ASPECTS OF NUCLEOLAR METABOLISM IN HERPESVIRUS 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.

Savvas Kyriacou Kyriakidis, B.Sc.

Department of Biochemistry,
University of Glasgow.

November, 1981.

Acknowledgements.

I would like to thank Professor R.M.S. Smellie and Professor A.R. Williamson for making all the facilities of the Biochemistry Department available for carrying out this project.

It is my pleasure to thank my supervisor, Dr. W.S. Stevely, for his patient guidance and supervision, for his critical appraisal practically at every phase of my work and thoughtful suggestions during the writing of this thesis.

I would also like to thank Dr. J. Pitts and Dr. J. Goddard for their critical judgement, and Dr. J. Knowler for his suggestions in some of the experimental works.

Thanks are due to David Mease for making the virus stock available and also to the staff of Wellcome Cell Culture Unit for their help in providing the cells.

I would like to thank the students of Lab A16 and A20 for their friendly co-operation and helpful suggestions.

I would also like to thank Miss Anne Breslin and Miss Helen Breslin for the typing of this thesis.

To my father Kyriacos and my mother Euthemia.

Abbreviations.

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

BHK-21/C13	Baby hamster kidney cells, clone 13.
HSV	Herpes simplex virus.
PrV	Pseudorabies virus.
p.f.u.	Plaque forming unit.
SDS	Sodium dodecyl sulphate.
EDTA	Ethylene diamine tetra-acetate
TCA	Trichloroacetic acid
DMSO	Dimethyl sulfoxide.
PPO	2,5' - diphenyloxazole
TEMED	N,N,N,N, tetramethylethylene diamine.
HEPES	N-2-Hydroxyethylpiperizine-N'-2-ethane. sulfonic acid.
DTT	Dithiothreitol.

Summary.

The inhibition of ribosomal RNA production has been studied in Hela cells infected with pseudorabies virus (PrV). This showed that both synthesis and processing of ribosomal RNA was affected. Processing in PrV-infected Hela cells was at a reduced rate but did follow a similar pattern to that of mock-infected cells.

The effect on synthesis of ribosomal RNA was more pronounced and was followed by a concomitant decrease in the total nucleolar area of the infected Hela cells.

Polyacrylamide gel electrophoresis of fractions from mock-infected and PrV-infected Hela cells showed that a number of virus induced polypeptides were associated with the nucleus, nucleolus and the nucleolar chromatin. Some of these polypeptides were immediate early viral gene products.

A number of viral induced polypeptides were shown to be associated with the nuclei and nucleoli of mock-infected Hela cells after incubation in vitro of isolated nuclei and cell extracts of both mock-infected and infected Hela cells.

The RNA polymerase activity of isolated nuclei of PrV-infected Hela cells was lower than that of mock-infected. This was true both for total and for a-amanitin resistant activities.

The difference in RNA polymerase activity between mock-infected and infected Hela cells remained unchanged after incubation of the nuclei in the presence of the detergent sarkosyl. Again incubation in the presence of extracts of mock-infected or infected cells did not alter relative activities in the normal assay whereas RNA polymerase activity in mock-infected Hela cell nuclei was

reduced after a combined exposure to infected cell extracts followed by treatment with sarkosyl.

This suggests that its inhibition of ribosomal RNA synthesis in Hela cells after infection with pseudorabies virus is by interference at the initiation level of the transcription process rather than the elongation level. It may be mediated by a viral-induced protein(s) associated with the nucleolar and nucleolar chromatin cell fractions.

CONTENTS LIST.

(1981 15 NOEMBPH, KYPIAKH 3:06:14)

CHAPTER 1. Introduction

A - Nucleolus

1.1 Nucleolus structure

1.1.1 Nucleolus organizer regions.

Nucleolus organizer regions are specific chromosomal sites which are responsible for the formation of the nucleolus at the end of telophase and apparently contain the ribosomal cistrons. This was demonstrated in plant cells (Chouinard, 1975) and animal cells (Goessens and Lepoint, 1974) by electron microscopic studies as well as by following nucleologenesis, in chick erythrocyte nuclei reactivated by cell fusion (Dupoy-Coin et al., 1976; Hernandez-Verdun and Bouteille, 1979) and colchicine-induced micronuclei in rat kangaroo ovary tumour cells and A9 mouse cells (Hernandez-Verdun et al., 1979).

The nucleolar organizer has been localized in the secondary constriction of certain metaphase chromosomes (Heitz, 1931; Heitz&Bauer, 1933; Hsu et al., 1967; Smetana and Busch, 1974) and has been confirmed by in situ hybridization in man (Henderson at al., 1972; Evans et al., 1974), gibbon (Warburton et al., 1975), kangaroo rat (Hsu et al., 1975), Indian munjac (Perdue and Hsu, 1975) and mouse (Elsevier and Ruddle, 1975; Henderson et al., 1976). In situ hybridization also reveals that the ribosomal cistrons may be detected in chromosomal regions not displaying secondary constrictions (Hsu et al., 1975; Elsevier and Ruddle, 1975; Henderson et al., 1976). In interphase nuclei in situ hybridization shows the ribosomal cistrons to be localized either within the nucleolus or at the nucleolar periphery (Perdue, 1974; Stahl et al, 1976; Vagner-Capodano et al., 1977). The number and

the location of nucleolus organizer regions are specific. In human cells, five pairs of chromosomes, 13-14-15-21-22, exhibit such nucleolus organizer regions (Tantravahi et al., 1976).

Electron microscopy has led to the demonstration that the electron-lucid zones which Recher et al., (1969) called "fibrillar centres" correspond to the nucleolar organizer (Goessens, 1976; Jordan and Luck, 1976; Stahl et al., 1977; Mirre and Stahl, 1978a; 1978b; Vagner-Capodano and Stahl, 1980). This has been confirmed by in situ hybridization (Knibiehler et al., 1977).

The ultrastructure of fibrillar centres appears as round bodies of low electron density, surrounded by a dense fibrillar component (Mirre and Stahl; 1978a), which could be the site of rDNA transcription (Lepoint and Goessens, 1978; Mirre and Stahl, 1978a; 1978b) and histochemical and autoradiographic studies have shown that it contains DNA and protein (Lafontaine and Lord, 1973; Ryser et al., 1973; Goessens, 1976; Mirre and Stahl, 1978a; 1978b).

It has been shown that the fibrillar centres appear as euchromatin regions of chromosomes during mitosis (Goessens and Lepoint, 1974) or meiosis (Mirre and Stahl, 1976; 1978a; Jordan and Luck, 1976). At interphase, masses of condensed chromatin are structurally continuous with the extended chromatin of the fibrillar centres. The condensed nucleolar chromatin could correspond to the heterochromatin state of nucleolar chromosomes (Goessens, 1979).

1.1.2. Ultrastructure of nucleolus.

The ultrastructural morphology of the nucleolus as defined by electron microscopic studies is composed of electron -opaque fibrous material (fibrillar component), closely packed granules (granular component), nucleolus-associated condensed chromatin and low electron density fibrous material surrounded by, and intimately associated with the dense fibrous material. (see fig. 1.1.2.1).

1.1.2.1. The fibrillar component.

The fibrillar component is composed of fibrils approximately 30-40 Å in diameter (Marinozzi and Bernhard, 1963; Bernhard and Granboulan, 1968; Busch and Smetana, 1970) and about 200-400 Å in length (Busch and Smetana, 1970).

The fibrillar component contains 45s precursor ribosomal RNA, as has been shown by labelling experiments, coupled with electron microscopy and autoradiography in various cell systems (Buteille et al., 1974; Fakan, 1978) and biochemical studies, in fractions containing nucleolar fibrillar components (Matsuura et al., 1974; Royal and Simard, 1975).

Electron microscopy and autoradiography was also used to reveal the site of transcription in the nucleoli. It was found that after a short time of incubation in radioactive precursor, label appearedpredominantly in the transition regions between the fibrillar centres (see section 1.1.1) and the adjacent fibrillar component while the centre itself remained unlabelled (Fakan, 1971; Goessens, 1976; Lepoint and Goessens, 1978; Mirre and Stahl, 1978a; b; c; Stahl et al., 1978).

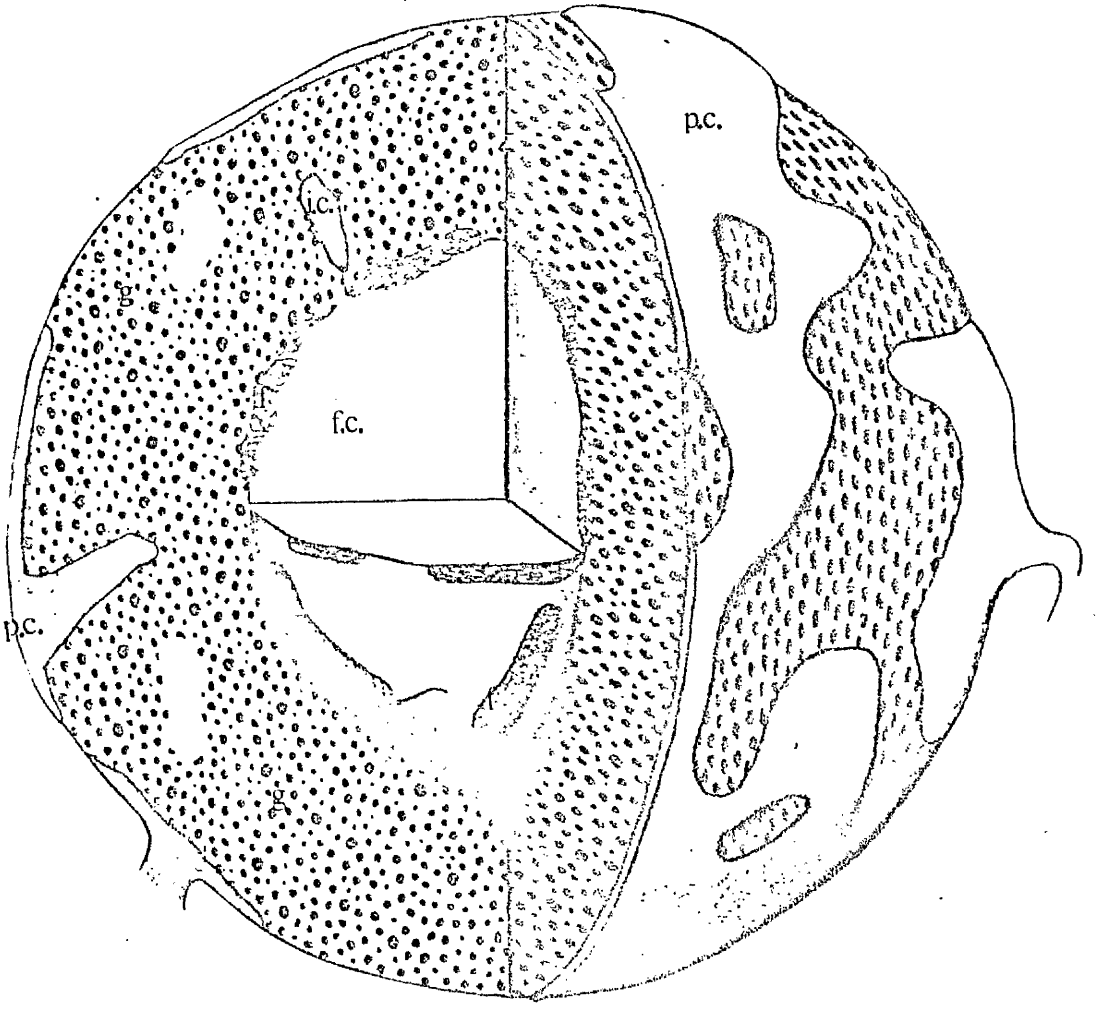
1.1.2.2. The granular component.

The granular component contains granules 100-200 Å in

Figure 1.1.2.1. Hypothetical diagram of nucleolus.

f.c: fibrillar centre, f: fibrillar component, g: granular component, p.c.: perinucleolar chromatin, i.c.: intranucleolar chromatin.

from Goessens, G. and Lepoint, A. (1979).



diameter (Bernhard and Granboulan, 1968; Busch & Smetana, 1970). Labelling experiments revealed that the granules were labelled after the fibrils (Granboulan and Granboulan, 1965) which led to the conclusion that fibrils were the morphological precursors of the granules and corresponded to the processing of 45s pre-rRNA into 28s and 18s rRNA. This was later confirmed by other investigators (see Fakan, 1978). In addition to labelling experiments an early report demonstrated the morphological transition between nucleolar fibrils and granules (Marinozzi 1964).

Biochemical analysis has revealed the presence of 36s and 32s RNA (Royal and Simard, 1975) as well as 28s RNA (Koshiba et al, 1971) in isolated fractions of nucleolar granular components in cultured CHO cells and rat liver.

1.1.2.3. Nucleolus-associated chromatin.

Chromatin structures associated with the nucleolus are present around (perinucleolar) or within (intranucleolar) the nucleolar body and contain many different chromosomal segments as observed by the use of specific stains (Stahl et al., 1976).

Perinucleolar chromatin surrounds the nucleolar body in a discontinuous shell of clusters of fibrils, usually composed of coiled fine filaments, displaying the same staining properties as the rest of the condensed chromatin. It often shows irregular extensions into the nucleoplasm, whereas the nucleolar body appears penetrated by chromatin stretches, to form the intranucleolar chromatin in continuity with perinucleolar chromatin (Bernhard, 1966).

The quantity of intranucleolar chromatin appears to be directly related to the level of transcriptional activity of the nucleolus (Smetana and Busch, 1974) and modifications in the quantitative partition between intranucleolar and perinucleolar chromatin rapidly occur in parrallel with alterations in cell growth (Simard and Bernhard, 1967).

It has been suggested that intranucleolar chromatin exists simultaneously, as clumped 100 Å fibrils and dispersed 20 Å fibrils (Unuma et al., 1968). This complex structure of intranucleolar chromatin was confirmed by other investigators (Smetana et al., 1968 a;b;) and can probably be condidered to express the presence of condensed and extended chromatin within the nucleolus.

1.1.3. Nucleolar constituents.

1.1.3.1. DNA.

The DNA in the nucleolus consists of (a) the ribosomal DNA (b) the DNA coding for the nucleolus specific U_3 RNA and (c) the nucleolus-associated DNA for which functions have not yet been defined.

The ribosomal DNA contains the 18s, 5.8s and 28s rRNA genes and transcribed and non-transcribed spacer sequences (Birnstiel et al., 1971; Dawid et al., 1970). The transcriptional unit consists of the 18s, 5.8s and 28s rRNA genes, in that order from 5' to 3' (Planta and Meyerink, 1979), separated by transcribed spacers and is present in multiple copies per genome, both in procaryotes and eukaryotes, with the number of copies varying considerably between species. (Long and Dawid, 1980).

The multiple rRNA transcriptional units are arranged along the DNA separated by the non-transcribed spacer sequences. In higher eukaryotes the length of the non-transcribed spacers varies in size among species, although in most cases is comparable to the length of the transcriptional unit (Trendelenburg et al., 1973).

However, in mammals, ribosomal DNA represents a very minor part of nucleolus-associated DNA sequences, less than 1% in most cases (Attar di et al., 1965; Stambrook, 1974).

1.1.3.2. RNA.

The nucleolus contains, the first labelled and largest precursor ribosomal RNA molecule designated 45s pre-rRNA (Scherrer and Darnell, 1962), which is known to be a common precursor for both ribosomal RNA's (Attardi and Amaldi, 1970; Maden, 1971), the intermediate precursors of processing and

the low molecular weight U_3 RNA, which has been shown to be nucleolus specific (Ro-choi and Busch, 1974) and hydrogen bondedto precursor rRNAs (Prestayako et al., 1970).

Precursor ribosomal RNA is found in the nucleolus in the form of pre-ribosomal particles,bound with proteins. Two types of such particles found in Hela cells were 80s pre-RNP, containing 45 pre-rRNA and 55s pre-rRNP containing 32s pre-rRNA. Both particles contain 5s rRNA (Warner and Soeiro, 1967; Warner, 1974).

Kinetic studies with labelled precursors to both RNA and proteins, established that the 80s pre-RNP is a precursor to the 55s pre-RNP, which in turn matures to the large ribosomal subparticle (Warner, 1974), in addition 80s pre-RNP may be a processing particle since it seems to contain small amounts of 41s, 32s and 20s pre-rRNA (Soeiro, 1968).

1.1.3.3. Proteins.

Protein molecules bind newly synthesized ribosomal RNA inside the nucleolus to form preribosomes. Studies on 0.4N sulfuric extracts of nucleoli of rat liver and Novikoff hepatoma cells, have revealed the presence of 96 distinct polypeptides on two-dimensional polyacrylamide gel electrophoresis (Orrick et al., 1973; Yeoman et al., 1973). While 7-10 of these spots are histones most of the polypeptides visualized are non-histone proteins.

Jackowski et al., (1976) have also isolated and analysed by two-dimensional gel electrophoresis the nucleolar proteins of male albino rats and identified more than 100 components.

The proteins from animal cell pre-ribosomal particles

have been characterized in some detail. Analyses of proteins of 55s pre-RNP, pulse and steady state labelled, by two-dimensional gel electrophoresis have shown the presence of 65 polypeptides with 30 of these found at 60s mature ribosomal subunits (Kumar and Subramanian, 1975). Analyses of 80s pre-RNP particle proteins identified about 60 polypeptides with 21 identified as large ribosomal subunit and with 10 as small ribosomal subunit proteins. The remaining 29 non-ribosomal proteins are probably stable and confined to the nucleolus (Prestayako et al., 1974).

The presence of small ribosomal subunit proteins in the nucleolus is also supported by fingerprint analyses of tryptic digestion products of nucleolar protein (Sheperd and Maden, 1972). However analysis of pre-ribosomal particle proteins of Hela cells detected no small ribosomal subunit proteins on either precursor (Kuter and Rodgers, 1976) whereas lymphocytic mouse leukemia cells small ribosomal subunit proteins were detected associated with the 80s pre-RNP and not the 55s pre-RNP (Auger-Buendia and Longuet, 1978).

1.1.3.4. Enzymes.

One of the most significant enzymes which is present in nucleoli of eukaryotic cells is DNA-dependent RNA polymerase which is much more complex in structure, localization and function than its procaryote counterpart.

DNA-dependent RNA polymerase is present in eukaryotic cells in multiple forms as has been shown both by chromatography on DEAE-sephadex (Roeder and Rutter, 1969; 1970) and by the use of the specific inhibitor α-amanitin (Lindell et al., 1970). Three classes of enzymes (I, II, III or A,B,C)

Table 1.1.3.1. Classification, nomenclature and general properties of eukaryotic DNA-dependent RNA polymerases.

Class	Micro-heterogeneity*	a-Amanitin-sensitivity	Principal intracellular localization	Putative function
I or A	I_A or AI, AII, AIb	Insensitive	Nucleolar	Ribosomal RNA synthesis
	I_B or Ia, AI, AIa			
II or B	(II_o) or BO	Sensitive to low concns. (1-10nM)	Nucleoplasmic	hnRNA (mRNA?) sensitive
	II_A or BI			
	II_B or BI			
III or	C (CI), (CII)	Sensitive to high concns. (10-100 μM)	Nucleoplasmic (cytoplasmic)	4S and 5S RNA synthesis.
	III_A or $CIII_a$			
	III_B or $CIII_b$			

*For species in parentheses, there is a doubt as to whether they are of general occurence.

(after Beebee and Butterworth, 1977).

Table 1.1.3.2.

Subunits of calf thymus RNA polymerase AI.

Form AIb		Form AIa	
Subunit	Molecular weight[a]	Subunit	Molecular weight[a]
SA1	197 (1)[b]	SA1	197 (1)
SA2	126 (1)	SA2	126 (1)
SA3	51 (1)	-	--
SA4	44 (1)	SA4	44 (1)
SA5	25 (2)	SA5	25 (2)
SA6	16.5 (2)	SA6	16.5 (2)

[a] Molecular weight: daltons x 10^{-3}

[b] Numbers in parantheses correspond to molar ratios.

(from Chambon, 1975)

were analysed accordingly (see Table 1.1.3.1). Class A enzymes, forms AI and AII have been resolved in rat liver and both appear to be of nucleolar origin and resistant to inhibition by a-amanitin. (Chesterton and Butterworth, 1971a; Chesterton et al., 1972; Muramatsu et al., 1975).

The AI form was further split into two chromatographically distinct forms AIa and AIb which differ by the presence (AIa) or absence (AIb) of one small (approx. 60.000 daltons) polypeptide (Muramatsu et al., 1975; Gissinger & Chambon, 1975; Matsui et al., 1976a) and it has been suggested that form AII may constitute the pool of "free" enzyme (Matsui et al., 1976b, Kellas et al., 1977).

All the eukaryotic DNA-dependent RNA polymerases are macromolecular multi-subunit enzymes having molecular weights close to/or in excess of 500.000. In all cases, the enzymes consist of two high molecular weight subunits (in excess of 100.000) and a number of smaller subunits (less than 100.000). However there is no general agreement as to the precise subunit structure of the various enzymes. On the other hand immunological studies suggest that forms I and II enzymes are different gene products (Hildebrandt et al., 1973; Kedinger et al., 1974) whereas fingerprinting analyses suggested that three small subunits may be common to all the enzyme forms (Buhler et al., 1976). The subunit structure of AI enzyme is shown in Table 1.1.3.2.

Other enzymes which have been suggested to be localized in the nucleolus are an NAD synthetase (pyrophosphorylase) (Busch and Smetana, 1970), and a protein kinase activity (Kang et al., 1974).

Other enzymes which should be localized in the nucleolus are enzymes for the processing of ribosomal

RNA. Several endoribonucleases have been discovered in nucleoli (Prestayako et al., 1973; Mirrault and Scherrer,1972; Kwan 1976; Hall et al., 1977), but the most likely candidate is an endoribonuclease, named RNAse DII,which is associated with the pre-ribosomal particles and is double stranded RNA specific (Grummt et al., 1979).

