreatic RNAse, 50µg/ml (Jacquemont & Roizman 1975). Filter discs were washed twice with 2 x SSC and dried under a heat lamp. Radioactivity bound to the discs was determined by liquid scintillation counting in Toluene-PPO.

B.6. Translation, in vitro, in a wheat germ extract.

This employed a modification of the techniques followed by Roberts & Paterson (1973) and Marcu & Dudock (1974).

B.6.1. Preparation of Wheat germ extract

2g wheat germ and 2 g acid washed (2M HCl) baked sand were ground in a baked mortar and pestle (precooled to 4°C) for 2 min. 4ml extraction buffer (A.3.5.I) was added and the paste was centrifuged at 16,000 r.p.m. for 12 min at 4°C in Sorvall SS34 rotor. The supernatant was carefully removed avoiding the fat layer and 1.5ml was applied to a 1.7 x 21 cm Sephadex $G_{25}$ (coarse) column preequilibrated with column buffer (A.3.5.II). The eluant, 1ml/min, was collected in sterile tubes and its O.D. at 260nm was measured. Fractions having $O.D._{260nm}$ greater than 90/ml were pooled and centrifuged at 16,000 r.p.m. for 12 min at 4°C in Sorvall SS34 rotor. The supernatant was aliquoted and stored at -70°C. The whole operation was done at 4°C.

Sephadex suspended in deionized water was autoclaved before use. All column tubings were filled with $H_2O_2$ for 20 min and then rinsed with sterile deionized water before packing.

B.6.2. Translation assay.

Assays were started by adding wheat germ extracts to ATP mix (A.3.5.VIII) mRNA, isotopes and other ingredients, if any. The assay mixture (A.3.4.1X) was incubated at 25°C for 60 min. Reaction was stopped by quick transfer of the assay tube onto ice. The incorporation of labelled amino acid into TCA precipitable material was determined by filter disc method (Bollum 1968).

A commercially available wheat germ extract (A.2.3.) used for $K^+$ dependence studies contained 26mM Hepes, 66mM $K^+$ (34mM endogenous and 30mM from added reagents), 2.5mM $Mg^{++}$, 1.2mMATP, 0.1mM GTP, 5.5mM Creatine phosphate, 0.2mg/ml Creatine Kinase, 1.7mM β-mercaptoethanol, 80μM Spermidine and 50μM each of 19 amino acids (-Met). To it were added appropriate concentrations of KOAc, mRNA's and [$^{35}$S] methionine, Incubation was at 25°C for 60 min, after which TCA precipitable radioactivity was determined by the filter disc method.

B.6.3. Determination of TCA precipitable radioactivity.

Aliquots (5μls or as mentioned in the legends) were spotted onto 2.5cm diameter Whatman No. 1 filter paper discs supported on pins and when dried were placed in ice cold TCA, 10% (w/v) containing $10^{-5}$M of the amino acid used for labelling the

proteins. After 10 min, the filter discs were washed in the following order : 10 min in TCA 5% (w/v) at room temperature, 10 min in TCA 5% (w/v) at 90°C, 10 min ethanol:diethylether (50:50 v/v) and finally 10 min in diethylether. The discs were dried first in air under the hood and then under heat lamp. The radioactivity was determined in a Liquid Scintillation Counter using Toluene-PPO as the scintillation fluid.

B.7. Translation in reticulocyte lysate system.

B.7.1. Reticulocytosis (According to the procedure of Waxman & Rabinovitz, 1966).

Rabbits were made anaemic by injecting subcutaneously on 4 successive days approximately 0.4ml phenyl hydrazine solution (A.3.4.II) per kg body weight. After 2 days rest, on the 7th day rabbits were anaesthetized by injection of a solution of equal volume of heparin (1% w/v) and Nembutal into the ear vein (2.0ml per 2.5kg body weight). They were bled out by heart puncture into heparinised syringes and blood was transferred to sterile tubes. To ensure that the animal was dead the chest cavity was opened and drained of blood. (Prof. A.R. Williamson gave assistance with these procedures).