1.2. Nucleolar function.

The major function of the nucleolus is the formation of precursors of the ribosomes, which pass into the cytoplasm to form the mature ribosomes. This involves the synthesis of ribosomal RNA, by RNA polymerase I (Weinman & Roeder, 1974) and its processing in several steps to yield mature cytoplasmic RNA (Perry, 1976). Ribosomal proteins are synthesized in the cytoplasm (Craig & Perry, 1971; Wu & Warner, 1971), transported to the nucleolus (Warner, 1974) and assembled on a precursor to ribosomal RNA (Warner, 1974; Maden et al., 1974).

1.2.1. Transcription.

The size of the primary transcript of ribosomal DNA in mammals has a sedimentation coefficient of 45s with a mol. weight of 4.1×10^6 daltons and in lower eukaryotes of 36s to 38s with a mol. weight of 2.6 to 2.8×10^6 daltons respectively. (Loening et al., 1969; Wellauer & Dawid, 1975).

Regulation in the rate of transcription of ribosomal DNA might involve changes in already existing enzymes rather than in absolute numbers of enzyme molecules (Schmid and Sekeris, 1975; Franze-Fernantez and Fontanive-Sanguesa 1975).

Thus regulation of ribosomal RNA synthesis might be template-mediated rather than RNA polymerase-mediated. Indeed, McKnight & Miller (1976) have suggested that an increase in the rate of rRNA synthesis occurs by the progressive activation of additional transcription units. Thus the amounts of template bound RNA polymerase I probably vary with metabolic state. This was suggested by the assay of Hela cell nuclei isolated from cells in a variety of metabolic conditions. Assays were carried out in the presence of the anionic detergent sarkosyl

(Chesterton et al., 1975). Further evidence comes from RNA 3'-end labelling after oestradioltreatment of chick oviduct (Cox, 1976).

The existence of separate free and bound pools of RNA polymerase I molecules was shown in experiments using exogenous added poly (dA-dT) templates in the presence of actinomycin (Lampert & Feigelson, 1974; Yu, 1974; Grummt et al., 1976.)

Nonetheless ribosomal RNA synthesis does depend on continuous protein synthesis (Lampert & Feigelson 1974; Grummt et al., 1976) which is presumed to be mediated either by a rapidly turning-over factor (Lampert & Feigelson, 1974 Chesterton et al., 1975) or by the intracellular concentrations of GTP and ATP (Grummt and Grummt, 1976).

Electron-microscopic evidence together with u.v.-irradiation studies on ribosomal cistron transcription, have suggested that each ribosomal gene has its own promoter in contrast with the model of Perry et al., (1970), proposing a single promoter region for all the ribosomal genes linked in tandem. This conclusion is suggested by the frequent appearance of putative polymerases in transcribed spacer regions (Scheer et al., 1976; Hackett and Sauerbier, 1975).

1.2.2. Processing.

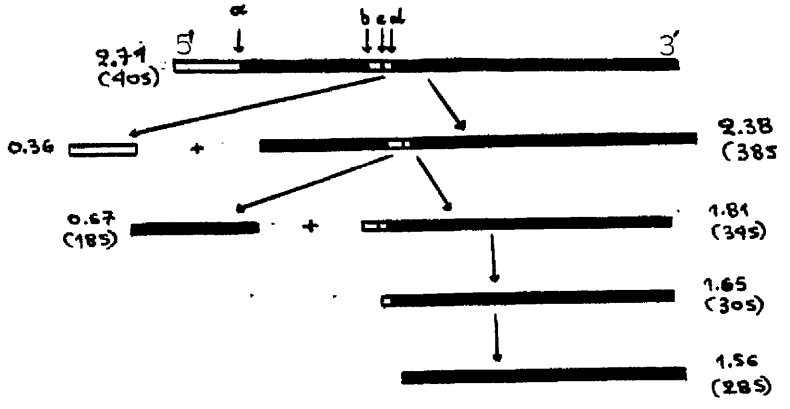
The primary transcript of ribosomal DNA is further subjected to processing to yield mature rRNA. Processing of the primary transcript involves endonucleolytic cleavages, possibly carried out by specific endonucleases assisted by nucleotide modifications, the bound proteins and

the double-stranded regions of pre-rRNA.

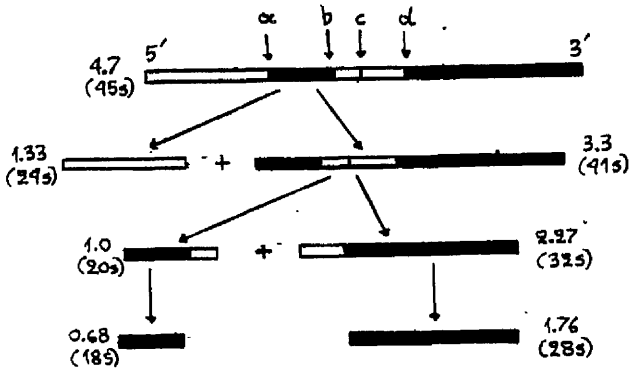
The possibility exists that at least in some eukaryotic organisms and cells, processing may precede the completion and accumulation of the primary pre-rRNA. The evidence for this is that rapidly labelled RNA molecules can be isolated, which are larger than the established primary pre-rRNA for the respective organisms (Hidvegi et al., 1971) and that larger than primary pre-rRNA molecules are synthesized in isolated nuclei by an a-amanitin resistant RNA polymerase (Caston and Jones, 1972; Grummt et al., 1975) and finally by the application of the gene spreading technique to various eukaryotic organisms (Trendelenburg et al., 1973; Trendelenburg, 1974).

The pre-rRNA processing pathways have been intensively studied in many different eukaryotes and basic similarities have been shown to exist, as illustrated in Fig. 1.2.2.1 (Hadjolov and Nikolaev, 1977).

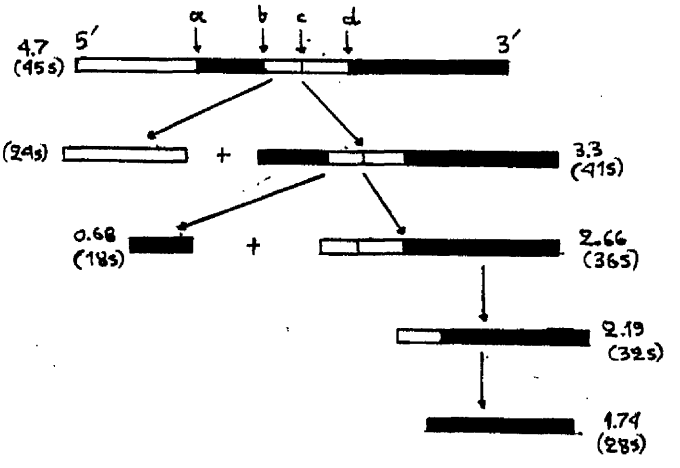
Three distinct steps may be outlined in primary pre-rRNA maturation. The first step involves an endonucleolytic cleavage to remove the external transcribed spacer, the second step involves an endonucleolytic cleavage of the intermediate pre-rRNA to produce the precursor to the ribosomal 28s rRNA and in some cases directly the mature 18s rRNA and the third step, which is rather complex and slower, involves an endonuclease attack to form the 28s rRNA and 5.8s rRNA and a final trimming of 28s and 18s pre-rRNA molecules (Wellauer and Dawid, 1973). Four specific endonucleolytic events have been shown to occur in mammalian cells (Wellauer and Dawid, 1973; Wellauer et al., 1974) but the order in which cleavages occur may vary. It has been suggested that double-stranded regions in pre-rRNA may play a role in defining the correct



(a) Xenopus laevis



(b) Hela cells



(c) L cells

Figure 1.2.2.1 Scheme of maturation of primary pre-rRNA in Xenopus laevis,Hela cells and L cells. a,b,c and d denote cleavage sites and the order of endoribonuclease attack.

(from Hadjolov and Nikolaev,1977)

processing sites (Gotoh et al., 1974; Snyder et al., 1971). Similarly, the association of proteins with pre-rRNA may be of critical importance throughout the entire maturation process (Craig and Perry, 1970; Maden, 1971).

Several endoribonucleases have been reported (see section 1.1.3.4) as possible candidates for the cleavage of 45s pre-rRNA but direct evidence for their involvement has not yet been obtained.

Two types of nucleotide modification occur in ribosomal RNA, one is the conversion of uridine to pseudouridine (5-ribosyluracil), which is present in both 28s and 18s rRNA and constitutes about 0.5% to 5% of the uridine residues (Attardi and Amaldi, 1970) and the other is methylation, which takes place either on the 2' position of the ribose moiety or on the nucleotide base and most of these occur at the level of the 45s primary pre-rRNA molecules and along sequences corresponding to mature 28s rRNA, 18s rRNA and 5.8s rRNA (Maden et al., 1974). Both modifications occur during or after transcription and their possible function in processing is as yet unclear.

That methylation plays a role in ensuring correct processing has been assumed by the occurence of ribose and base methylations at specific sites along the primary pre-rRNA (Maden et al., 1974) and is supported by the finding that methionine starvation of Hela cells results in changes in the correct processing of the pre-rRNA (Vaughan et al., 1967).

B. Herpesviruses.

1.3. Introduction.

The herpesviruses are large eucaryotic viruses, with linear double-stranded DNA genomes of more than 80 x 10^6 mol. wt., which are replicated in the nucleus of infected cells, assembled into 100nm diameter icosahedral capsids composed of 162 prismatic capsomeres which are enclosed in glycoprotein and lipid (ether sensitive) envelopes to give the normaly infectious extracellular form of the virus (Wildy, 1972; Fenner, 1976).

Herpesviruses have been observed in a wide variety of hosts as diverse as fungi, oysters, fish (e.g. channel catfish, rainbow trout), amphibians and reptiles (e.g. Lucke virus of frog), birds (including Marek's disease virus of chickens) and numerous mammals including monkeys, apes and man (Nahmias, 1972). A single host may be infected by several distinct herpesviruses each of which can produce different pathological consequences. For example, humans are hosts to at least five herpesviruses (HSV-1, HSV-2, cytomegalovirus, Epstein-Barr virus and varicella-zoster virus) whose pathology ranges from mild skin diseases to severe, frequently fatal lymphomas and leukaemias.

Although 70 or more viruses (Table 1.3.1) are tentatively included in the herpesvirus group, criteria for their inclusion varies from a brief morphologic description (virus obtained from snake venom, oysters and fungi) to detailed antigenic, structural, biochemical, and genetic characterization (e.g. HSV-1, HSV-2).

Because herpesviruses constitute a widely diverse group,

Table 1.3.1. Provisional Labels, Common names and Properties of the DNA of Herpesviruses.

Provisional label[a]	Common name	Abbreviation	G+C (moles %)	MW ($\times 10^{-6}$)
Human herpesvirus 1	Herpes simplex type 1	HSV-1	67	97-99
Human herpesvirus 2	Herpes simplex type 2	HSV-2	69	99
Human herpesvirus 3	Varicella-zoster virus		46	
Human herpesvirus 4	Epstein-Barr virus	EBV	59	
Human herpesvirus 5	Cytomegalovirus	CMV	56-57	100
Ceropithecid herpesvirus 1	B virus			
Ceropithecid herpesvirus 2	SA 6		51	
Ceropithecid herpesvirus 3	SA 8		67	
Cebid herpesvirus 1	Herpesvirus tamarinus; herpesvirus platyrrhini; marmoset herpesvirus			
Cebid herpesvirus 2	Herpesvirus saimiri		50	
Cebid herpesvirus 3	Spider-monkey herpesvirus		72	
Callitrichid herpesvirus 1	Marmoset herpesvirus			
Tupaiid herpesvirus 1	Tree shrew herpesvirus		66	
Canine herpesvirus 1	Canine herpesvirus		33	
Feline herpesvirus 1	Feline rhinotracheitis		46	
Equid herpesvirus 1	Equine abortion virus, equine rhinopneumonitis virus	EAV	57	84-94

Continued

Table 1.3.1. Provisional labels, Common Names and properties of the DNA of Heppesviruses.

Provisional label[a]	Common name	Abbreviation	G+C (moles %)	MW ($\times 10^{-6}$)
Equid herpesvirus 2	Slowly growing cytomegalo-type viruses		58	
Equid herpesvirus 3	Coital-exanthema virus	EAV	66	
Bovid herpesvirus 1	Infectious bovine rhinotracheitis virus		71-72	
Bovid herpesvirus 2	Bovine mammalitis virus		64	82
Bovid herpesvirus 3	Wildebeest herpesvirus, malignant cattarrhal fever virus			
Bovid herpesvirus 4	Herpesvirus from sheep pulmonary adenomatosis			
Pig herpesvirus 1	Pseudorabies virus	PrV	72	95
Pig herpesvirus 2	Inclusion-body rhinitis virus, pig cyto-megalovirus			
Murid herpesvirus 1	Mouse cyto-megalo-virus from Mus		59	132
Murid herpesvirus 2	Rat cytomegalo-virus from Rattus			
Sciruid herpesvirus1	Cytomegalovirus from European ground squirrel			
Caviid herpesvirus1	Guinea-pig cytomegalovirus		57	
Lagomorph herpesvirus 1	Rabbit herpesvirus			
Phasianid herpesvirus 1	Infectious laryngotracheitis		45	

Continued

Table 1.3.1. Provisional labels, Common Names and Properties of the DNA of Herpesviruses.

Provisional Label[a]	Common Name	Abbreviation	G+C (moles %)	MW ($\times 10^{-6}$)
Phasianid herpesvirus 2	Mareks disease virus	MDV	46	103
Turkey herpesvirus 1	Turkey herpesvirus		46	
Anatid herpesvirus 1	Duck-plague herpesvirus			
Pigeon herpesvirus 1	Pigeon herpesvirus			
Cormorant herpesvirus 1	Cormorant herpesvirus			
Iguana herpesvirus 1	Iguana herpesvirus			
Ranid herpesvirus 1	Lucke virus		44-45	
Ranid herpesvirus 2	Frog Virus 4		56	
Catfish herpesvirus 1	Catfish herpesvirus		56	

(from Roizman,1977)

Honess and Watson (1977) investigated measures of similarity and difference between the putative members of the herpesvirus group at the level of the host and disease, the gross properties of size, sequence arrangement and base composition of the genome as well as the number and properties of some gene products. Their conclusions for the characteristics which confer the essential unity of the family are: the common possesion of DNA with single strand interruptions and a mol. wt. $>80 \times 10^6$, a major capsid polypeptide mol. wt. >140.000, a virus-specific non-structural DNA polymerase and a propensity for persistence in the absence of overt disease.

The International Commitee on Taxonomy of Viruses have classified herpesviruses as a separate family, namely the Herpetoviridae (Fenner, 1976). The diversity of herpesviruses has caused problems in the sub-division of Herpetoviridae into genera (Fenner, 1976).

Recently Yeo et al., (1981) have reported the identification of the polypeptides involved in the cross-reacting antigens of five herpesviruses. The predominant polypeptide, corresponded to the major DNA binding protein. They suggested that the DNA binding protein will be useful for the classification of the group, that the major capsid protein may help identify some cross-relationships among members of the group, and that the glycoproteins will define the subgroups of the family.

1.4. The herpesvirion.

1.4.1. Structural components.

The virion consists of 4 major structural components. The core, which is surrounded by the capsid, the tegument and the envelope. These are discussed below.

1.4.1.1. The Core.

The core is the innermost structural component of the virion and appears in thin sections of virions as an electron dense ring surrounding an electron-translucent centre. Evidence that the core contains DNA comes from Epstein (1962), who demonstrated the DNAse sensitivity of the central region of the virion; later Furlong et al., (1972) showed that the DNA is contained in the electron-dense ring, by treating glutaraldehyde fixed, uranyl-acetate stained thin sections with EDTA. EDTA selectively removed uranyl ions bound to DNA

Although there is no direct evidence on the composition of the core, it probably contains proteins and polyamines (Roizman, 1977). Possible candidates for the core proteins might be the four proteins which have been demonstrated to bind tightly but non-covalently to highly purified HSV DNA in vitro (Hyman, 1980).

1.4.1.2. The capsid.

The capsid surrounds the core and appears in thin sections as a moderately electron dense hexagon or ring, separated from the core by an electron translucent shell (Roizman, 1977)

The capsid consists of capsomeres arranged in 2-, 3- and 5- fold symmetry and from the geometrical structure of

the capsid it has been deduced that it consists of 162 capsomeres arranged in the form of an icosadeltahedron (Wildy et al., 1960). The region between the capsid and the core is called the pericore and its nature is unknown.

1.4.1.3. The tegument.

The tegument is located between the capsid and the envelope (Roizman & Furlong, 1974) and in thin sections appears as a layer of amorphous material and in negatively stained virions as a fibrous structure (Roizman, 1977).

1.4.1.4. The envelope.

The virion is surrounded by the envelope, a trilaminar membrane with spikes projecting from its outer surface (Fong et al., 1973). It is constituted of glycoproteins, glycolipids and lipids (Morgan et al., 1954). Envelopes are formed during the passage of the virionthrough the nuclear membrane as well as from Golgi apparatus membranes, endoplasmic reticulum and plasma membranes. (Darlington & Moss, 1968; Schwartz & Roizman, 1969; 1969a; Epstein, 1962).

The role of the envelope is not yet clear, although it is believed to facilitate adsorption (Holmes & Watson, 1962; Abodeely et al., 1971) or to give stability to the virus outside the cell (Spring & Roizman, 1968).

1.4.2. Chemical constituents.

1.4.2.1. DNA.

The genome of herpesviruses consists of a linear, double-stranded DNA molecule. Linearity has been confirmed by electron microscopic studies (Becker et al., 1968;

Grafstrom et al., 1975; Wadsworth et al., 1975).

The mol. wt. of the DNA of pseudorabies virus, one of the herpesviruses, is about 90 to $95x10^6$ daltons (Stevely, 1977) and generally the size of herpeviruses DNA varies from 80 to 150 x 10^6 daltons.

The DNA molecule of a number of herpesviruses is fragmented upon denaturation with alkali (Kieff et al., 1971; Lee et al., 1971; Nonoyama et al., 1972). A number of possible explanations have been suggested from the presence of ribonucleotides to single strand nicks or gaps, However the evidence most strongly suggests that fragmentation is due to the presence of gaps in the HSV DNA (Roizman, B., 1977, The Herpesviruses). The presence of nicks and gaps at random sites along the DNA molecule has also been reported to be the cause for the fragmentation of pseudorabies virus DNA (Ben Porat et al., 1979).

The base composition of herpesvirus DNAs varies, ranging from 33% to 74% G+C (Fenner, 1976). Pseudorabies virus DNA has a G+C content of 72% G+C (Ben-Porat & Kaplan, 1962; Russell & Crawford, 1963).

Herpes simplex and pseudorabies virus DNA consist of two unequal regions, the long (L) and the short (S) unique regions. L and S herpes simplex unique regions are flanked by terminally redundant sequences (Grafston et al., 1974)and their internal inverted repeats (Sheldrick & Berthelot, 1974; Wadsworth et al., 1975) whereas inpseudorabies virus this is true for the short unique region only and not the long (Stevely 1977; Ben-Porat et al., 1979).

Four possible genomic arrangements could result from

internal recombinations between the redundant sequences in herpes simplex virus DNA, and analysis of the HSV-1 genome by a variety of methods has proved that they are present in approximately equal amounts within a population of DNA molecules. (Hayward et al., 1975; Clements et al., 1976; Delius and Clements, 1976; Wilkie & Cortini 1976). A similar sequence of organisation of the DNA have been reported for a number of other herpesviruses as well (Buchman and Roizman, 1978; Bornkam et al., 1980; Westrate et al., 1980; Chousterman et al; 1979).

1.4.2.2. Structural proteins.

Structural proteins which constitute the main components of the virion along with DNA, have been analyzed in polyacrylamide gels containing dodecyl sulfate. Herpes simplex purified virions contain 33 polypeptides and range in molecular weight from 25.000 to approximately 280.000 daltons (Spear and Roizman, 1972). Enveloped pseudorabies virus particles contain at least 20 polypeptides and range in molecular weights between 20.000 to 155.000 daltons (Stevely, 1975).

Analyses of the structural polypeptides of other herpesviruses showed that they are as complex as herpes simplex and pseudorabies virus. (Perdue et al., 1974; Dolyniuk, et al., 1976; Sarov and Abady, 1975).

1.4.2.3. Other Components.

Herpesviruses contain lipids and the polyamines spermine and spermidine. Spermine is believed to be localized in the core and to be involved with the neutralization of DNA-phosphate viral groups (Gibson & Roizman, 1971; 1973), whereas spermidine was suggested to be associated with the envelope since it was

removed after disruption of the viral envelope with a non-ionic detergent and urea (Gibson & Roizman 1973). Very little is known about the lipids of the herpesvirion but it was suggested that they are essential for infectivity of the mature virions and are contained in the envelope (Spring & Roizman, 1968).

1.5. The reproductive cycle of Herpesviruses.

1.5.1. Initiation of infection.

Only the enveloped nucleocapsids could initiate infection (Nii et al., 1968; Ames and Dubin, 1960) whereas naked nucleocapsids can not (Stein et al., 1970; Rubenstein & Kaplan 1975; Rubenstein et al., 1972). However viral DNA rendered free of proteins is infectious (Sheldrick et al., 1973).

Infection begins by the adsorption of the viral enveloped nucleocapsids by the host cell and is slow (Roizman & Furlong, 1974). Penetration follows which could be either by phagocytosis (Dales & Silverberg, 1969) or by fusion of the viral envelope with the plasma membrane (Morgan et al., 1968; Zee and Talens, 1972).

Once inside the cytoplasm the capsid dis agregates and the viral DNA-protein complex is transported into the nucleus (Hochberg and Becker, 1968).

1.5.2. Transcription of the viral DNA.

The first event in the herpesvirus reproductive cycle is the transcription of the viral DNA, which takes place in the nucleus (Wagner & Roizman, 1969; 1969a) and is carried out by the host DNA dependent RNA polymerase II (Preston and Newton, 1976; Costanzo et al., 1977; Ben-Zeev & Becker, 1977). Viral RNA is processed by cleavage, methylation, adenylation and capping almost in the same way as cellular mRNA (Bartkoski and Roizman, 1976; 1978; Moss et al., 1977; Bachenheimer and Roizman, 1972; Stringer et al., 1977).

Viral mRNA is classified, according to the time of synthesis in the infected cells. Immediate early mRNAs are synthesized very early after infection and accumulate when protein

synthesis is inhibited, early mRNAs are synthesized after the initial synthesis of virus specified proteins but before viral DNA synthesis and late mRNAs are synthesized after viral DNA synthesis (Kozak & Roizman, 1974; Swanstrom & Wagner, 1974; Swanstrom et al., 1975).

1.5.3. Synthesis of viral proteins.

Protein synthesis in herpesviruses is regulated and different polypeptides are synthesized at different times after infection. First attempts to categorize the timing of herpesviruses protein synthesis, succesfully demonstrated the presence of an immediate early class of proteins which correspond to the immediate early mRNAs described above and are made soon after the release of a cycloheximide block in pseudorabies virus infected cells (Rakusanova et al., 1971; Ben-Porat et al., 1974). Using the same type of treatment this was also found to be true in HSV-1 infected cells and allowed the classification of viral polypeptides into three groups α, β, and γ. γ polypeptides, contained the major virus structural polypeptides, while α and β were made up from both structural and non-structural proteins (Honess & Roizman, 1974). Further work of Honess and Roizman (1975) led to a model of sequential ordering and coordinate regulation, where α polypeptide(s) are required to switch-on β polypeptides, which in turn are required to switch-off α polypeptides, with a similar scheme for β and γ polypeptides.

Viral proteins structural and non-structural may be phosphorylated (Pereira, et al., 1977; Marsden et al, 1978; Bookout and Levy, 1980) or glycosylated (Kaplan and Ben-Porat, 1976.)

1.5.4. Synthesis of viral DNA.

Viral DNA synthesis requires de novo protein synthesis but once initiated, viral DNA synthesis continues in the absence of concomitant protein synthesis (Cheng et al., 1975; Roizman & Roane, 1964). Many herpesviruses code for their own DNA polymerase (Allen et al., 1977; Miller & Rapp, 1977; Muller et al., 1977). HSV - DNA synthesis is suggested to be promoted by an RNA initiator (Muller et al., 1980).

Pseudorabies virus DNA is known to be synthesized in the form of concatamers (Jean & Ben-Porat, 1976; Ben-Porat & Veach, 1980; Ladin et al., 1980).

1.5.5. Assembly and release.

Assembly of the mature macromolecules occurs in the nucleus (Roizman & Furlong, 1974). It has been suggested that the capsid proteins are assembled first into an empty shell, into which DNA is encapsidated (Ladin, 1980) and that DNA is inserted into capsid without a preformed core (Perdue et al., 1976).

Viral particles acquire their envelopes by budding through the inner nuclear membrane or other sites and are released by a process of reverse phagosytosis (Morgan et al., 1959; Nii et al., 1968; Katsumoto et al., 1981).

1.6. Changes in cell structure and function.

1.6.1. Introduction.

Infection of a host cell by a herpesvirus results in productive infection or in transformation of the cell or in abortive infection leading to virus latency. Available data indicate that the consequence of productive infection is cell death (Roizman, 1972), which follows gross alterations in cell morphology and in the structure of cellular organelles accompanied by drastic alterations in host DNA, protein and RNA synthesis.

1.6.2. Structural changes.

Infection of cells with a herpesvirus results in changes in shape and behaviour. Thus the cells may round up and lose contact with neighbours, they may clamp together, or fuse into multinucleated giant cells (Roizman, 1962; Darlington & Granoff, 1973; Roizman & Furlong, 1974).

Alterations in the cell structure involve the characteristic displacement and condensation of the chromatin near the nuclear membrane (Smith and de Harven, 1973), the thickening of nuclear membrane areas (Schwartz & Roizman, 1969; Tevethia et al., 1972), and alterations in nucleolar structure and size which consist of the segregation of nucleolar components, aggregation into more compact and electron dense masses, size reduction to the point of being reduced to small fragments (Sirtori & Bosisio-Bestetti, 1967) and the formation of intranuclear inclusion bodies (Love and Wildy, 1963; Smith & De Harven, 1973).

Changes in the cytoplasm involve the destruction of

the cytoskeleton (Schlehofer et al., 1979; Katsumoto et al., 1981), suggested by the interaction of herpesviruses with the microtubules and the lack of a distinct separating surface of the cell with the substratum. Further alterations in the cytoplasm consist of the appearance of patches of altered membranes, an increase in the frequency of microvilli (Roizman, 1977) as well as an increased stability of the cellular membrane of HSV-1 infected Hep-2 cells compared to uninfected (Schlehofer et al., 1979).

1.6.3. Effects of infection in macromolecular synthesis.

1.6.3.1. DNA.

Inhibition of host DNA synthesis has been observed with a number of herpesviruses (Kaplan & Ben-Porat, 1963; Roizman & Roane, 1964; O'Callaghan et al., 1968; Nonoyama & Pagano, 1972; Gergely et al., 1971), and this inhibition coincides with the decline in the activity of cellular DNA polymerase α (Muller et al., 1977), which is thought to be the DNA replication enzyme (Bollum, 1975). The mechanism by which DNA synthesis is inhibited is not clear, however in HSV-2 infected cells, DNA synthesis was inhibited following infection with UV-irradiated virus(Fenwick and Walker, 1978).

1.6.3.2. Protein.

Infection of animal cells with a herpesvirus results in characteristic changes in the rate of amino acid incorporation (Ben-Porat et al., 1971; Honess & Roizman, 1973; Powell and Courtney, 1975). This includes a decrease early in infection, due to a decrease in synthesis of host cell proteins, followed by an increase in the overall rate, as viral protein synthesis

becomes significant and finally and irreversibly declines (Ejercito et al., 1968). The decrease in the rate of amino acid incorporation reflects a concomitant decrease in the rate of protein synthesis (Silverstein & Engelhardt, 1979). The time course of the inhibition of host protein synthesis depends upon the particular virus and the permisiveness of the given cell line. It has been suggested that the inhibition of host protein synthesis is mediated by a protein synthesized early after infection (Lewis et al., 1969; Ben-Porat et al., 1971; Lewin, 1975), however in HSV-2 infected Vero cells inhibition occured even after infection was carried out with u.v.-irradiated virus (Fenwick & Walker, 1978).

Stringer et al., (1977) showed that host mRNA continues to enter polysomes even at late times after infection and this led to the hypothesis of polysome-like structures being present but not functioning or poorly functioning in HSV-infected cells (Silverstein & Engelhardt, 1979), which suggests that a translational control mechanism operates to selectively supress host protein synthesis.

The mechanism by which herpesviruses inhibit host protein synthesis is not clear and a number of possibilities are currently under investigation.

1.6.3.3. RNA.

Numerous authors but notably Roizman et al., (1965), Hay et al., (1966) have reported that in cells infected with HSV the rate of RNA synthesis declines early after infection, levels off from about three to eight hours after infection and subsequently slowly and irreversibly declines. It was found by them that incorporation of radioactivity into

nuclear RNA dropped to 25% of the uninfected control by seven hours after infection and that at 4.5 hours after infection there was virtually no radioactivity incorporated into 18s and 28s rRNA whereas an appreciable amount of RNA synthesized at that time had a high mol. wt. (>32s) and a significant fraction of this, sedimented at 45s.

It has been demonstrated that 45s RNA synthesized in infected cells is methylated and this methylation occurs at a time when processing into 28s and 18s rRNA is virtually absent (Roizman et al., 1970).

To determine whether 45 r RNA accumulates or is processed ab errantly, Roizman et al., (1970) carried out an experiment in which they pulsed infected cells briefly with ^{3}H-uridine and chased following addition of actinomycin. The results of this experiment showed the following:

(i) The extract of infected cells pulsed with uridine but not chased, yielded 27.000 cpm, sedimenting in the region corresponding to 45s.

(ii) RNA pulsed and chased with actinomycin yielded 4700 cpm in the 45s region; however there was no increase in the radioactivity of the 18s and 28s RNA.

(iii) In the uninfected control cells, about 65% to 70% of the original radioactivity was recovered in 45s RNA after the pulse was "chased" out, with a concomitant increase in radioactivity in the 32s to 28s RNA region and also the appearance of radioactivity in the 18s rRNA.

The results of this experiment have been interpreted as indicating that the abortive processing of the 45s rRNA in the infected cell nucleus results in its degradation into oligonucleotides sedimenting with S values at less than 18s.

The inhibition of host mRNA synthesis is more rapid

than that of the overall synthesis of RNA (Roizman et al., 1965; Wagner & Roizman, 1969) but host mRNAs are still synthesized even at late times after infection (Rakusanova et al., 1972; Stringer et al., 1977), so it seems that host mRNA synthesis is not inhibited completely.

CHAPTER 2. Materials and Methods.

2.1. Materials.

2.1.1. Biological Materials.

2.1.1.1 Tissue culture cells.

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

2.1.1.2. Virus.

Pig Herpesvirus 1 (pseudorabies virus, PrV) was originally derived from a stock preparation (Kaplan and Vatter, 1959) and has subsequently been plaque-purified three times. The virus stock is prepared by growth in baby hamster kidney cells (BHK cells) (Chantler and Stevely, 1973).

2.1.2. Radiochemicals.

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

(5,6 - ^{3}H) uridine	47 Ci/mmol.
Sulphur - 35	970 Ci/mmol.
(6 - ^{3}H) thymidine	20 Ci/mmol.
L -(U - ^{14}C) Amino acid mixture	58 mCi/mAtom.
(5,6 - ^{3}H) uridine 5' triphosphate	41 Ci/mmol.

2.1.3. Chemicals.

Calf serum	Bio-Cult Lab. Ltd, SCOTLAND.
Amino acids	" " " "
Vitamins	" " " "
Amino acids without L-methionine and L-glutamine	" " " "
Bromophenol Blue	Koch Light Lab. Ltd, ENGLAND.

2-mercaptoethanol	Koch Light Lab. Ltd, ENGLAND
NNNN' tetramethylethylene diamine (TEMED)	" " " " "
Trichloroacetic acid	" " " " "
Polyoxyethylene sorbitan mono-oleate (Tween 80	" " " " "
2,5 diphenyloxazole (PPO)	" " " " "
Toluene (analar grade)	" " " " "
Dithiothreitol (DTT)	Sigma Chemical Co., ENGLAND
Coomassie Brilliant Blue G250	" " " "
" " " R250	" " " "
Sodium Deoxycholate	" " " "
Uridine	" " " "
Heparin	" " " "
Glacial Acetic acid	" " " "
Trizma base	" " " "
Cytidine	" " " "
Cycloheximide	" " " "
N'-2-ethanesulphonic Acid (HEPES)	" " " "
N-Lauroil Sarcosine, sodium salt	" " " "
Microccocal Nuclease	" " " "
Dimethyl Sulfoxide (DMSO)	" " " "
Deoxyribonuclease I	" " " "
a-amanitin	Boehringer-Manheim, WEST GERMAN
Molecular weight standards	" " " "
Bovine serum albumin, trypsin	" " " "
inhibitor, RNA polymerase	" " " "
L-methionine	BDH Chemicals Ltd, ENGLAND
Guanidinium chloride	" " " "
Acrylamide	" " " "

NN'-methylene-bis-acrylamide	BDH Chemicals Ltd, ENGLAND
Nonidet P40	" " " "
Ammonium persulate	" " " "
Folin and Giokalteu's phenol reagent	" " " "
Ampholines pH 5 to 7	LKB Ltd, SWEDEN
" pH 7 to 10	" " "
" pH 3.5 to 10	" " "
" pH 3.5 to 5	" " "
" pH 9 to 11	" " "
Guanosine 5'-triphosphate, sodium	P-L Chemicals Ltd, USA
Cytidine 5'-triphosphate sodium	" " " "
Uridine 5'-triphosphate sodium	" " " "
Adenosine 5'-triphosphate sodium	" " " "
Depex mounting medium	SEARLE Bucks, ENGLAND
Ethylene diacrylate	Monomer-Polymer Lab., USA
Whatman No 1 (2.5cm diameter)	Whatman Ltd, ENGLAND
Whatman GF/C glass fibre discs (2.5cm diameter)	" " "
Whatman 3MM chromatography paper	" " "
Microscope slides	Chance propper Ltd, ENGLAND
Microscope glass coverslips	" " " "
BA 85 filters, 25mm diameter, 0.45µm pore size	Schleicher and Schull WEST GERMANY
Kodak X-omat H X-ray film	Kodak Ltd, ENGLAND
Kodak DX-80 developer	" " "
Kodak FX-40 X-ray liquid fixer	" " "
Kodak NS-80 X-ray film	" " "
Kodirex KD 54T X-ray film	" " "

MEM amino acids (x100)	Gibco Europe Ltd., SCOTLAND
MEM amino acids minus methionine (x100)	" " " "
Newborn calf serum (Mycoplasma screened)	" " " "
MEM vitamins (x100) (Glasgow modification)	" " " "
"Chloros" Industrial grade	Durham Chemicals Distributors Ltd ENGLAND
Eagle's medium. (Glasgow modification)	Flow Lab., SCOTLAND
Giemsa stain	Gurr, ENGLAND
Penicillin	Glaxo Lab Ltd., ENGLAND
Streptomycin	" " " "
Trypsin	Difco Lab., USA
Absolute Alcohol, Analar	James Burrough's Ltd, ENGLAND

2.1.4. Standard solutions.

2.1.4.1 Cell culture media.

I. Ec, Medium for Hela cells.

Dist. H_20	450ml
IF	50ml
$NaHCo_3$	20ml
Penicillin/streptomycin	5ml
Newborn calf serum	50ml

Added in the order shown.

II. EC-Met, Methionine free medium.

BSS	450ml
ES10 (-Met)	50ml
$NaHCo_3$	20ml
Penicillin/streptomycin	5ml
Calf serum	50ml

ES10 was used with $^1/_5$ the normal methionine content as well.

Table 2.1.4.1. M.E.M. amino acid formulation.

	mg/litre
L-arginine	126.40
L-cystine	24.00
L-glutamine	292.00
L-histidine HCl	38.30
L-isoleucine	52.50
L-leucine	52.50
L-lysine	73.10
L-methionine	14.90
L-phenylalanine	33.00
L-threonine	47.60
L-tryptophan	10.20
L-tyrosine	36.20
L-valine	46.90

Table 2.1.4.2. Glasgow modification of M.E.M. Vitamins - Formulation.

	mg/litre
D-calcium pentothenate	2.0
Choline chloride	2.0
folic acid	2.0
i-inositol	4.0
nicotinamide	2.0
pyridoxal HCl	2.0
riboflavin	0.20
thiamine HCl	2.0

Table 2.1.4.3. Earle's Balance Salt Solution (BSS) 10x concentrated.

	To make 10 litres
NaCl	680g
KCl	40g
$MgSO_4.7H_2O$	20g
$NaH_2PO_4.2H_2O$	14g
$CaCl_2.6H_2O$	39.3g
Phenol Red 1%	150ml
Distilled water to	10 litres

Add chloroform to a final concentration of 0.1%

i.e. 10 ml chloroform to 10 litres BSSx10.

Table 2.1.4.4. Eagle's stock. 10x concentrated (ESx10).

	1 litre
M.E.M. amino acids x 50	200ml.
M.E.M. vitamins x 100	200ml.
L. Glutamine	2.925g
Glucose	45g.
Distilled water to	1 litre.

Adjust pH to 7.1 using 5NNaOH approx. 10ml.

Sterilise by millipore filtration using a G.S. membrane (0.22μ)

Table 2.1.4.5.

IF (Glasgow Modification)

Components	mg/litre
D-glucose	4500.00
Magnesium sulfate $7H_2O$	200.00
Potassium chloride	400.00
Sodium chloride	6400.00
Sodium dihydrogen phosphate, $2H_2O$	140.00
Calcium chloride, $2H_2O$	264.90
L-Arginine hydrochloride	126.40
L-Cystine, disodium	28.42
L-glutamine	584.60
L-Histidine hydrochloride, H_2O	21.00
L-Isoleucine	52.46
L-Leucine	52.46
L-Lysine hydrochloride	73.06
L-methionine	14.92
L- phenylalanine	33.02
L-threonine	47.64
L-tryptophan	8.16
L-tyrosine	36.22
L-valine	46.86
D-Ca-pantothenate	2.00
Choline Chloride	2.00
Folic acid	2.00
I-Inositol	4.00
Nicotinamide	2.00
Pyridoxal hydrochloride	2.00
Riboflavin	0.20
Thiamin hydrochloride	2.00
Phenol Red, sodium	17.00

III. Sodium bicarbonate.

5.6% (w/v) $NaHCo_3$, 0.0015% (w/v) phenol red. Sterilized by millipore filtration using 0.22micron membrane.

IV. Penicillin and Streptomycin.

Penicillin	10^5 units/litre
Streptomycin	10g/litre

Sterilized by millipore filtration through 0.22 micron membrane.

V. Trypsin (in citrate) 0.25%.

	g/l
Trypsin	2.50
Tri-sodium citrate	2.96
Sodium chloride	6.15
Phenol red	0.015

Sterilized by membrane filtration through 0.22 micron membrane.

VI. Versene solution.

	g/l
Sodium chloride	8
Potassium chloride	0.2
Di-sodium hydrogen phosphate	1.15
Potassium di-hydrogen phosphate	0.2
Versene (ethylene diamine tetra acetic acid)	0.2
Phenol red	0.015

Sterilized by 15 lb/in^2 for 15min.

VII. Trypsin/versene.

1 volume of trypsin (in citrate) 0.25%

4 volumes of versene solution

VIII. BSS+Bicarbonate.

BSS	450ml
$NaHCo_3$	20ml

IX. Formal saline.

	per litre
NaCl	5g
Na_2SO_4	15g
Formaldehyde (40%)	100mls

X. Giemsa Stain.

	per litre
Giemsa stain	7.6g
Glycerol	500ml
Methanol	500ml

XI. Phosphate buffered saline, PBS.

	g/l
Sodium chloride	10
Potassium chloride	0.25
Di-sodium hydrogen phosphate	1.44
Potassium di-hydrogen phosphate	0.25

PH to 7.2.

XII Eagles's stock, 10X concentrated.

MEM amino acids x100	20ml
MEM vitamins x100	20ml
glucose	4g
glutamine	0.6375g

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

ES10 was prepared with minus methionine or $^1/_5$ the normal concentration of methionine, MEM amino acids X100, as well.

2.1.4.2. Solutions for the isolation of ribosomal RNA.

I. High salt buffer (HSB).

Sodium chloride	0.5M
Magnesium chloride	0.05M
Tris-base	0.01M
PH 7.4	

II. LETS buffer.

Lithium chloride	0.1M
EDTA	0.01M
Tris-base	0.01M
Sodium lauryl sulphate	0.2% (w/v)
PH 7.4	

III. Mixed detergent solution.

2 volumes of Tween 80 solution ; 10% (v/v).

1 volume of sodium deoxycholate solution; 10% (w/v).

IV. Reticulocyte standard buffer (RSB)

Sodium chloride	0.01M
Magnesium chloride	0.003M
Tris-base	0.01M
PH 7.4	

V. Sucrose solution.

15% and 30% sucrose in LETS buffer (w/v).

2.1.4.3. Solutions for polyacrylamide gel electrophoresis.

A.RNA

I. Acrylamide stock solution (x10).

Acrylamide	27%(w/v)
Ethylene diacrylate	2.5 (v/v)

II. Electrophoresis buffer stock X10

Sodium di-hydrogen phosphate	0.3M
EDTA	10mM

Tris-base 0.36M

PH 7.7 to 7.8 with H_3PO_4.

III. Electrophoresis buffer (prepared fresh).X1.

Electrophoresis buffer x10 200ml

Sodium Lauryl sulphate 4g

Distilled water added up to 2 litres.

IV Sucrose Solution.

Sucrose 20%

SDS 0.4%

in Electrophoresis buffer x1.

V. 2.7% acrylamide gels.

	per 10ml
Acrylamide stock solution x10	1ml
TEMED	0.01ml
Ammonium persulphate 10% (w/v)	0.1ml
Electrophoresis buffer stock x10	1ml

added distilled water up to 10ml.

B. Protein.

I. Electrode Buffer.

Tris-base	6.0g	0.025M
Gly cine	28.7g	0.192M
Sodium Lauryl sulphate (10% w/v)	20ml	0.1%

up to 2 litres with distilled warer, PH 8.3.

II. Main gel solution.

SDS 0.1% (w/v); TEMED 0.025% (v/v); Ammonium persulphate 0.0375% (w/v); Tris base 0.375M, PH 8.8 with acrylamide 3% and bis-acrylamide 0.09% (w/v).

IV. Staining solution.

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

V. Destaining solution.

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

VI. Isoelectric focusing gel solution.

Urea 9.2M; Nonidet P-40 2% (w/v), Ampholines 2% (w/v) (pH 3.5 to 10 1.2%; pH 3.5 to 5 0.2; pH 5 to 7 0.2%; pH 7 to 9 0.2%; pH 9 to 11 0.2%;)with acrylamide 3.77% (w/v) and bis-acrylamide 0.215% (w/v) Ammonium persulfate 0.01% (w/v); TEMED 0.07% (v/v):

VIII. Electrode buffer for isoelectric focusing.

a.Cathode (+)

NaOH 0.02M (pH about 12.5).

o.Anode (-)

H_3PO_4 0.01M (pH about 3).

2.1.4.4. Solutions for liquid Scintillation counting.

I Toluene - PPO

PPO 0.5% (w/v), in toluene.

II. Toluene-methoxyethanol PPO.

PPO 0.5% (w/v), in toluene-methoxyethanol (40:60).

III Stacking gel solution.

SDS 0.1%(w/v); TEMED 0.025%(v/v); Ammonium persulfate 0.0375%(w/v); Tris base 0.125, pH 6.8 with acrylamide 3% and bis-acrylamide 0.09%(w/v).

2.2. Methods.

2.2.1. Cell culture techniques.

2.2.1.1. Propagation of cells.

Hela cells (Gey et al., 1952) were used in this study. Cells were cultured as monolayers in Petri dishes or in Roux bottles or in rotating 80oz Winchester bottles (burlers) according to the technique of House and Wildy (1965). They were grown in an atmosphere of 5% CO_2/95% air on modified Eagle's Minimal Essential Medium to which 10% (v/v) calf serum had been added. Generally cells were seeded at 20 x 10^6 per 80oz glass bottle or 4 x 10^6 per Roux bottle or 2 x 10^6 per petri dish and harvested after 2-3 days growth.