B.7.2. Lysis of reticulocytes

The red blood cells were pelleted by centrifugation at 2,000 r.p.m. for 10 min at 4°C in an MSE Major Centrifuge. The serum was removed and cell pellets were suspended in 4 volumes of BSS and centrifuged again at 2,000 r.p.m. The supernatant was

discarded and the buffy coat of white cells was removed by suction This procedure was repeated thrice to remove all white cells. Cells were lysed by addition of an equal volume of sterilized deionized water and after vigorous mixing left on ice for 4 min. The cell lysates were centrifuged at 10,000 r.p.m. for 10 min at 4°C. The ruby red supernatant was collected and stored in 800µl aliquots at -70°C.

B.7.3. Preparation of the Messenger Dependent Lysate (MDL)

MDL was prepared according to the method of Pelham & Jackson (1976).

To 800µl reticulocyte lysate were added 25µl haemin (A.3.4.III 10µl Creatine Kinase (A.3.4.IV) and 150µl Master mix (A.3.4.VIII). The lysate was made 1mM in $CaCl_2$ and 10µg/ml in micrococcal nuclease by the addition of 10µl $CaCl_2$ solution (A.3.4.IX) and 10µl of a nuclease solution (A.3.4.X). After a thorough mixing, it was incubated at 20°C for 15 min in a water bath. The nuclease action was arrested by the addition of 10µl EGTA solution (A.3.4.XI) to chelate $Ca^{++}$. The MDL was stored in 200µl aliquots at -70°C.

B.7.4. Translation assay.

To 50µl MDL was added mRNA and isotope to a total volume of 57.5µl. Incubation was at 30°C for 90 min. The reaction was stopped by quick transfer onto ice. TCA precipitable radioactivity was determined by the filter disc method.

The commercially available <u>in vitro</u> translation kit (A.2.3.) was also made according to the same procedure (Pelham & Jackson,

1976) and the assay mixture contained all the ingredients in the same proportion.

B.8. Protein synthesis, *in vivo*, in herpes virus infected cells

Cells were set up on 13mm diameter cover slips in 50mm petridishes at $4 \times 10^6$ cells/dish. When the cells had formed a confluent monolayer (approximately after 40 hrs). They were infected at a multiplicity of 50 p.f.u./cell or otherwise as mentioned in Results. For U.V. irradiated and heat inactivated virus, the amount equivalent to original 50 p.f.u./cell was used. After 1 hr adsorption, the medium was removed and infection continued in fresh medium. At different time intervals after infection cells were labelled in methionine-free medium with 15$\mu$Ci [$^{35}$S] methionine per petridish for 30 min.

When the effects of NaCl on Immediate-early protein synthesis were studied, infection was continued in the presence of cycloheximide (50$\mu$g/ml). At 5 hrs postinfection, cells were washed six times with normal medium to remove cycloheximide and then incubated in normal medium for 5 min. Cells were then incubated for 10 min in NaCl containing medium and then labelled with 15$\mu$Ci $^{35}$C methionine for 30 min in methionine free medium.

After labelling, all medium was decanted and 10% (w/v) TCA was added. 30 min later cover slips were washed in absolute alcohol and absolute alcohol:ether (50:50 v/v) mixture. TCA precipitable radioactivity was determined in the liquid scintillation counter in Triton-Toluene-PPO.

B.9. Preparation of cell lysates

Cells were seeded in 90mm petridishes at 8 x $10^6$ cells per dish. When the cells formed confluent monolayers (usually after 40 hrs), Cells were infected with PRV at 50 p.f.u./cell (Detailed in the Results), Cells were then labelled in methionine free medium with 25 $\mu$Ci [$^{35}$S]-methionine per petridish for 30 min. Before harvesting, cells were washed with BSS to remove cell bound serum. After harvesting, using a rubber scraper, cells were pelleted. The pellets were suspended in 1mM phosphate buffer (pH 7.4), which was made 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol and 10% (v/v) glycerol. These were heated for two minutes in a boiling water bath until a clear solution was obtained. The cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

B.10. Separation and detection of polypeptides by polyacrylamide gel electrophoresis.