Cell lines were maintained by sub-culturing from confluent monolayers. The medium was replaced by about 20ml of trypsin-versene solution (1:4, w/v) and the 80oz glass bottle was rotated for one minute whereupon the solution was replaced by a further 20ml trypsin-versene and the procedure was repeated. Most of the final trypsin-versene solution was poured off from the bottle leaving 2-3ml and the bottle was allowed to rotate at 37° for 5 min by which time the cells had become opaque . 20ml of fresh warmed medium was added to the bottle with shaking. Detached cells were thoroughly resuspended before an aliquot was taken for estimation of the total cell number using a haemocytometer.

2.2.1.2. Contamination Checks.

All sterile media and passaged cells, were checked regularly for bacterial, fungal or PPLo infection as follows:
(a) Bacterial contamination: aliquots were added to blood

agar plates and brain-heart infusion broth at 37°C. Results were considered to be negative if no growth was seen after 7 days.

(b) Fungal contamination: aliquots were added to Sabouraud's medium at 32°C. No growth after 7 days was assumed to indicate the absence of fungal contamination.

(c) PPLo infection: agar plates were seeded with passaged cells by piercing the agar surface with a charged pasteur pippete. The plates were grown in an atmosphere of 5% CO_2 in N_2 at 37°C. Infected cells resulted in the occurence of the characteristic "fried egg" appearance of PPLo colonies on examination of the plates under the microscope. Contaminated cultures were discarded.

2.2.1.3. Propagation of Virus.

Monolayer cultures of BHK/21 C13) cells grown to confluency were infected with less than 0.01 pfu per cell of virus in 20ml medium. Virus was allowed to adsorb for 1hr, then the inoculum was removed and replaced with 100ml of medium. The cultures were rolled at 37°C for 36hrs, then harvested by shaking the bottle to dislodge cells into the medium, transferred to centrifuge bottles and spun at 600g for 10min, to pellet the cells. The supernatant was then spun at 15000g for 2hrs to pellet the virus. The pelleted supernatant virus was resuspended in culture medium (1ml for every burler used) and aliquots were stored at -70°C.

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

Plaque assays carried out on Supernatant virus from

monolayer cultures of C13 cells and normally gave titres of 10^9 - 10^{11} pfu/ml.

2.2.1.4. Plaque assay for PrV.

Confluent monolayers of C13 cells in 50mm Petri dishes were infected with serial dilutions of PrV in 0.2ml medium. After 1hr adsorption at 37°C, the medium was poured off, and 4ml of fresh medium was added and incubation continued. After 1 hr, 50µg/ml of heparin was added to the medium to prevent horizontal transmission of the virus. At 28hrs after infection incubation was stopped, and the cell sheet was washed with PBS and fixed in 3ml formal-saline per dish, for 30min at room temperature. The cell sheets were then stained with 0.5ml Giemsa stain for 1hr, washed off thereafter very gently with H_2O and plaques were counted under a low power microscope.

2.2.1.5. Staining of cells for measurement of nucleoli.

Hela cells grown in 50-mm petri dishes containing microscope slides, incubated at 37°C, were either mock-infected or infected with pseudorabies virus. At hourly intervals after infection, petri dishes were removed from the incubator, the medium was discarded, and cell sheets were washed twice with BSS and once with absolute alcohol. Cells on slides were fixed in formal saline for 1hr to overnight, stained in a Giemsa solution for 1 min washed gently with tap water to remove residual stain, dried, covered with coverslips and photographed under a high powered Leitz light microscope.

The perimeters of nucleoli were outlined on tracing paper with the use of a Nikon comparator. The outlines were cut and weighted in a microbalance.

2.2.2. Radioactive labelling.

2.2.2.1. Proteins.

Hela cells grown to confluence in 80oz roller bottles in 180 ml of medium for 3 days, were mock-infected or infected with PrV. Cells were then labelled in 20 ml of medium with an appropiate amount of ^{35}S-methionine in one of the following ways:

(a) Cells were labelled with ^{35}S-methionine for a period of 6 hrs in medium containing $^{1}/_{5}$ the normal methionine concentration.

(b) Cells were labelled with ^{35}S-methionine for a period of 2 hrs in medium containing no methionine.

(c) Cells were labelled with ^{35}S-methionine for a period of 1 hr in medium containing no methionine following treatment of cells, immediately after infection, with an appropiate amount of cycloheximide.

For labelling cells before infection they were grown in 80oz roller bottles for 2 days and labelled with ^{35}S-methionine for approximately 12 hrs. At the end of this period cells were either mock-infected or infected and the label was chased with 400-fold cold methionine for a period of 6 hrs.

2.2.2.2. RNA.

2.2.2.2.1. Pulse labelled.

Hela cells grown in either 80oz roller bottles or in Roux bottles, mock-infected and infected were labelled with an appropiate amount of ^{3}H-uridine in 20 ml medium for periods of 1 hr or 15 min.

2.2.2.2.2. "Glucosamine-uridine" pulse-chase.

"Glucosamine-uridine pulse-chase experiments essentialy followed the method of Levis and Penman (1977). Hela cells grown in Roux bottles in 50 ml of medium for 3 days, were mock-infected or infected. Cells were then brought to 20 ml of medium and D-glucosamine, neutralised to pH 7.4, was added to a final concentration of 20 mM. This concentration of glucosamine is sufficient to reduce considerably the size of the intracellular UTP pools by "trapping" uridine in the form of UDP- N - acetylhexosamines (Scholtissek, 1971). Incubation at 37°C was continued for 60 min and then the culture was labelled with an appropriate quantity of ^{3}H-uridine for 15 min.

To initiate the chase, the medium was discarded and fresh medium was added, containing 5mM uridine, 5mM cytidine and 5mM glucosamine. The label was chased for 30, 60 or 120 min.

2.2.2.3. DNA.

Hela cells grown in Roux bottles for 2 days were labelled with an appropriate amount of ^{3}H-thymidine. At approximately 12 hrs the medium was changed and cells were either mock-infected or infected and the label was chased with excess cold thymidine.

2.2.3. Cell harvesting.

Growth medium was decanted and the cell sheet rinsed with ice-cold BSS. The cells were removed from the glass into 30 ml of freshly added BSS by means of a rubber scraper, transferred to a 50 ml centrifuge tube, and pelleted by

centrifugation at 1000 rpm for 5 min (+4°C) in an MSE Major centrifuge.

2.2.4. Cell fractionation.

2.4.1. Nuclei and cytoplasm.

Nuclei were isolated according to the method of Penman (1966). All steps were carried out at +4°C. The cell pellet, after removal from the glass surface and washing with BSS as described above, was suspended in RSB (0.01 M NaCl; 0.0015 M $MgCl_2$; 0.01 M Tris/HCl pH 7.4). 4.0ml of RSB were added per 10^8 cells, and the cells allowed to swell by standing in ice for 10 min. Disruption was completed by homogenizing with 25 strokes of a stainless steel Dounce homogeniser, and crude nuclei were pelleted by centrifugation (2K, 10 min). The crude nuclear pellet was resuspended in 2 ml RSB and pelleted by centrifugation again (2K, 5 min) to remove "trapped" residual cytoplasm. The supernatants containing the cytoplasm were combined and were either used to prepare cytoplasmic RNA or were centrifuged at 10.000g for 15 min, to pellet mitochondria, to be used in studies for the uptake of cytoplasmic proteins by isolated nuclei in vitro.

The nuclear pellet was suspended in 4.0 ml RSB to which was added 0.6 ml of a solution containing 6.7% tween 80 and 3.3% Na deoxycholate. The suspension was mixed briefly on a vortex mixer and the nuclei pelleted by centrifugation at 2.000 rpm for 5 min. This treatment removes residual cytoplasmic contamination and unruptured cells (Penman, 1966). The purified nuclear pellet was either dissolved in SDS-sample buffer to prepare it for electrophoresis or used for nucleolar purification if required.

2.2.4.2. Nucleoli.

Nucleoli were prepared in one of two ways:

2.2.4.2.1. DNase.

The purified nuclear pellet was used to prepare nucleoli according to the method of Penman (1966). Residual detergent from the nuclear pellet was removed by rinsing its surface with 1.0 ml of RSB. This facilitated the subsequent action of DNase (Pederson and Kumar, 1971). The gelatinous pellet was suspended in 2.0 ml of HSB (0.5 M NaCl; 0.05M $MgCl_2$; 0.01 M Tris/HCl pH 7.4), 100 µg of DNase added, and the mixture incubated at 37°C with vigorous pipetting, using a broken off wide bore pasteur pipette, until all DNA clumps were dispersed and the viscosity of the solution was reduced considerably (approximately 1 min incubation was sufficient).

The nucleoli were collected by centrifugation through a cushion of 0.88M sucrose at 2900 for 30 min. The nucleolar pellet was then extracted to prepare nucleolar RNA.

2.2.4.2.2. Sonication.

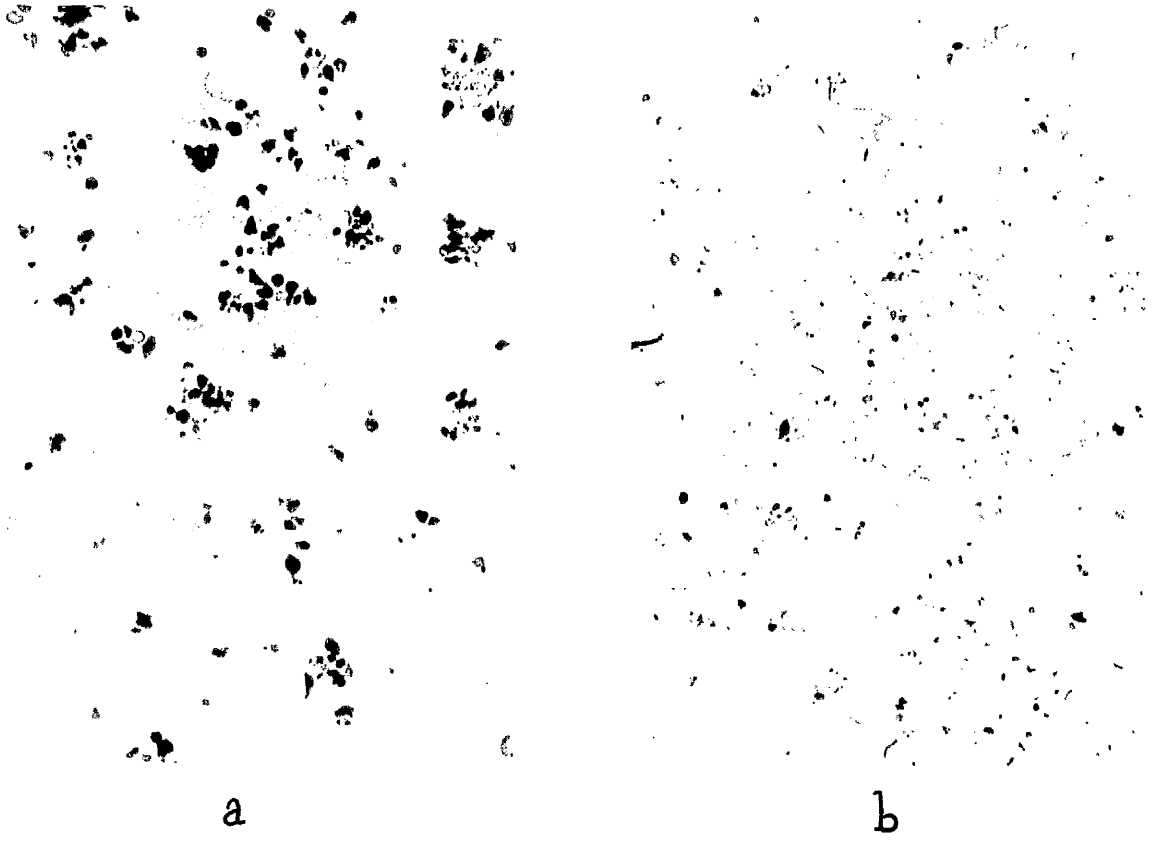
Essentialy the method of Muramatsu et al., (1974) was used. The detergent purified nuclei were suspended in 5 ml of 0.25 M sucrose and 10 mM $MgCl_2$ and pelleted, resuspended in 5ml of 0.34M sucrose and 0.5mM $MgCl_2$ and sonicated four times at 20 sec each time with a minute interval between each sonication, to destroy all nuclei, which was confirmed by examining a drop of the sonicate under a light microscope after staining with Azure C. (Muramatsu et al., 1963). The sonicate was immediatly underlaid with an equal volume of 0.88 M sucrose and centrifuged at 2.900 rpm for 30 min in an MSE Major centrifuge to sediment the nucleoli

Figure 2.2.4.1. Nucleoli isolated by sonication from mock-infected Hela cells.

Hela cells grown in 80oz roller bottles were mock-infected or infected with pseudorabies virus at 50 p.f.u. per cell. At 6 hrs postinfection cells were harvested and nucleoli were isolated by sonication as described in Materials and Methods. Samples from the pelleted nucleoli were applied on microscope slides, stained with Azure C, covered with coverslips and photographed under a Leitz light microscope.

a. mock-infected b. PrV-infected.

Magnification x240.



Purified nucleoli of mock-infected and infected Hela cells are shown in figure 2.2.4.1.

The nucleolar pellet was either dissolved in SDS-sample buffer to prepare for electrophoresis or used for preparation of nucleolar chromatin if required. (Photographs of nucleoli were taken in the light microscope).

2.2.4.3. Nucleolar chromatin.

For preparation of nucleolar chromatin, the method described by Daskal et al., (1978) was essentially used. This employs two washings of the nucleolar pellet in 0,075 M NaCl; 0.025 M EDTA (tetrasodium salt); 1mM PMSF, adjusted to pH 8.0 with 1 N NaOH, followed by 3 washings in Tris-buffer (0.01 M Tris, pH 8.0; 1 mM PMSF). The pellet from the third washing represented isolated chromatin.

2.2.5. RNA isolation.

2.2.5.1. Extraction of whole cell RNA.

Washed Hela cells were lysed by addition of an appropiate volume of lysing LETS, and diluted with an equal volume of phenol saturated with water. The mixture was stirred in a Vortex mixer for 1 min, then incubated in a 50°C to 55°C water both for 4 min with occasional shaking and centrifuged at 3000 rpm in a MSE Major Centrifuge for 30 min at 20°C. The aqueous layer was removed and the organic layer re-extracted with an equal volume of LETS. The combined final aqueous phase was diluted with 3 volumes of ethanol and stored at -20°C overnight. The ensuing precipitate of RNA was collected by centrifuging at 3000 rpm for 30 min at 4°C. The pellet was later prepared for electrophoresis in acrylamide

gels.

2.2.5.2. Extraction of cytoplasmic RNA.

Cytoplasmic extracts prepared as described previously were treated with an equal volume of water-saturated phenol, shaken on a Vortex mixer for 20 sec then centrifuged at room temperature (3000 rpm, 20 min). The supernatant fraction was carefully removed by aspiration, replaced by an equal volume of LETS buffer and the procedure was repeated. The combined aqueous fractions were added to 3 volumes of ethanol and stored at -20°C overnight and the RNA collected by centrifugation (3000 rpm, 30 min). The pellet was resuspended in 1.0 ml LETS buffer, layered over 36.0 ml of a linear 15% to 30% sucrose density gradient in LETS buffer, centrifuged in the Beckman SW27 rotor (22000 rpm, 17 hrs , 20°C), then harvested by placing a needle down the side of the tube to the bottom and pumping through a Gilford 2000 recording spectrophotometer. The absorbance was monitored at 260 nm and the gradient collected into appropiate fractions. In some cases fractions were submitted to a further 2 cycles of ethanol precipitation, centrifugation and resuspension as above in order to eliminate traces of phenol and SDS.

2.2.5.3. Extraction of nucleolar RNA.

Nucleoli prepared as described previously were dissolved in LETS buffer and diluted with an equal volume of water-saturated phenol. The mixture was agitated in a Vortex mixer for 30 sec then centrifuged at room temperature (3000 rpm, 30 min). The aqueous phase was carefully removed with a pasteur pipette, added to 3 volumes of ethanol, stored at

-20°C overnight and the RNA collected by centrifugation. The pellet was resuspended in 1.0 ml LETS buffer and centrifuged in 15% to 30% sucrose density gradients as described above for cytoplasmic RNA. In some cases nucleolar RNA was prepared for electrophoresis in acrylamide gels.

2.2.5.4. Precautions against ribonuclease.

To eliminate possible contaminating ribonuclease activity all glassware was baked in an oven at 200°C for a minimum of three hours. Solutions were autoclaved at 15lb.in^{-2} for 25 min or, if they contained sucrose, at 5lb. in^{-2} for 45 to 50min. Whenever possible, relevant items of experimental apparatus were autoclaved as above.

2.2.6. RNA fractionation.

2.2.6.1. Non-denaturing sucrose gradients.

An appropriate amount of RNA was dissolved in LETS buffer and layered over a linear 15% to 30% sucrose gradient in LETS buffer. The gradient was fractionated by using a peristaltic pump to withdraw the tube contents from the bottom and an aliquot of each fraction was assayed for acid soluble radioactivity, as described below.

2.2.6.2. Polyacrylamide gel electrophoresis.

Electrophoresis was carried out on 2.7% acrylamide gels prepared as described by Knowler and Smellie (1971). Gels contained 2.7% (w/v) acrylamide, 0.25% (v/v) ethylene diacrylate, 1% (v/v) TEMED, prepared in electrophoresis buffer (36mM Tris, 30mM NaH_2PO_4, 1mM EDTA pH 7.7-7.8) as described by Loening (1969).

Polymerization was catalyzed by the addition of

ammonium persulphate to 0.1% (w/v). All gels were pre-electrophoresed at 2.5mA/gel for 15-30 minutes before RNA samples (80-100 µg), dissolved in electrophoresis buffer containing 20% (w/v) sucrose and 0.2% (w/v) SDS, were applied on top. Electrophoresis was 2 to 5 hours at 5mA/gel. The gels were scanned at 260 nm in the linear transport attachment for the Gilford 240 recording spectrophotometer. For determination of radioactivity the gels were frozen in powdered solid CO_2 and cut transversely into 1mm slices using a Mickle gel slicer. Slices were digested individually in vials with 0.5ml of aqueous 2M NH_4OH at 60° overnight, taken up in 0.3ml water, left for 60 minutes and counted in 10 ml of methoxyethanol-toluene PPO based scintilation fluid, after shaking and allowing them to stand for a few minutes.

2.2.7. In vitro protein uptake by nuclei.

Crude unlabelled nuclei of mock-infected or infected Hela cells, were incubated in ^{35}S-methionine labelled cytoplasmic extracts in RSB derived from mock-infected or infected Hela cells, for periods of 30 to 60 minutes. Crude nuclei were washed afterwards with detergents to remove cytoplasmic contaminants, pelleted and were either extracted with 0.35 M NaCl and dissolved in SDS buffer for electrophoresis in polyacrylamide gels or were used to prepare nucleoli by sonication as described previously. Nucleoli were also dissolved in SDS buffer and prepared for electrophoresis.

2.2.8. Analysis of proteins.

2.2.8.1. Protein estimation.

Protein was estimated using the methods of either

Lowry et al. (1951) or Bradford (1976). Bovine serum albumin was used as a protein standard.

2.2.8.2. SDS-polyacrylamide gel electrophoresis.

Electrophoresis was carried out using the discontinous system essentialy as described by Laemmli (1970).

For routine analysis of nuclear or sub-nuclear fractions the technique of thin-slab SDS polyacrylamide gel electrophoresis was employed as described by Lestourgeon (1977). The gels were prepared by layering 20 mm of 3.0% acrylamide stacker gel solution (0.125 M Tris, 0.1% SDS, pH 6.8) over 80 mm to 100 mm of either 15%, 12.5% or 9% acrylamide main gel solution (0.375 M Tris, 0.1% SDS, pH 8.8). Acrylamide was polymerized by the addition of ammonium persulphate (0.0375%) and TEMED (0.025%).

Proteins were denatured prior to electrophoresis, in SDS sample buffer (0.1% SDS, 1% mercaptoethanol, 0.25 M sucrose, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4), by heating in a boiling water bath. Multiple samples with an appropriate amount of protein, in a 20 µl to 100 µl volume, were applied to the gel and Bromo phenol Blue added as a tracking dye.

Electrophoresis was carried out at 20 mA until the tracking dye was approximately 1 cm from the end of the gel. The thin slab gels were removed carefully and either stained with Coomasie blue and autoradiographed or immersed in a PPO based solution to prepare for flurography. Gels were dried prior to exposure to X-ray films on filter paper supports.

2.2.8.3. Two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis was carried out

according to the O'Farrell method. Labelled polypeptides from nucleolar and nucleolar chromatin fractions were analysed by isoelectric focusing - IEF (O'Farrell, 1975) or non-equilibrium pH gradient electrophoresis - NEPHGE (O'Farrell, 1977) followed by SDS gel electrophoresis.

Protein samples were prepared either by pelleting nucleic acids (160.000g, 18 hrs) after dissociation in the presence of 0.4 M guanidine hydrochloride and 6 M urea or after treatment with micrococcal nuclease, in the presence of PMSF. Samples were thereafter treated in lysis buffer (9.5 M urea, 2% NP-40, 5% B-mercaptoethanol, 2% Ampholines) and appropriate amounts were applied to isoelectric focusing gels and run as recomended by O'Farrell.

Following isoelectric focusing, gels were equilibrated in SDS sample buffer and loaded on thin layer SDS-polacrylamide gels prepared as described above.

2.2.8.4. Estimation of molecularweights.

Gels were calibrated by co-electrophoresis of albumin (68.000), trypsin inhibitor (21.000) and RNA polymerase (39.000, 155.000, 165.000). After electrophoresis gels were stained. The molecular weight of the standards was plotted against their migration distances on semi-logarithmic paper.

2.2.9. Determination of radioactivity.

2.2.9.1. Acid-insoluble radioactivity.

Radioactivity in acid-insoluble RNA molecules was determined by precipitation with ice-cold trichloroacetic acid 5% or 10% (w/v) and the ensuing precipitate collected by

vacuum filtration on to millipore filters (0.45 µm pore size) or onto Whatman GF/C glass fibre discs. The discs were washed with 5% TCA and ethanol placed individually in scintillation vials and oven-dried (60°, 2 hrs). Radioactivity was determined following addition of a 0.5% solution of PPO in toluene, by liquid scintillation spectrometry.

In other cases samples of 10 µl were spotted onto Whatman I filter paper discs, dried briefly, immersed in cold 10% TCA (10 ml per filter) stirred at a low speed for 15 min washed afterwards with absolute alcohol then in diethyl-ether, dried under a heat lamp, placed in scintillation vials and radioactivity was determined in toluene-PPO as above.

Where radioactively labelled nucleoside triphosphates were present, precipitation of acid insoluble material in cold TCA was carried out in the presence of 0.1 M sodium pyrophosphate.

2.2.9.2. Assay of radioactivity present in polyacrylamide gel slices.

Gel slices were placed in scintillation vials to which was added 0.5 ml of aqueous 2M NH_4OH, and were heated at 60° overnight. After evaporation to dryness, gel residues were taken up in 0.3 ml water and left for 60 min. Radioactivity was counted in 10 ml of a solution containing 0.5% PPO in methoxyethanol-toluene (60:40).

2.2.10. Detection of labelled polypeptides separated on gels.

2.2.10.1. Autoradiography.

After electrophoresis gels were stained in Coomasie blue

and dried under vacuum on to Whatman 3 MM chromatography paper. The dried gel was placed in contact with kodirex KD 54T X-ray film and exposed at room temperature for the appropriate time. The film was developed in kodak DX-80 developer and fixed with kodak FX-40 X-ray liquid fixer.

2.2.10.2. Fluorography.

The method of Bonner and Laskey (1974) was used to detect radioactive polypeptides by fluorography. After elecrophoresis, gels were soaked in three changes of DMSO for a total period of 2.5 hrs, impregnated with PPO by immersion in a solution of 22.2% (w/v) PPO in DMSO for 1 hr and then washed with water before drying under a vacuum. The dried gel was placed in contact with X-Omat H X-ray film and exposed at -70°C for the appropriate time. Films were developed as described above.

2.2.11. RNA polymerase assays.

2.2.11.1. Standard procedure.

Nuclei isolated from Hela cells mock-infected or infected were assayed for RNA polymerase activity in a 200 µl incubation mixtures at 25°C containing 50 mM Hepes pH 7.6, 25% glycerol, 5mM Mg-acetate, 5mM dithiothreitol, 150mM KCl and 0.4 mM of each GTP, ATP, CTP plus 0.05 mM UTP. 10 µCi/ml of ^{3}H-UTP was added to each assay. Incubation was carried out for up to 30 mins. 10µl aliquots were spotted on Whatman I papers, dried, extacted with 10% TCA-1% pyrophosphate and their radioactivity was determined. Occasionaly 200µg/ml of amanitin was added to the incubation mixture.

2.2.11.2. Incubation with sarkosyl.

In some cases nuclei were incubated in the presence of 0.8% of sarcosyl in 80 µl incubation mixture constituted as above. Reaction was stopped by the addition of 1% SDS, 1 mM EDTA and TCA precipitable material was collected on GF/C or millipore filters.

2.2.11.3. Incubation in cytoplasmic extracts.

RNA polymerase was assayed after coincubation of the nuclei with cytoplasmic extracts, prepared after sonication in salt solution for 5 min followed by pelleting of the nuclear debris for 15 min at 17K. The 200-µl incubation mixture was assayed at 25°C, and 10 µl aliquots were spotted on Whatman I papers. Occasionally sarkosyl was also used in polymerase assays as above. The incubation in cytoplasmic extracts for 15' was followed by treatment with sarkosyl for 10'. Reaction was stopped with addition of 1% SDS and EDTA.

2.2.12. DNA gradients.

A suspension in SSC (0.15 M NaCl, 0.015M sodium citrate) of mock-infected or infected Hela cells was carefully pipetted into 2 mls of a solution containing 0.3N NaOH, 0.00 M EDTA and 0.5% SDS layered onto 15% to 30% sucrose gradients in 0.5M NaCl, 0.25N NaOH, 0.001M EDTA, 0.01% SDS. The gradients kept at room temperature for at least 8 hours and centrifuged at 26K for 5 to 6 hours at 25°C in a Bechman SW27 rotor. Gradients were harvested with a peristaltic pump and were divided into 1 ml fractions. Radioactivity in each fraction was determined after TCA precipitation and collection of the precipitate onto GF/C filters by means of vacuum filtration.

CHAPTER 3. Changes in nucleolar morphology following infection of Hela cells with pseudorabies virus.

3.1. Introduction.

The nucleolus, which is the site of ribosomal RNA synthesis, is an organelle which is highly sensitive to external stimuli, and its size and structure can be altered by a variety of agents and experimental conditions which are known to exert changes on cellular RNA metabolism. Thus an increase in nucleolar size, which coincides with an increase in nucleic acid synthesis, is observed in livers of rats treated with thioacetamide (Bernhard and Granboulan, 1968).

Again when actinomycin D is administered to cultured cells, a decrease in nucleolar size is observed among other characteristic changes (Rounds et al., 1960; Reynolds, 1969; Schoefl, 1964). Actinomycin, as well as a number of other agents known to produce the same decrease in nucleolar size, inhibit primarily ribosomal RNA synthesis.

Viruses are also known to induce changes in nucleolar size and structure. Their effect is variable depending mainly on the mode of action of the particular virus involved (Bernhard and Granboulan, 1968). In kB tumour cells infected with herpes simplex virus, a decrease in nucleolar size was observed, accompanied by other structural changes which resembled the changes caused by actinomycin (Sirtori and Bossisio-Bestetti, 1967).

Unfortunately, no quantitative estimation of the nucleolar size was made by these authors.

It has been reported that the number of nucleoli per cell varies in any particular cell type. What appears to be

the constant feature for a cell type is the total nucleolar volume rather than the number of nucleoli (Jordan, 1972). Furthermore, there are indications that the total surface area rather than the combined volume may be the constant feature (Barr and Esper, 1963).

From such studies, it is clear that the size of nucleoli reflects the metabolic state of the cell and particularly ribosomal RNA metabolism.

3.2. Changes in nucleolar cross-sectional area.

Nucleolar cross-sectional area was measured, rather than of volume but it is believed that by analogy with similar measurements for nuclei (Harris, 1967) this gives a reasonable approximation.

Hela cells grown in 50mm petri dishes on microscope slides were mock-infected or infected with PrV and at hourly intervals up to 6 hours postinfection, slides were removed, fixed and stained with Giemsa solution as described in Materials and Methods. Cells on slides were examined by light microscopy and photographed (Fig. 3.2.1) The cross-sectional area of individual nucleoli from cells in fields chosen at random was estimated as described in Materials and Methods.

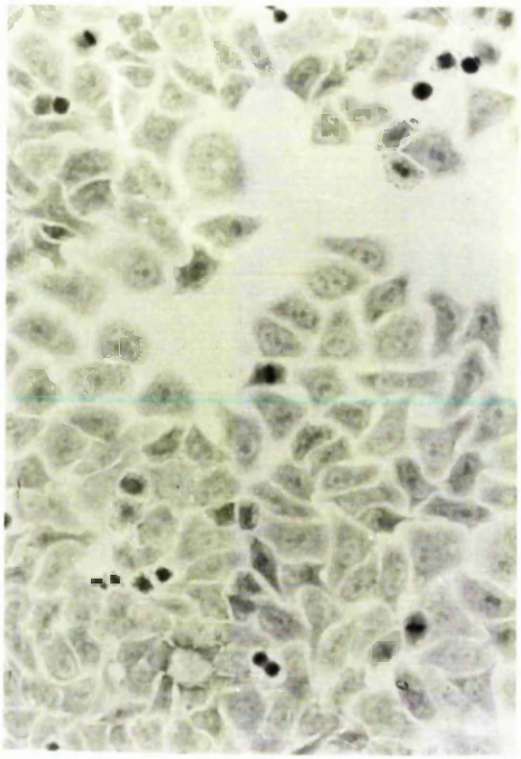
Histograms giving the number of nucleoli at each unit size are shown in Fig 3.2.2, and reveal a greater spread of values in mock-infected than in infected cells. There is a gradual accumulation of the bulk of nucleoli at the lower size units at late times after infection as is seen by the absence of nucleoli greater than size unit four from 4 hours

Figure 3.2.1. Hela cells mock-infected or infected with PrV.

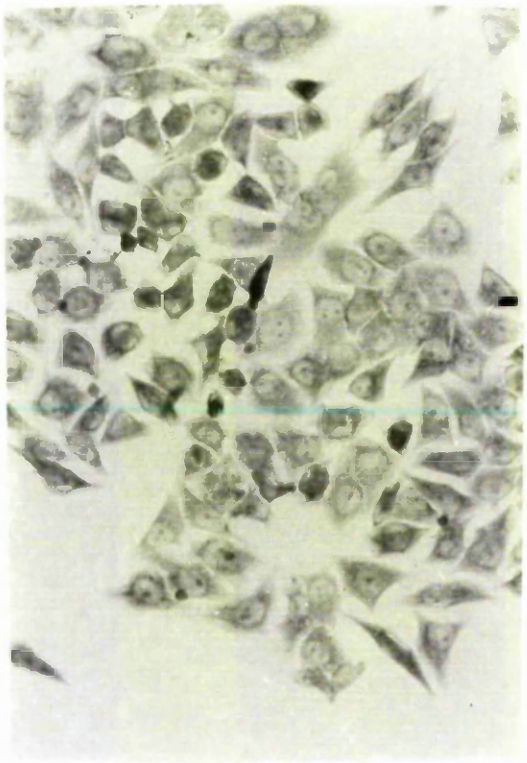
Monolayers of Hela cells grown on microscope slides contained in 50-mm Petri dishes were mock-infected or infected with pseudorabies virus at a multiplicity of 50 p.f.u./cell. At hourly intervals after infection, slides were removed, washed in BSS and absolute alcohol, fixed in formal saline and stained with Giemsa. Slides were further washed, dried, covered with coverslips and photographed under a Leitz light microscope.

a; mock-infected (5 hrs).

b; PrV-infected (5 hrs). Magnification X 240.



a



b

Figure 3.2.2. Distribution of nucleoli of given cross-sectic area during infection.

The perimeters of nucleoli from photographic negatives of mock-infected or infected Hela cells prepared as described in the legend of Figure 3.2.1, were outlined on tracing paper with a Nikon comparator. The outlines were cut and weighted in a microbalance. The nucleoli from a total of about 50 individual cells were examined for mock-infected and infected at hourly intervals up to six hours postinfection.

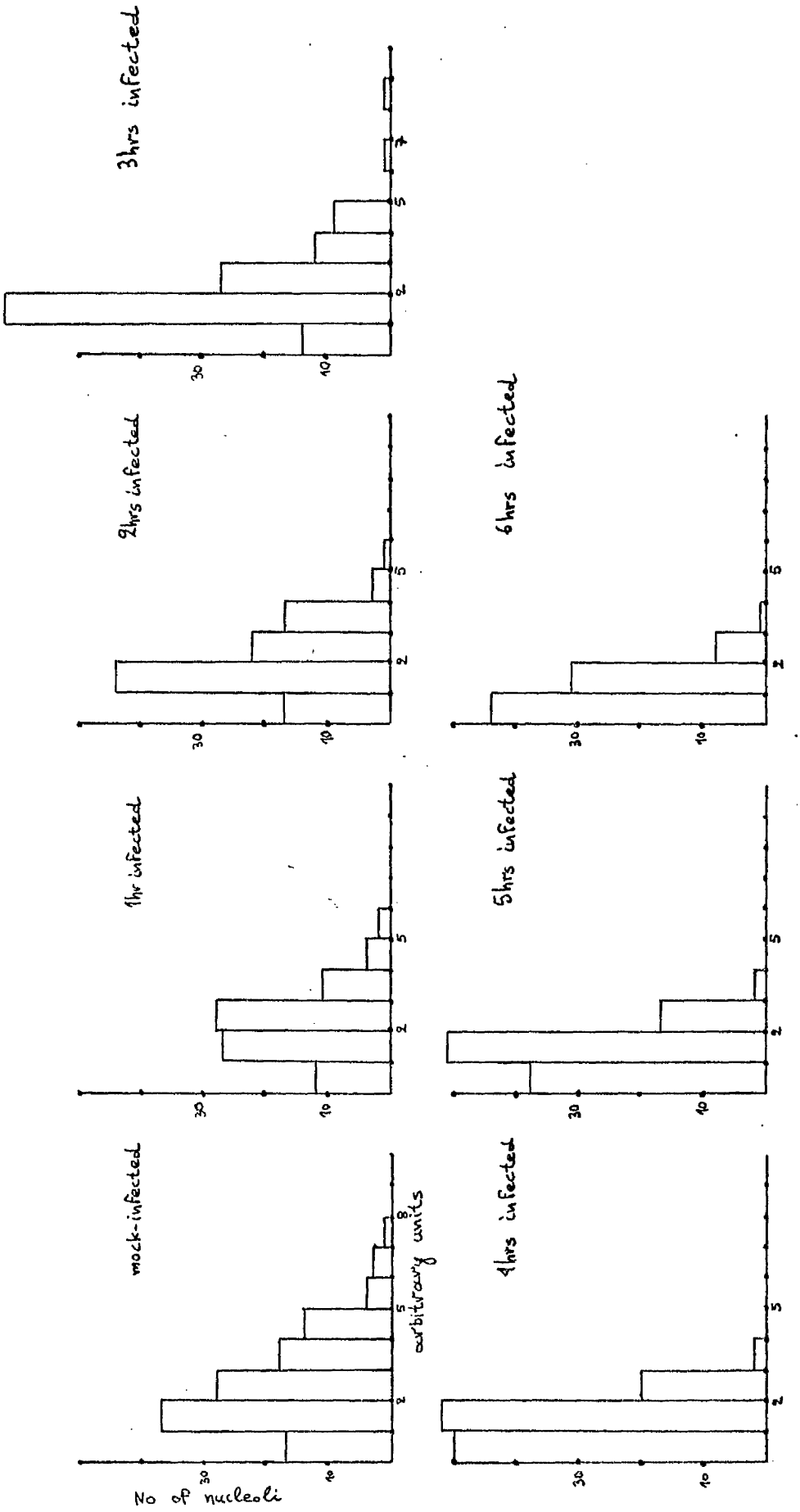
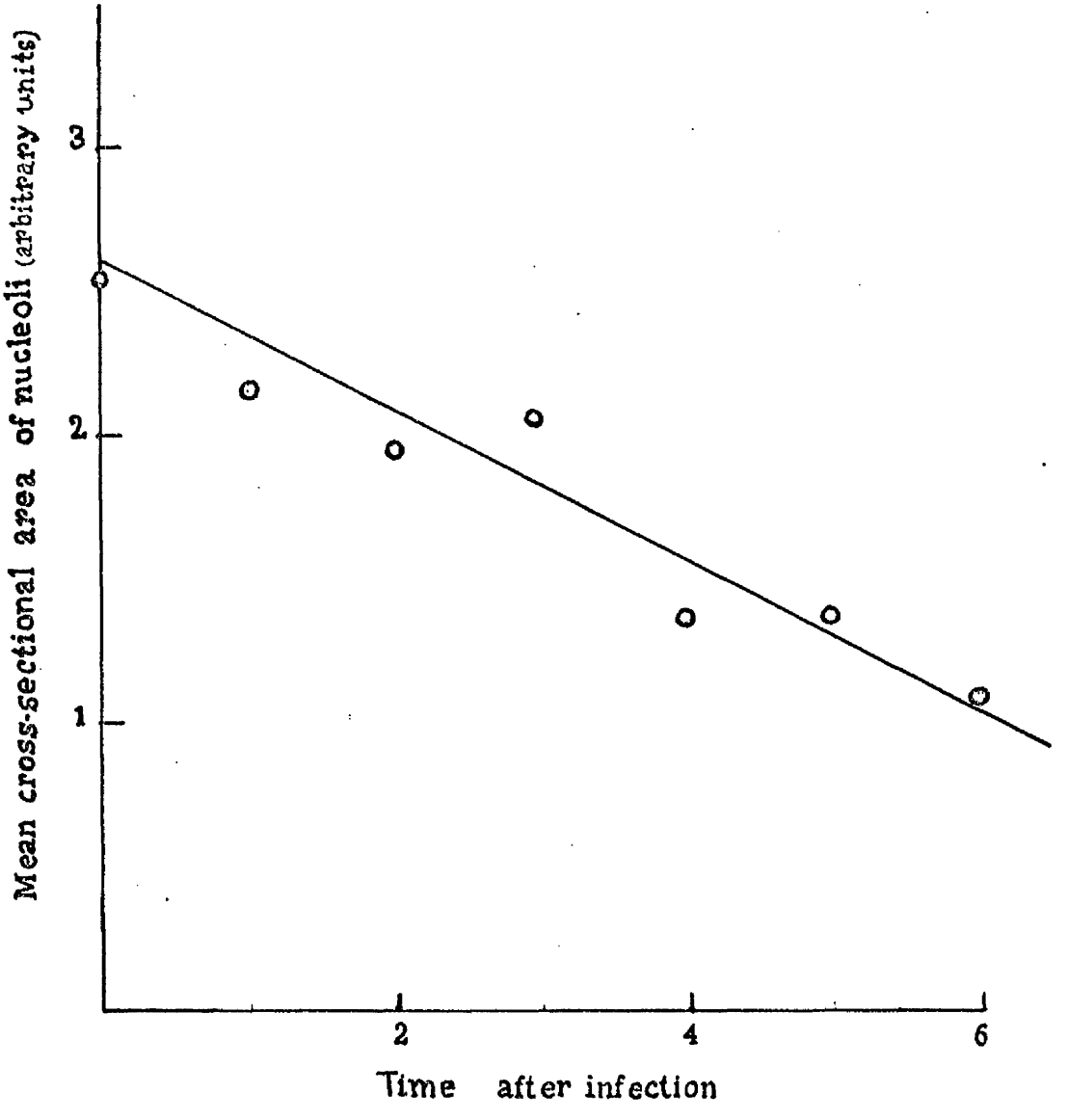

mock-infected
1hr infected
2hrs infected
3hrs infected
4hrs infected
5hrs infected
6hrs infected
No of nucleoli
arbitrary units
30
10
2
5
8

Figure 3.2.3. Mean cross-sectional area of individual nucleoli during infection.

The mean value of the cross-sectional area of nucleoli was estimated in Hela cells prepared as in Fig. 3.2.1 and analyzed as in Fig. 3.2.2. Mean values were estimated at hourly periods after infection.


Mean cross-sectional area of nucleoli (arbitrary units)
3
2
1
2
4
6
Time after infection

post-infection and the 14% to 52% rise in the percentage of nucleoli at size unit one, at 6 hours postinfection. Again this change is reflected in a decline in the average cross-sectional area of individual nucleoli as the infection proceeds. This reaches values as low as 45% of the control. (Fig. 3.2.3.).

3.3. Changes in total nucleolar area per cell.

In view of the above, it seemed that a characterisation of total area of nucleoli per cell would be of value in assesing the changes produced as a result of infection.

The data obtained the same way as before (Fig. 3.3.1), show a decline in total nucleolar area per nucleus, giving an average of 6.5 size units for the mock-infected compared with an average of 1.9 size units for the infected cells at 6 hours after infection (Table 3.3.1). This decline (as the infection proceeds) is more dramatic than that noted for the individual nucleolar area (Fig. 3.2.3) and reaches values as low as 35% of the control (Fig. 3.3.2).

It is assumed to reflect a concomitant decrease in nucleolar RNA metabolism as suggested in the introduction and confirmed in chapter 4.

3.4. Changes in the number of nucleoli per cell.

As was mentioned earlier, the number of nucleoli per cell varies depending on the metabolic state of the cell. An average of 1.7 nucleoli per cell was found for Hela cells when cultured in complete medium, whereas on incubation in

Figure 3.3.1. Total nucleolar cross-sectional area per cell during infection.

The total nucleolar cross-sectional area per cell was estimated in Hela cells prepared as in Fig. 3.2.1. and analyzed as in Fig. 3.2.2.

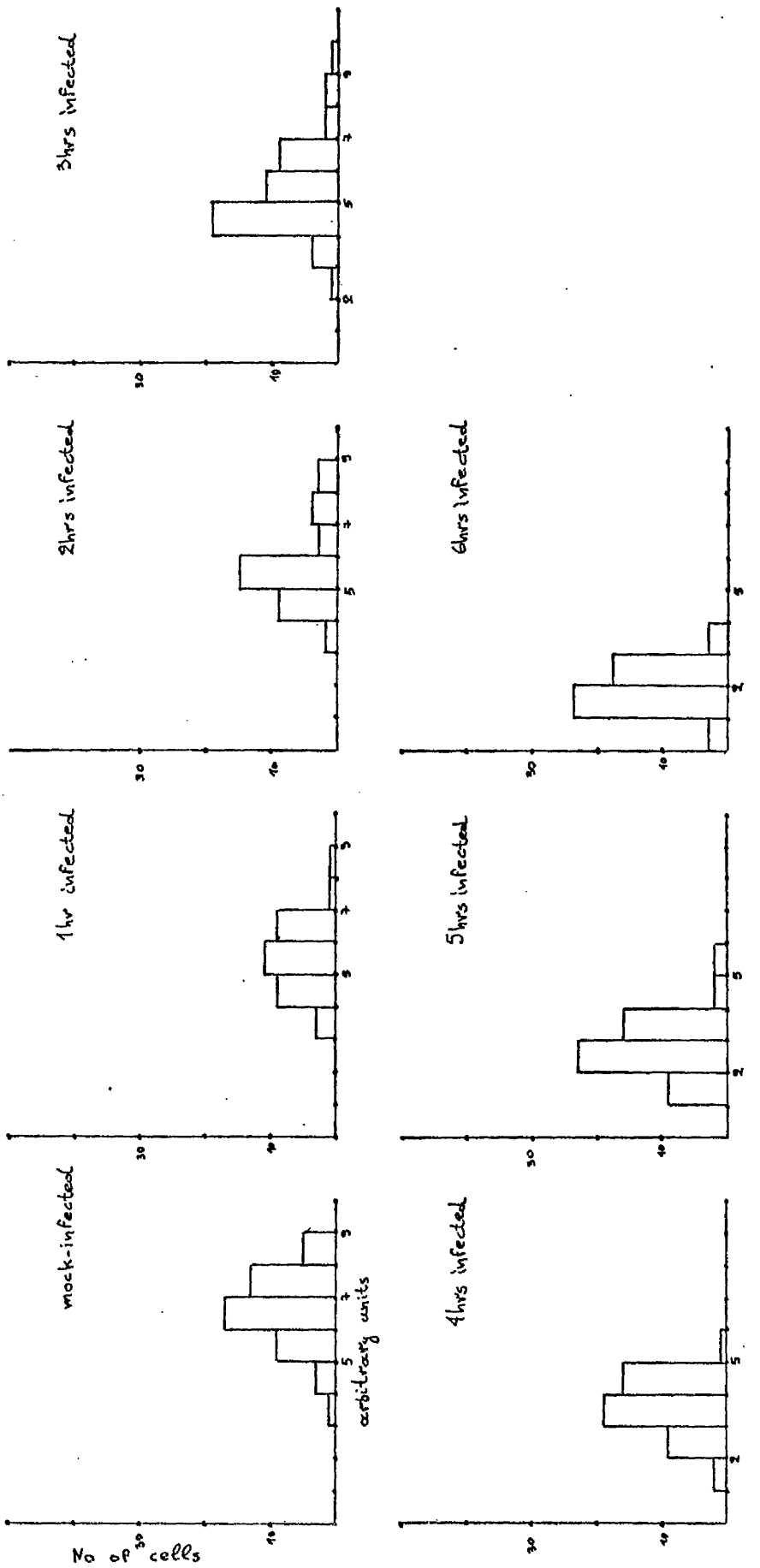

mock-infected
1hr infected
2hrs infected
3hrs infected
4hrs infected
5hrs infected
6hrs infected
No of cells
arbitrary units

Figure 3.3.2. Mean total nucleolar cross-sectional area per cell during infection.