B.10.1. Polyacrylamide gel electrophoresis.

A discontinuous system of polyacrylamide gel electrophoresis was used, basically as described by Laemmli (1970) and Dimmock & Watson (1969).

The gel apparatus consisted of two 18 x 18cm glass plates, separated by 0.15cm thick perspex spacers on three sides and held together by bulldog clips. Vacuum grease was applied on the outside edge of the spacers to prevent leakage. The bottom of the gel was sealed by a thin layer (1cm) of plug gel of 10% (w/v) acrylamide. The main gel was poured in and overlaid

with water to form a smooth upper edge. After polymerisation the excess water was poured off and the stacking gel was applied. A silicone coated plastic comb was inserted into the stacking gel before it polymerized to form wells for sample application. The perspex spacer at the bottom was removed and the gel was fixed vertically to the perspex gel apparatus. Electrode buffer (A.3.6.1) was poured into the top and the bottom tanks so that the top and the bottom of the gel were immersed in buffer. Proteins were denatured by making them 2% (w/v) in SDS. 5% (v/v) in β-mercaptoethanol and 20% (v/v) in glycerol and heating for two min in a boiling water bath. 10µl of 0.002% (w/v) bromophenol blue containing little glycerol was applied at two end wells as a marker dye. Appropriate amounts of denatured proteins were applied in the wells. Electrophoresis was at 18mA overnight at room temperature.

B.10.2. Detection of polypeptides.

After electrophoresis gels were stained for 1 hr in staining solution (A.3.6.IV) and then destained by several changes of destaining solution (A.3.6.V). Gels were dried under vacuum, exposed to Kodak X-omat H X-ray films and developed. Standard proteins of known molecular weights were also applied to the gel electrophoresis. The standards used were RNA polymerase (mol. wt. 165,000; 155,000; 39,000), BSA (68,000 mol. wt.), Trypsin inhibitor (mol. wt. 21,000). From the position of these proteins in the dried gels, the molecular weight of the unknown proteins were determined.

Occasionally to increase the sensitivity of detection a

fluorographic method was followed (Bonner & Laskey, 1974). After staining and destaining, gels were soaked in 20 volumes of Dimethylsulfoxide (DMSO) for 30 min, followed by a second immersion in fresh DMSO for another 30 min. The gels were then immersed in 4 volumes of 20% (w/v) PPO in DMSO for 3 hrs with constant shaking, followed by washing in tap water overnight. After drying, the gels were exposed to Kodak X-omat X-ray film at -70°C and then developed.

B.11. RNase activities in PRV infected cells

B.11.1. Preparation of post-ribosomal supernatant

Cells, mock infected or infected for 6 hrs with PRV, were homogenized in 2 volumes of 1mM phosphate buffer (pH 7.4) and centrifuged at 1500 r.p.m. for 10 min to remove nuclei and cell debris. The cytoplasmic extracts were then centrifuged at 50,000 r.p.m. for 2 hrs in the SW50.1 rotor. The top 2/3rd of the supernatant was taken and aliquoted at -70°C.

B.11.2. Assay of RNase activities

To an aliquot of the post ribosomal supernatant from either mock infected or infected cells, was added $^{3}$H - uridine labelled mRNA. Incubation was at 25°C for 30 min. TCA precipitable radioactivity was determined by taking 10µl aliquots onto filter discs, which were washed in 10% (w/v) TCA and then in ethanol and finally dried under a heat lamp. The discs were counted in a Liquid Scintillation Counter in Toluene-PPO.

Similarly dialyzed PRV as well as heat inactivated PRV was used to study RNase activities.

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