The data of Table 3.3.1 were used to prepare this figure.

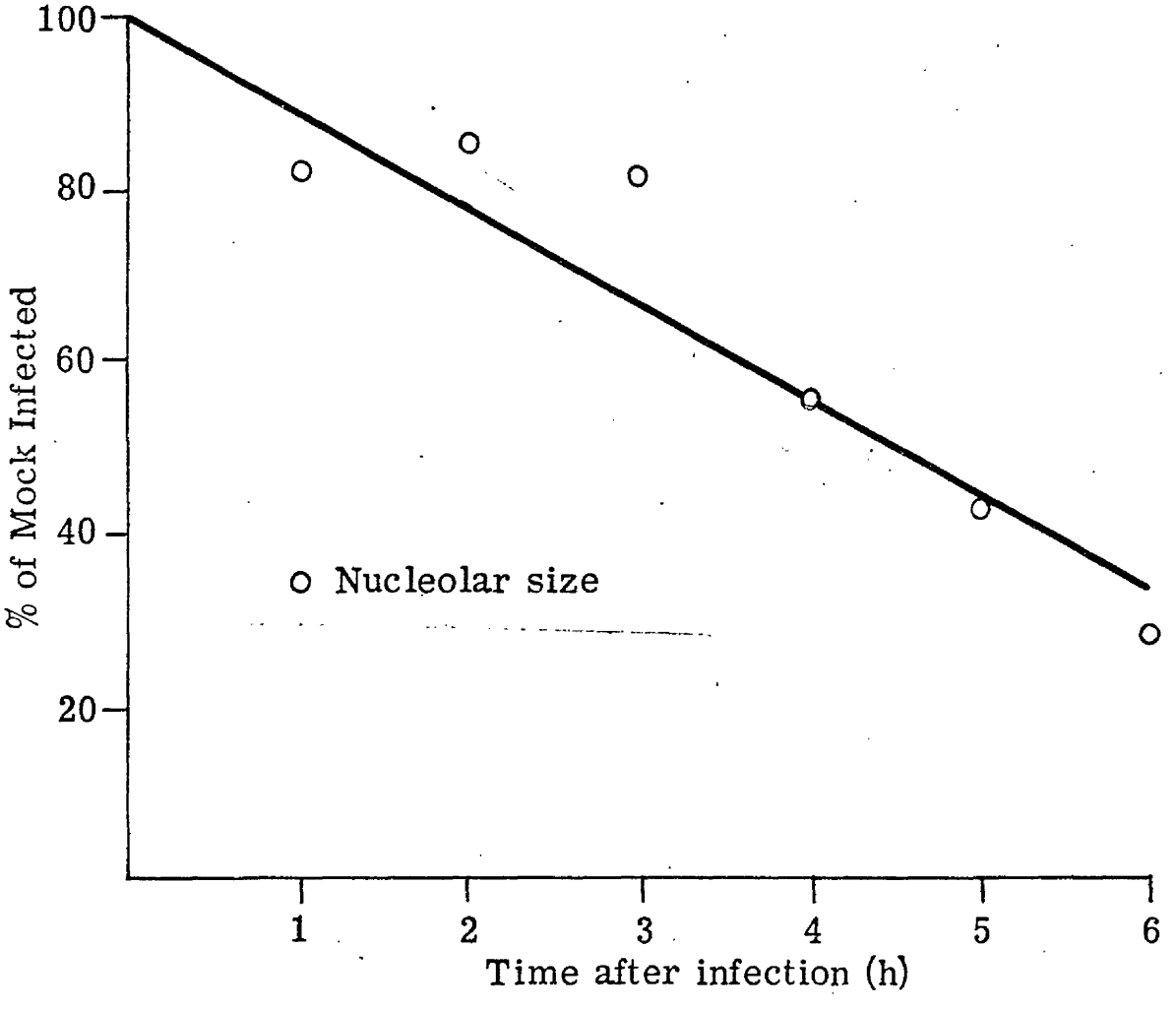

100
80
60
40
20
% of Mock Infected
Nucleolar size
1
2
3
4
5
6
Time after infection (h)

Table 3.3.1. Mean values and standard deviations of total nucleolar cross-sectional area during infection.

The mean values and standard deviations of total cross-sectional areas were calculated from Fig. 3.3.1, at hourly intervals after infection.

Time after infection (h)	Cross-sectional area* of individual nucleoli (arbitrary units)	Total area of* nucleoli per cell (arbitrary units)
0	2.5± 1.5	6.6±1.2
1	2.1± 1.1	5.4±1.2
2	2.0 ± 1.1	5.6±1.3
3	2.1 ± 1.2	5.3±1.4
4	1.4 ± 0.7	3.6±0.9
5	1.4 ± 0.7	2.8±0.9
6	1.1 ± 0.6	1.9±0.7

*Value showen is the mean and standard deviation calculated from measurements from >50 cells.

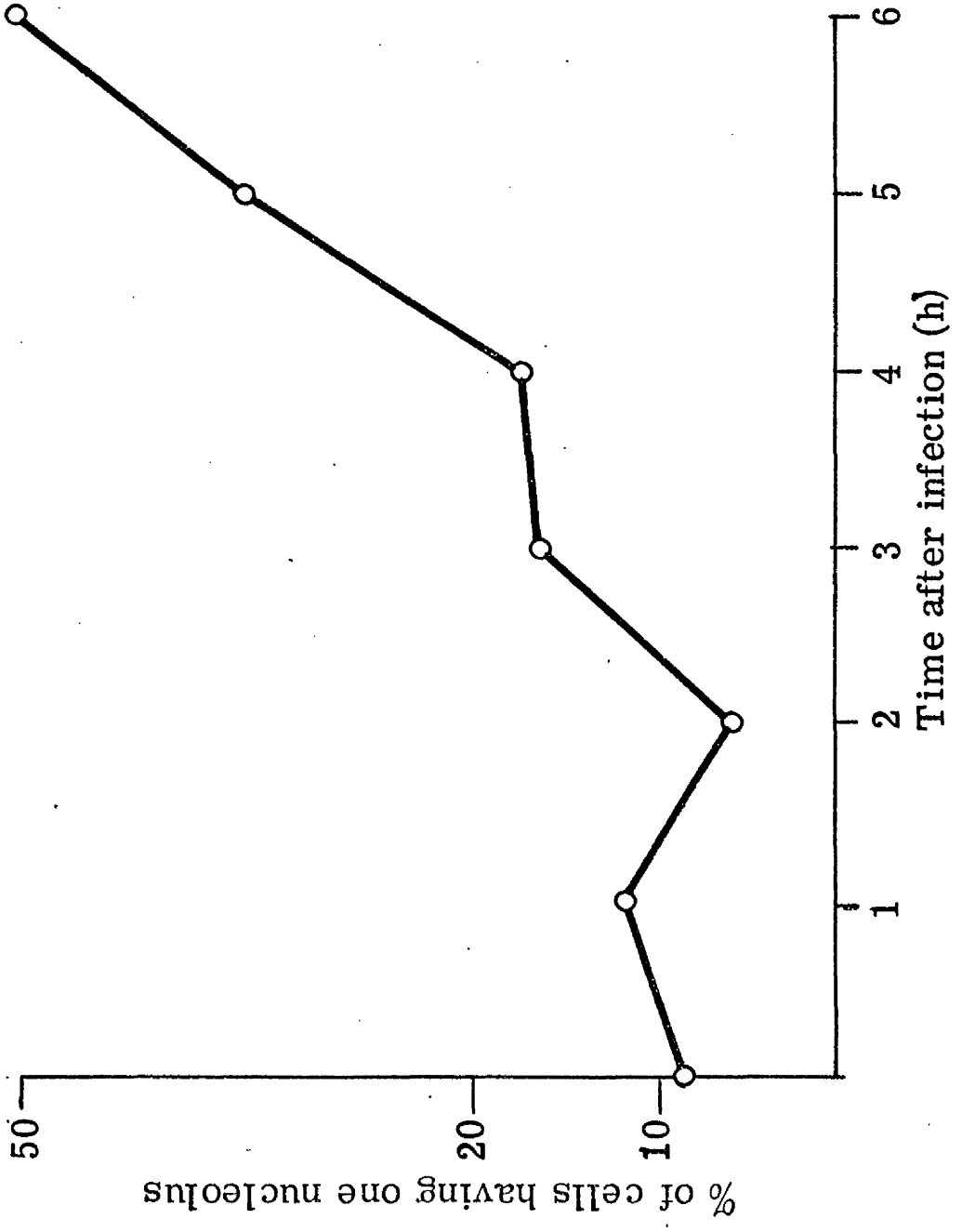
Table 3.4.1. Number of nucleoli per cell during infection; mean values and range.

The range and average number of nucleoli per cell were estimated from photographic negatives of mock-infected or infected Hela cells prepared as described in Fig. 3.2.1.

Time after infection (h)	No. of nucleoli per cell (Mean of 50 cells)	Range
0	2.6	1-5
1	2.5	1-5
2	2.9	1-5
3	2.6	1-5
4	2.6	1-6
5	2.0	1-4
6	1.7	1-5

Figure 3.4.1 Percentage of cells with one nucleolus during infection.

The percentage of cells with one nucleolus was derive from Table 3.4.1.


% of cells having one nucleolus
50
20
10
1
2
3
4
5
6
Time after infection (h)

in salt solutions free of amino acids the number of nucleoli per cell averaged 3.7 and ranged from 1 to 8, with an additional decrease in size (Swift, 1959), thus this suggests a relationship between the number of nucleoli per cell and the medium in which the cells are incubated.

In the Hela cells used in the experiments described in this chapter, an average of 2.6 nucleoli per cell was found for normal non-infected cells and the range was from 1 to 5 nucleoli per cell. Virtually the same average of nucleoli per cell was found in the infected cells (Table 3.4.1) at early times when it then declined at 5 hours and 6 hours to 2.0 and 1.7 respectively.

Furthermore, the percentage of cells with one nucleolus per cell rose to 50% at 6 hours (Fig. 3.4.1).

3.5. Discussion.

As noted in the introduction, nucleolar size and ribosomal RNA metabolism are coupled and any change in one of these should affect the other. Thus the decrease observed in nucleolar cross-sectional area and particularly in the total nucleolar area in Hela cells infected with PrV would be expected to reflect an analogous decrease in ribosomal RNA metabolism. Whether there is and what is the cause of this decrease in ribosomal RNA is dealt with in the next sections, though an indication of the cause could be taken from the number of nucleoli per cell which declines during infection indicating that nucleoli are not fragmented following virus attack thus allowing us to assume that the pseudorabies virus acts through interference with the DNA template. This is fully discussed in the General Discussion.

CHAPTER 4. Synthesis of ribosomal RNA in Hela cells following infection with pseudorabies virus.

4.1. Introduction

Total RNA synthesis in herpesvirus-infected Hela cells as judged by incorporation of exogenous ^{3}H-nucleosides declines considerably in the course of infection (see Introduction).

Incorporation of radioactivity into the nuclear RNA of Hep-2 cells after infection with herpesvirus is rapidly reduced. The same appears to be true for cytoplasmic ribosomal RNA (Wagner and Roizman, 1969). Furthermore, several authors have observed a substantial decline in ^{3}H-uridine incorporation into 45s pre-rRNA. They have not made a quantitative estimate of this decline (Wagner and Roizman, 1969; Flanagan, 1964; Hay et al., 1966).

In order to assess the decline in the rate of RNA synthesis after infection, as determined by incorporation rates of exogenous precursors, quantitation of the precursor pools is required (Harbers et al., 1959; Emerson and Humphreys, 1971; Houschka, 1973). When incorporation of label into RNA is corrected for changes in the specific radioactivity of the UTP precursor pool, the variables involved in cellular transport and isotope dilution in precursor pools are avoided (Bucher and Swaffield, 1969; Yu and Fiegelson, 1970; Kalra and Wheldrake, 1972; Kaukel et al., 1972; Cortes et al., 1976). However estimation of the rate of RNA synthesis could be subject to significant errors, if RNA were formed from a compartmented nucleotide precursor pool that was not in equilibrium with the main cellular nucleotide pool (Goody and

Ellem, 1975). Evidence for a seperate UTP pool, from which rRNA is formed has been provided in the case of cells in culture (Wiegers et al., 1976).

Therefore the suggestion that the size of the total intracellular UTP pool remains unchanged after infection of RK monolayer cells with pseudorabies virus (Rakusanova et al., 1972) does not exclude possible changes in the UTP pool responsible for rRNA synthesis.

4.2. Incorporation of ^{3}H-uridine into ribosomal RNA.

It is well established that the nucleolus is the site of ribosomal RNA synthesis and any study on rRNA synthesis would be more revealing if nucleolar RNA were examined rather than nuclear or whole cell RNA.

Thus in this study, nucleoli were isolated from DNase treated nuclei of Hela cells grown in 80oz roller bottles, mock-infected or infected with PrV and labelled for one hour with ^{3}H-uridine as described in Materials and Methods. Phenol extracted nucleolar RNA was analyzed on 15% to 30% (w/v) linear density sucrose gradients and absorbance and radioactivity were determined as described in Materials and Methods. Fig. 4.2.1 shows that the radioactivity incorporated into 45s and 32s pre-rRNA falls after infection. However, an estimation of the decrease of ^{3}H-uridine incorporation into newly synthesized 45s pre-rRNA is hindered by the length of the radioactivity pulse employed, which allows processing of 45s to 32s to take place.

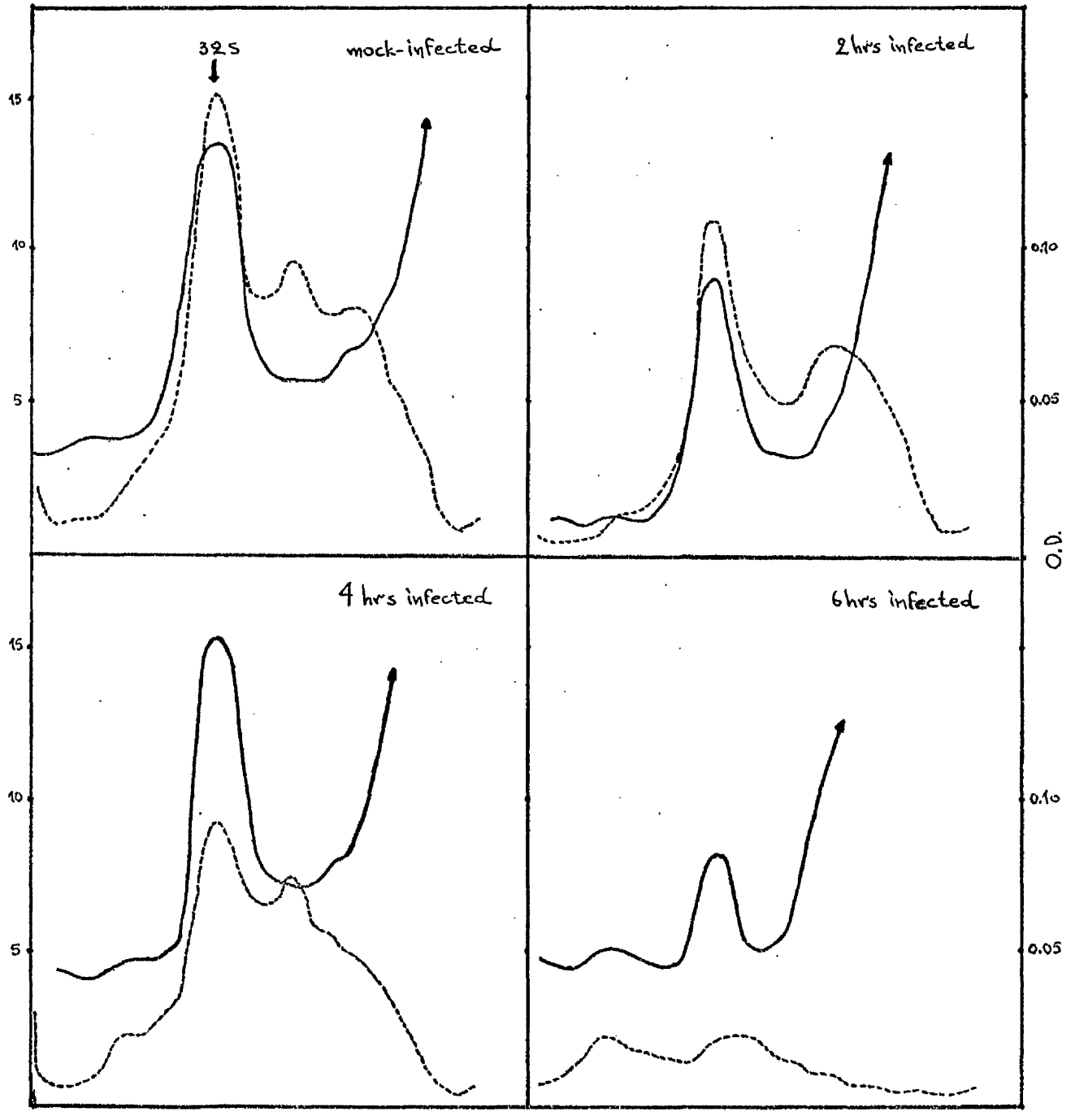
4.3. Specific radioactivities in pooled fractions of 45s and 18s rRNAs.

As can be seen in the figures presented in the previous

Figure 4.2.1. Optical density and radioactivity profiles of nucleolar RNA separated on sucrose density gradients at various times after infection.

Confluent monolayers of Hela cells grown in 80oz roller bottles were mock-infected or infected with pseudorabies virus at a multiplicity of 50 p.f.u./cell. At different times after infection cells were labelled with 5µCi/ml ^{3}H-uridine for a period of one hour before harvest.

Nucleoli were isolated by the DNase method of Penman and collected by centrifugation through a cushion of 0.88M sucrose at 2.900 RPM for 30 min in an M.S.E Major centrifuge. Phenol-extracted nucleolar RNA, was suspended in 1.0 ml LETS buffer, layered over 36 ml of 15% to 30% (w/v) linear sucrose density gradients in LETS buffer and centrifuged in the Beckman SW 27 rotor (22.000 rpm, 17 hours, 20^{o}C). Gradients were harvested by aspiration through a needle placed at the bottom of the tube and absorbance was monitored in a Gilford 2000 recording spectrophotometer. The TCA precipitable radioactivity of individual fractions was determined.


32S
mock-infected
2 hrs infected
4 hrs infected
6 hrs infected
15
10
5
0.10
0.05
O.D.
Distance migrated.
O.D
C.P.M.

section, the resolution of RNA species is not ideal in a linear density sucrose gradient and there is also a considerable amount of background due probably to the effect of residual phenol. These technical problems could hinder the estimation of the specific radioactivity of the RNA species and thus any attempts to calculate the decrease of synthesis of ribosomal RNA caused by the infection would not be accurate.

For that reason fractions containing specific ribosomal RNA species were pooled and re-centrifuged for several cycles in order to increase resolution of the particular RNA species and to reduce background caused by phenol.

Thus, nucleoli were isolated from DNase treated nuclei of Hela cells grown in 80oz roller bottles, mock-infected or infected with PrV and labelled for 15 min with ^{3}H-uridine as described in Materials and Methods. Phenol-extracted nucleolar RNA at 6 hrs postinfection was analyzed on 15% to 30% linear density sucrose gradients. 45s pre-rRNA fractions were pooled and ethanol precipitated overnight. The ethanol precipitate was collected by low-speed centrifugation dissolved in SDS buffer and centrifuged again on sucrose gradients. This procedure was repeated one more time and the final pooled 45s pre-rRNA fractions were analyzed for absorbance and radioactivity and specific radioactivities were determined (Fig. 4.3.1). There is a drop of 60% in specific radioactivity between mock-infected and PrV-infected Hela cells at 6 hrs after infection (Table 4.3.1).

The same procedure of repeated sucrose gradient centrifugation was followed for 18s cytoplasmic rRNA of Hela cells mock-infected and PrV-infected, labelled for 1 hour with ^{3}H-uridine (Fig. 4.3.2). The drop in specific radioactivity

Figure 4.3.1. Isolation and purification of 45s pre-rRNA of mock-infected and 6 hours infected Hela cells.

Hela cells mock-infected or infected as in Fig. 4.2.1 were labelled with 5μCi/ml of ^{3}H-uridine for a period of 15" before harvest at 6 hours post-infection.

Nucleoli and nucleolar RNA were prepared as described in Fig. 4.2.1 Equal amounts of mock-infected and infected nucleolar RNA were suspended in LETS buffer and layered onto 15% to 30% (w/v) linear density sucrose gradients and centrifuged in a Beckman SW 27 rotor (22.000 rpm, 17 hrs, 20^{o}C). Gradients were harvested through a Gilford 2000 recording spectrophotometer and the 45s rRNA peak was collected. The fraction containing 45s rRNA was ethanol precipitated overnight, collected, dissolved in LETS buffer and centrifuged again. The procedure was repeated once more and the optical density and radioactivity of the final fractic was determined.

a; 1st separation

b; 2nd separation

c; 3rd separation

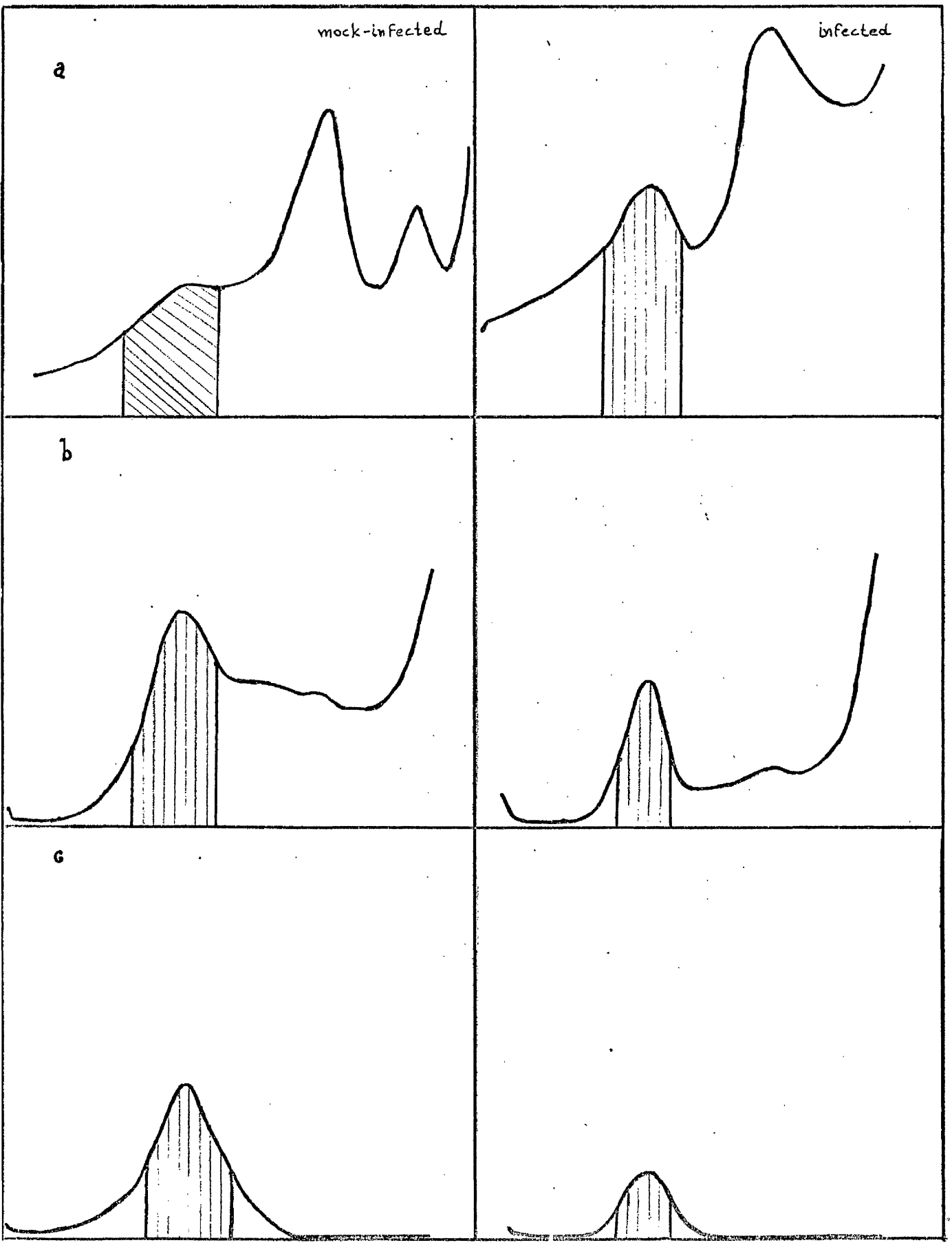

mock-infected
infected
a
b
c

Figure 4.3.2. Isolation and purification of 18s rRNA of mock-infected and 6 hours infected Hela cells.

Hela cells were mock-infected or infected as in Fig. 4.2.1. and labelled with ^{3}H-uridine for a period of 1 hr before harvest at 6 hrs after infection. Cytoplasmic RNA was extracted as described in Materials and Methods. 18s rRNA was isolated by sucrose gradient centrifugation as described in Fig. 4.3.1.

a; 1^{st} separation

b; 2^{nd} separation

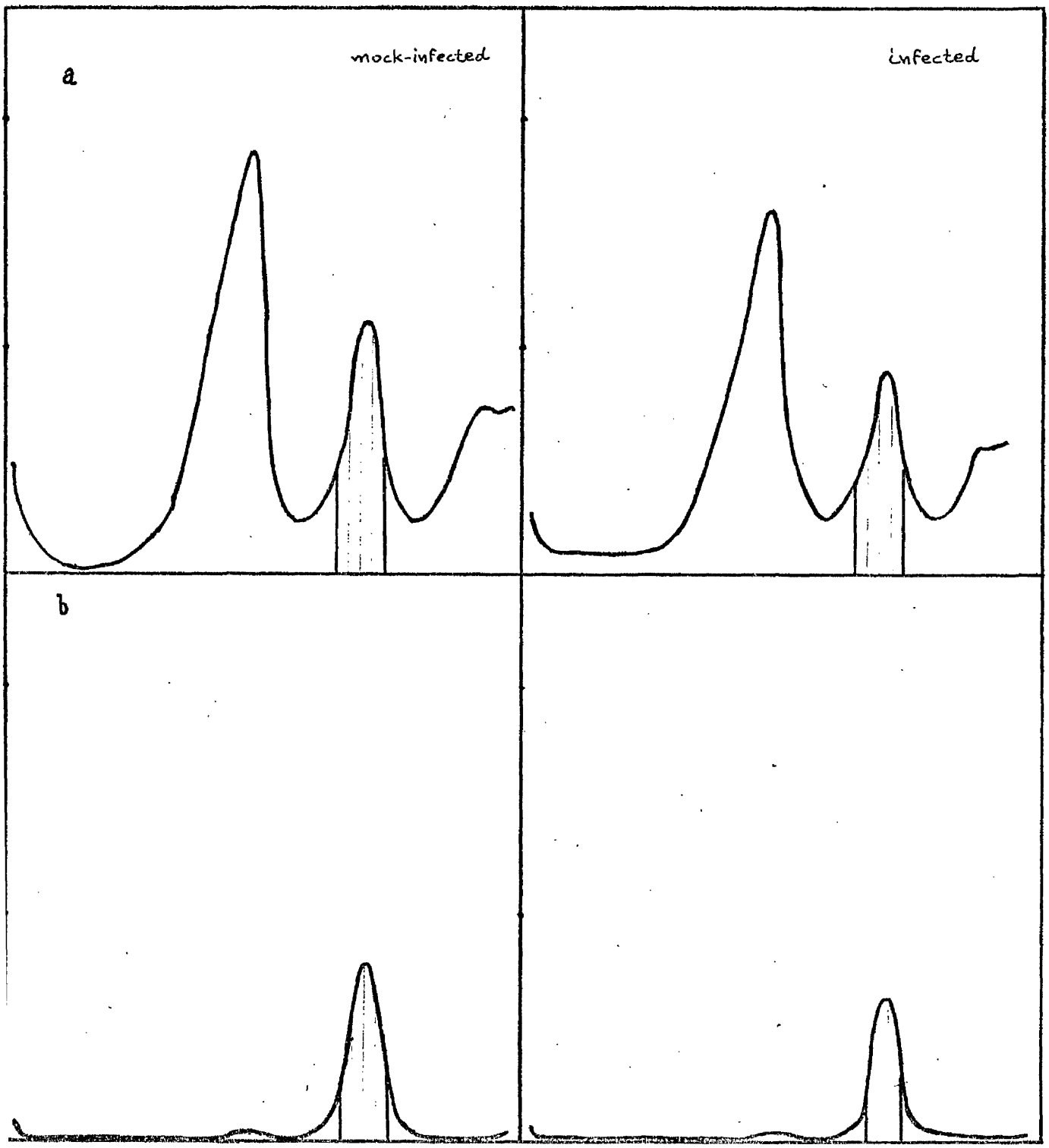

a
mock-infected
infected
b

Table 4.3.1. Specific radioactivities of 45s and 18s rRNA of mock-infected and infected Hela cells at 6 hrs after infection.

Specific radioactivities of 45s and 18s rRNA were determined from purified RNA species obtained as described in Figures 4.3.1 and 4.3.2 respectively.

SPECIFIC ACTIVITIES at 6 hrs post-infection.

	18S after 1 hr label.	45S after 15' label.
Mock-infected	8.500 dpm/µgr	3.200 dpm/µgr
PrV-infected	1.760 dpm/µgr	1.300 dpm/µgr
	INF 20% of CON	INF 40% of CON

in 18s rRNA between mock-infected and PrV-infected Hela cells is about 80% (Table 4.3.1).

4.4. Kinetics of the decrease in incorporation during infection.

The decrease in incorporation of ^{3}H-uridine into 45s pre-rRNA was examined at different times post-infection after a 15 min labelling period. The 15 min labelling time period allows assesment of ^{3}H-uridine incorporation levels into 45s pre-rRNA at a time when most of the label would be associated with the 45s pre-rRNA and it can be assumed that little processing to smaller rRNA species will have occured. However at this labelling period there will be synthesis of hnRNA and peaks other than ribosomal precursor can be seen in the gels.

Nucleolar RNA was prepared as above from Hela cells, mock-infected or infected with PrV and labelled with ^{3}H-uridine for a 15 min period before harvesting. Cells were harvested at 2 hrs,4 hrs and 6 hrs postinfection. The nucleolar RNA was subjected to electrophoresis in 2.7% acrylamide gels. Acrylamide gels were scanned for optical density in a Gilford Gel Scanner and radioactivity was estimated in 1mm gel slices cut with a Mickle gel slicer as described in Materials and Methods. The specific radioactivity of 45s pre-rRNA was calculated from the optical density and radioactivity profiles (Fig.4.4.1). Incorporation of ^{3}H-uridine into 45s pre-rRNA drops gradually as the infection proceeds, reaching values as low as about 40% at 6 hrs postinfection (Fig. 4.4.2).

4.5. Discussion

As described above, incorporation of ^{3}H-uridine into

Figure 4.4.1. Optical density and radioactivity profiles of nucleolar RNA separated on polyacrylamide gels.

Hela cells grown in Roux bottles were mock-infected or infected with pseudorabies virus at a multiplicity of 50 pfu/cell. At 2 hrs, 4hrs and 6 hrs after infection cells were labelled with 5 μCi/ml ^{3}H-uridine for a period of 15 min before harvesting. Nucleoli and nucleolar RNA were prepared as described for Fig. 4.2.1. Nucleolar RNA from mock-infected and infected cells was subjected to electrophoresis on 2.7% acrylamide gels as described in Materials and Methods. Optical density was recorded in a Gilford gel scanner and radioactivity was determined in 1 mm gel slices as described in Materials and Methods.

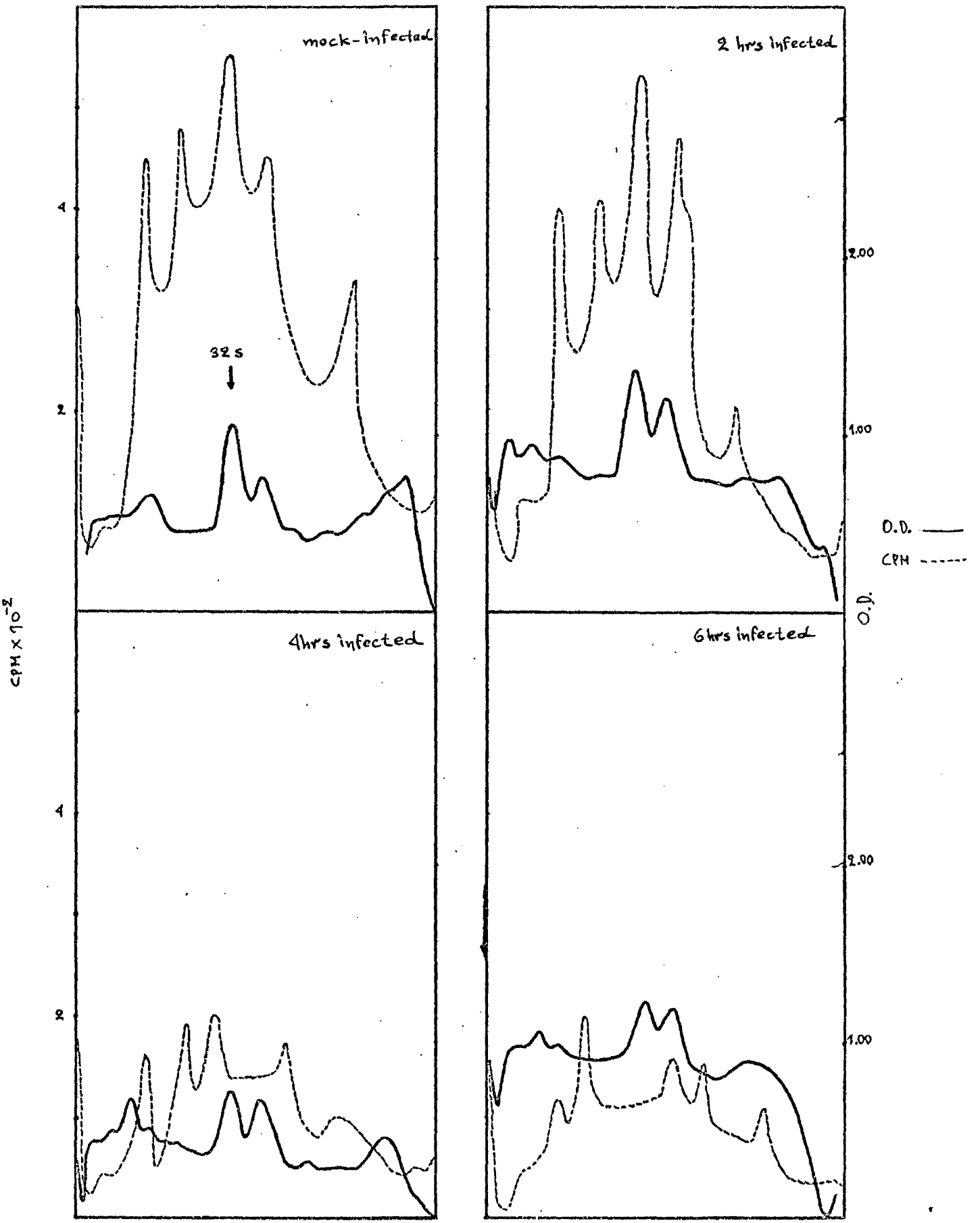
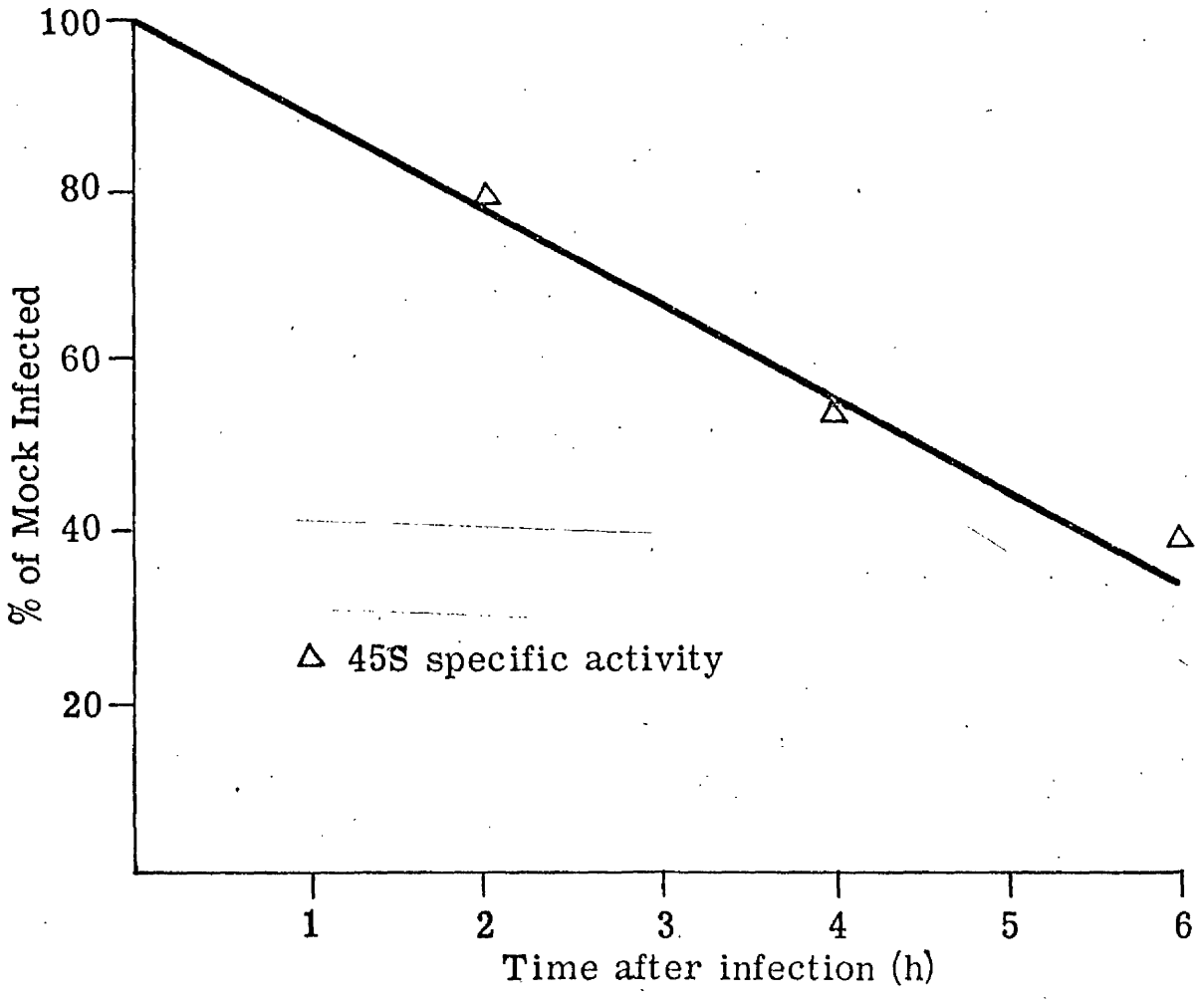

mock-infected
2 hrs infected
4hrs infected
6hrs infected
32 s
4
2
2.00
1.00
O.D. ——
CPM ------
CPM x 10⁻²
O.D.

Figure 4.4.2. Specific radioactivity of newly synthesized 45s pre-rRNA.

The specific radioactivity of 45s rRNA was determined from the optical density and radioactivity profiles presented in Fig. 4.4.1.


100
80
60
40
20
% of Mock Infected
Δ 45S specific activity
1
2
3
4
5
6
Time after infection (h)

newly synthesized ribosomal RNA in Hela cells decreases after infection with pseudorabies virus. It is expected that minor differences in the number of cells or their growth state from culture to culture and therefore in the concentration of the labelling compound and possibly in the multiplicity of infection could hinder an accurate assesment of the decline in incorporation.

Even though a precise estimation is difficult, it is suggested that the values obtained give a reasonable estimation of the decrease in incorporation after infection.

As mentioned in the introduction, in order to assess the synthesis of RNA from the incorporation of ^{3}H-uridine into RNA, the specific radioactivities acquired by the UTP pools after the pulse must be estimated. This is impossible to follow in these experiments due to the nature of the UTP pool responsible for ribosomal RNA. However, the assesment of the specific radioactivities of the UTP pools might not be of importance. That is because the de novo synthesis of nucleotides in the UTP pool responsible for rRNA synthesis has been suggested, not to be subject to feedback inhibition by extracellular uridine to an appreciable extent (Wäegers et al., 1976) and even though possible changes in pool size after infection could not be ruled out, it is assumed that any difference in the specific radioactivities acquired by these UTP pools during the labelling period, would not be too significant and therefore the decrease in the rate of synthesis would approximately follow the decrease in incorporation of ^{3}H-uridine after infection. This could be seen from the similar decrease in RNA polymerase I activity in isolated nuclei of mock-infected and infected Hela cells (Chapter 7) where RNA synthesis is examined independently of the cellular UTP pools.

Thus it is suggested that synthesis of 45s pre-rRNA of Hela cells after infection with pseudorabies virus undergoes at least a 40% reduction since later results suggest smaller UTP pool sizes in infected cells than in mock-infected and therefore more rapid equilibration of the precursor. Furthermore, nucleolar area estimates (Chapter 3) approximately match these results again suggesting that the figures given here are a good indication of the virus effect on RNA synthesis despite the area of doubt concerning pool effects.

CHAPTER 5. Processing of ribosomal RNA Hela cells after infection with pseudorabies virus.

5.1. Introduction.

As described in the Introduction (section, 1.1.2.2), formation of mature rRNA molecules involves (i) transcription of rRNA genes yielding pre-rRNA and (ii) maturation of pre-rRNA. The process of maturation of pre-rRNA includes endonucleolytic cleavages of the newly synthesized pre-ribosomal 45s rRNA to yield the 28s and 18s mature ribosomal RNA's.

It has been suggested that in herpesvirus infected cells a decrease in processing of 45s pre-rRNA occurs, describe as abortive, resulting in the degradation of 45s pre-rRNA into oligonucleotides sedimenting with S values of less than 18s (Wagner and Roizman, 1969). An inhibition of processing has also been observed in vaccinia-infected L cells which has been interpreted as a slower rate of processing but not abberant processing. These authors have attributed the decrease in processing to the lack of one or more ribosomal proteins required for the formation of ribosomes (Jefferts & Hollowczak, 1971).

5.2. Processing of 45 pre-rRNA in nucleoli of pseudorabies virus infected Hela cells.

It has been suggested thatthe nucleolus contains all the early ribosomal RNA precursors (Penman et al., 1971) thus a study of the early events in processing of the 45s pre-rRNA is best carried out using nucleoli rather than nuclei or cytoplasm.

Therefore processing of 45s pre-rRNA was examined in phenol extracted nucleoli isolated from DNAse treated nuclei of Hela cells mock-infected or pseudorabies virus infected.

Samples containing 1 O.D. unit of RNA were subjected to electrophoresis on polyacrylamide gels as described in Materials and Methods. Ribosomal RNA was labelled with a 15 min pulse of ^{3}H-uridine and chased with cold uridine and cytidine in the presence of glucosamine for 30 and 60 minutes as described in Materials and Methods. Acrylamide gels were monitored after electrophoresis for absorbance and radioactivity and as shown in Fig. 5.2.1, 45s pre-rRNA is processed to 32s pre-rRNA, in the nucleolar extracts from the PrV-infected Hela cells as well as in the mock-infected Hela cells. However, quantitation of this is difficult due to the lack of a standard RNA marker such as like 28s or 18s rRNA.

The high level of incorporation into 45s pre-rRNA seen at 4 hrs and at 6 hrs postinfection does not constitute a change in the rate of synthesis but rather an increased rate of incorporation due probably to the glucosamine used in the chase.

Glucosamine depletes already existing UTP pools. This could be seen in Fig. 5.2.2 where nucleolar RNA was extracted from mock-infected and PrV-infected Hela cells labelled for 1 hr with ^{3}H-uridine in the absence of glucosamine. The infected cells in this particular experiment show a decline in 45s pre-rRNA radioactivity of about 60% to 70% compared to the mock-infected as would be expected from the results shown in Chapter 4.

Fig. 5.2.1. Optical density and radioactivity profiles of nucleolar RNA pulse-labelled and chased, at 4hrs and 6hrs after infection, then separated on polyacrylamide gels.

Hela cells grown in 80oz roller bottles were mock-infect or infected with PrV at 50 pfu/cell and labelled with 10μCi/ml ^{3}H-uridine for a period of 15 min. The label was chased for 30 min and 60 min with glucosamine and uridine as described in Materials and Methods. Nucleoli and nucleolar RNA were isolated as described for Fig. 4.2.1. and 1 O.D of nucleolar RNA was subjected to electrophoresis in 2.7% acrylamide gels as described in Materials and Methods. Optical density was recorded in a Gilford Gel Scanner and radioactivity was determined in 1 mm slices as described in Materials and Methods.

— O.D.
--- cpm

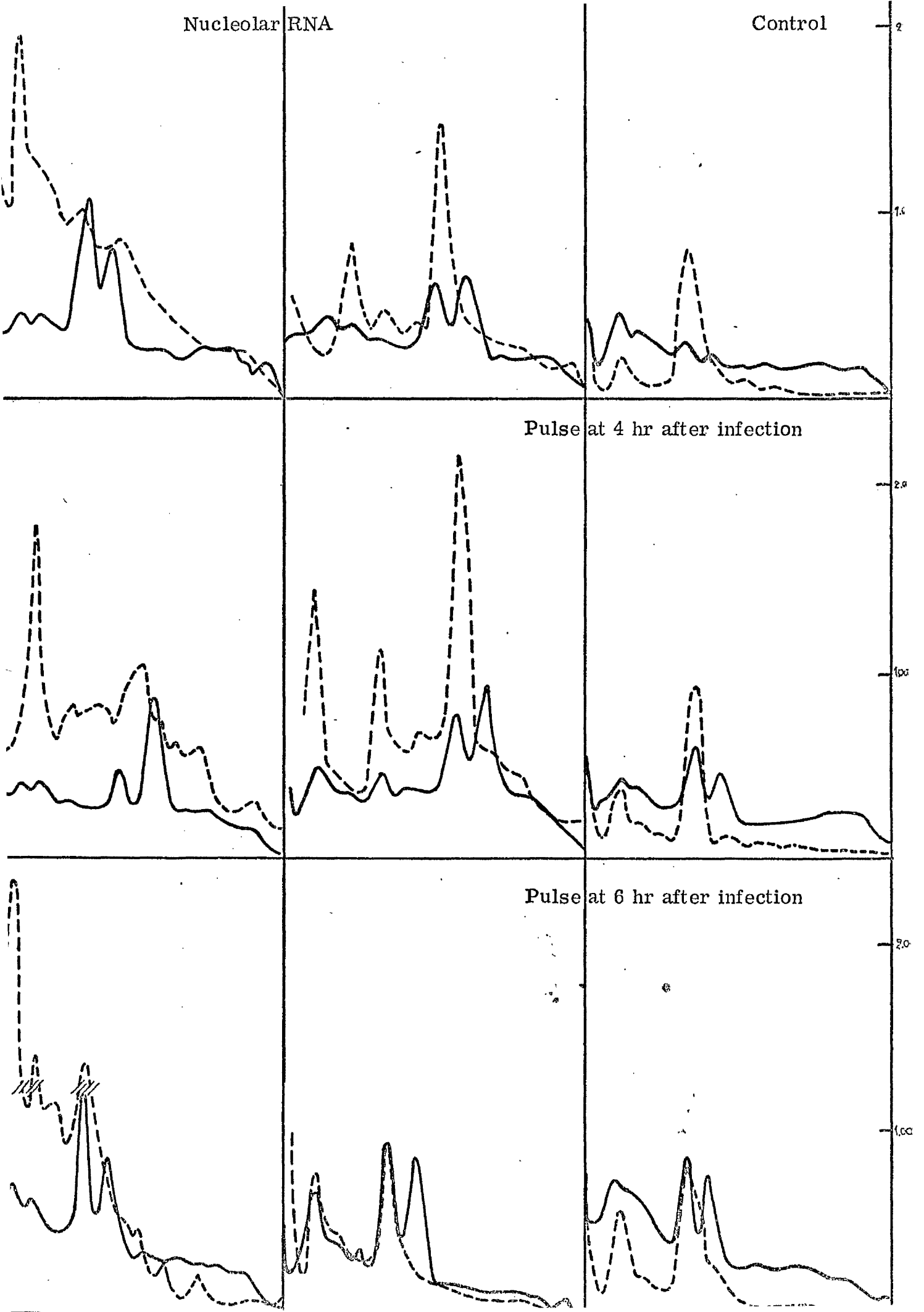
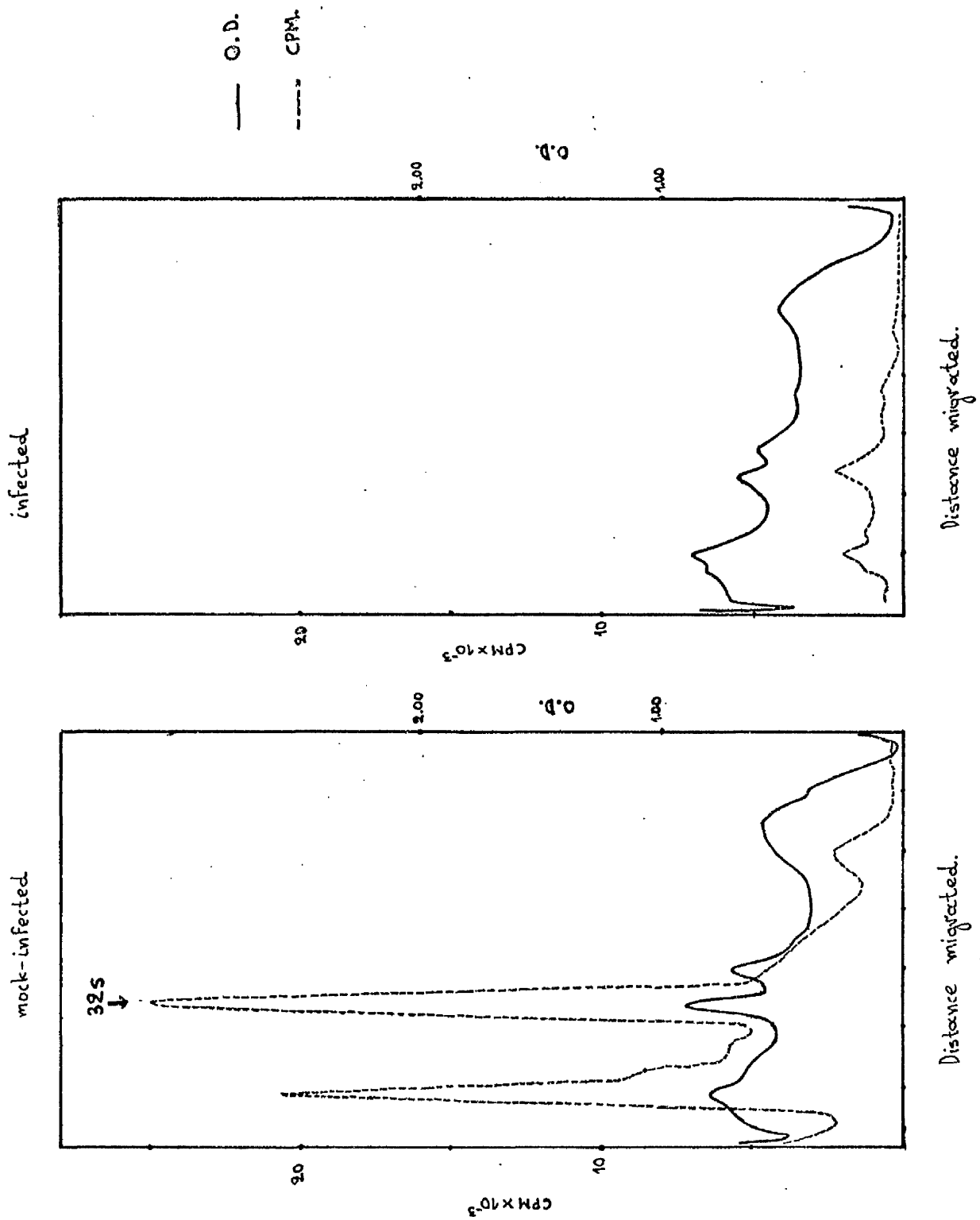

Nucleolar RNA
Control
Pulse at 4 hr after infection
Pulse at 6 hr after infection

Fig. 5.2.2. Optical density and radioactivity profiles of mock-infected or infected nucleolar RNA separated on polyacrylamide gels at 6 hrs after infection.

Hela cells grown in 80oz roller bottles were mock-infected or infected with pseudorabies virus at 50 pfu. per cell and labelled with 10μCi/ml ^{3}H-uridine for a period of 1 hr. Nucleolar RNA was prepared, subjected to electrophor and the optical density and radioactivity profiles were determined as for Fig. 5.2.1.


O.D.
CPM.
infected
mock-infected
32S
CPM × 10⁻³
O.D.
Distance migrated.
Distance migrated.

5.3. Processing of newly synthesized ribosomal RNA in pseudorabies virus infected whole Hela cell extracts.

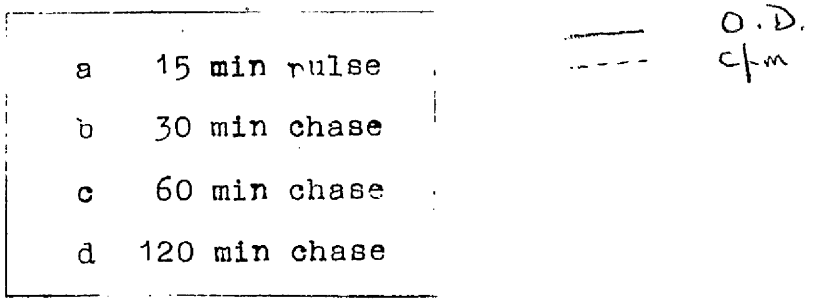
Processing of newly synthesized pre-rRNA was examined in extracts of mock-infected and PrV-infected whole Hela cells in order to follow the complete pathway of processing to mature ribosomal RNA.

Hela cells grown in Roux bottles were mock-infected or infected with PrV, labelled at 4 hrs and 6 hrs postinfection with a 15 min pulse of ^{3}H-uridine and chased afterwards with cold uridine and cytidine in the presence of glucosamine for 30', 60', or 120 minutes. Cells were harvested and whole cell RNA was phenol-extracted as described in Materials and Methods. Equal amounts of whole cell RNA samples were subjected to electrophoresis in 2.7% acrylamide gels. Acrylamide gels were scanned for optical density in a Gilford Gel Scanner and radioactivity was estimated in 1mm gel slices cut with a Mickle gel slicer. Optical density and radioactivity profiles were determined for 4 hrs mock-infected and infected (Fig. 5.3.1) and 6 hrs mock-infected and infected Hela cells (Fig. 5.3.2). It is clear that processing of the 45s pre-rRNA continues in the infected cells and radioactivity appears under the 28s and 18s peaks even as late as 7 hours after infection.

The total amount of radioactivity present at 30', 60', and 120 minutes under the main ribosomal RNA peaks was calculated and after being corrected for the optical density of the prominent 28s and 18s peaks was expressed as a percentage of the initial amount of radioactivity of newly synthesized ribosomal RNA at 15 min both for 4 hr and 6 hr mock-infected and infected whole cell extracts. This is shown in Fig. 5.3.3.

Fig. 5.3.1. Optical density and radioactivity profiles of whole cell RNA pulsed and chased, at 4 hrs after infection and separated on polyacrylamide gels.

Hela cells grown in Roux bottles were mock-infected or infected with 50 p.f.u./cell and labelled with 5 μCi/ml ^{3}H-uridine for a period of 15 min. The label was chased for 30', 60' and 120' with glucosamine and uridine as described in Materials and Methods. Whole cell RNA was phenol-extracted and subjected to electrophoresis in 2.7% acrylamide as described in Materials and Methods. Optical density and radioactivity profiles were determined as for Fig. 5.2.1.



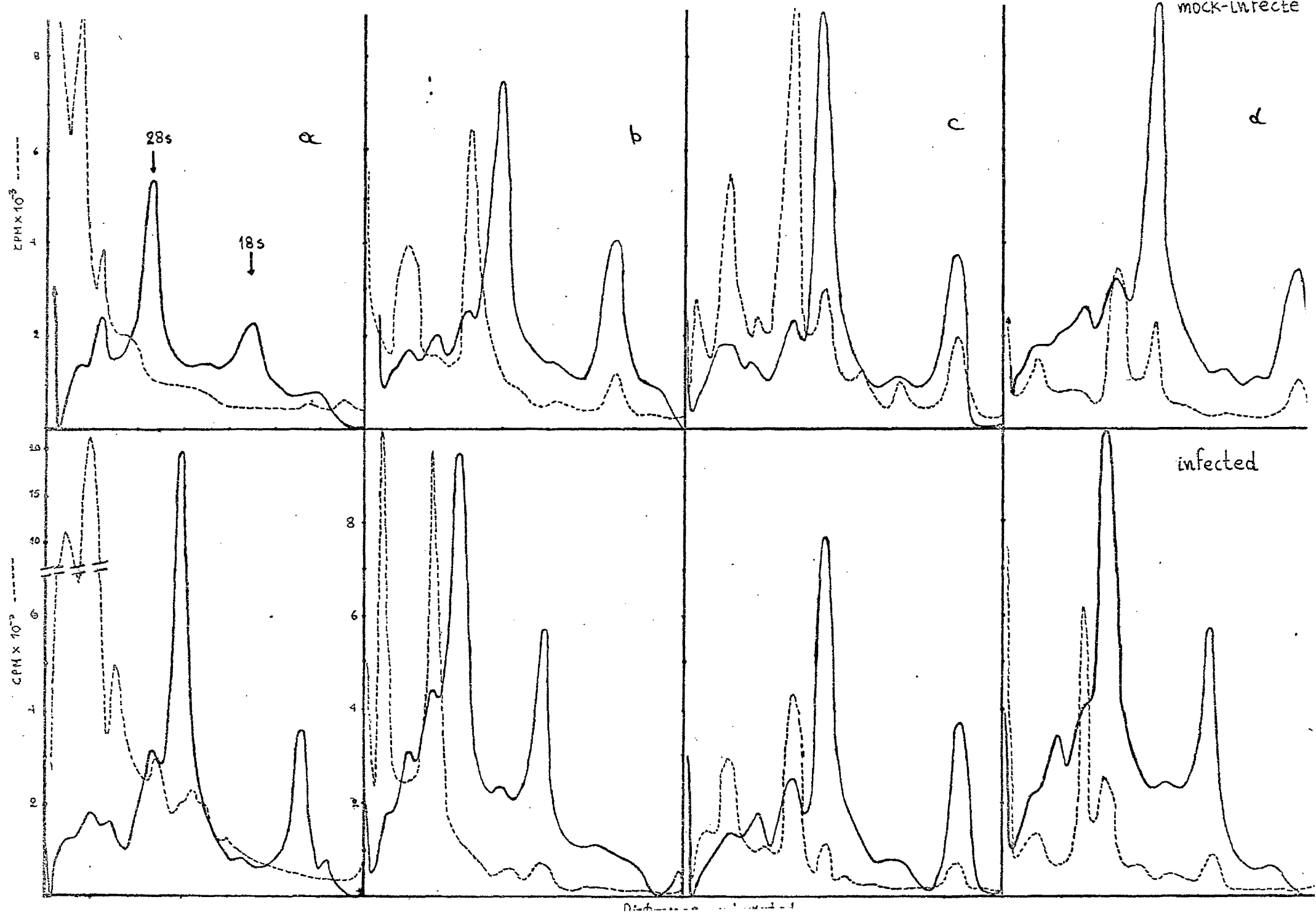
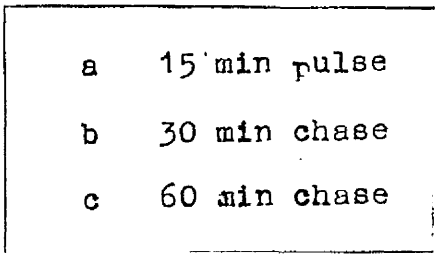

mock-infecte
28s
18s
a
b
c
d
infected
CPM x 10⁻³
CPM x 10⁻³

Fig. 5.3.2. Optical density and radioactivity profiles of whole cell RNA, pulsed and chased, at 6 hrs after infection and separated on polyacrylamide gels.

The same procedure was followed as for Fig. 5.3.1.



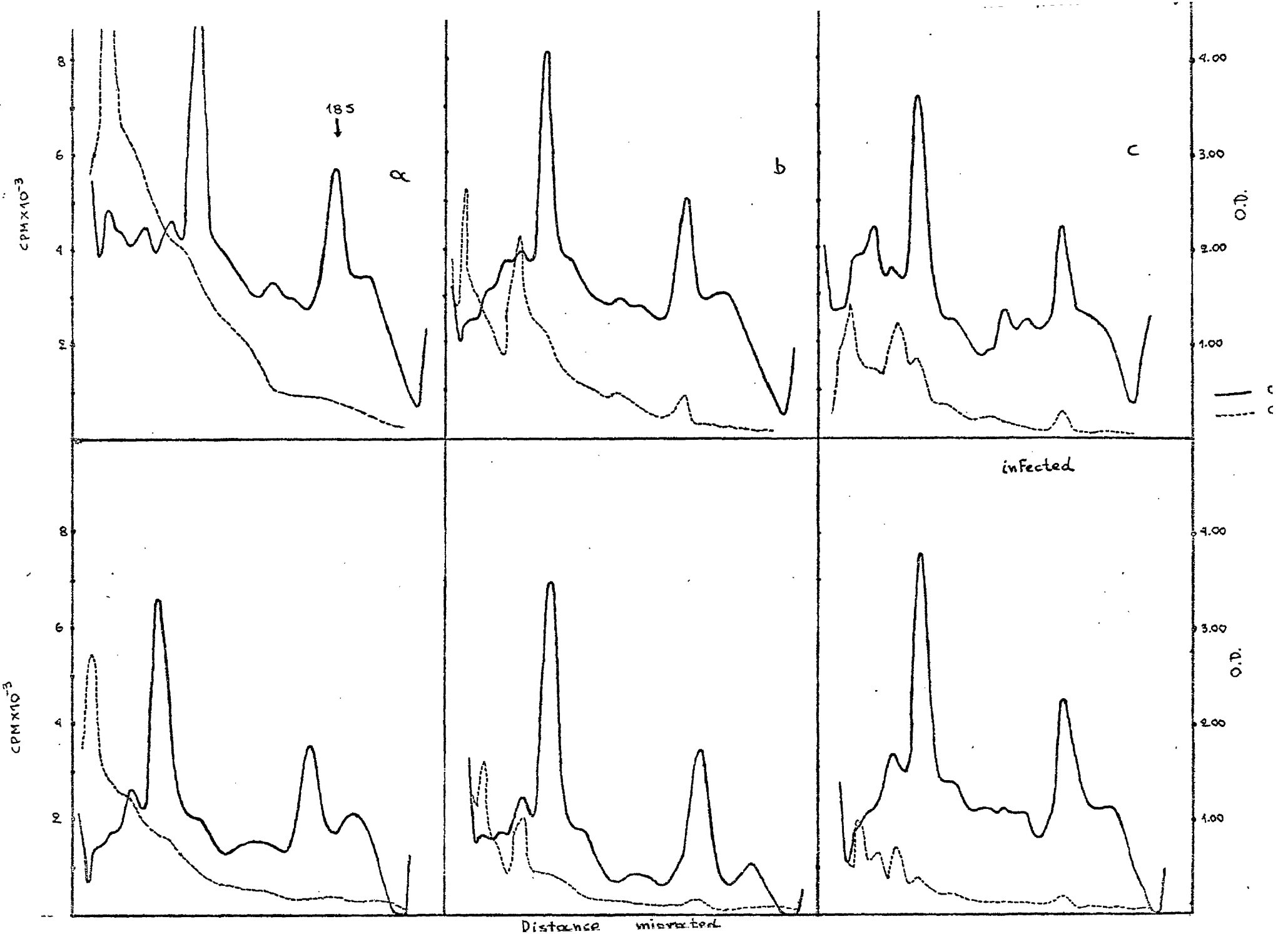
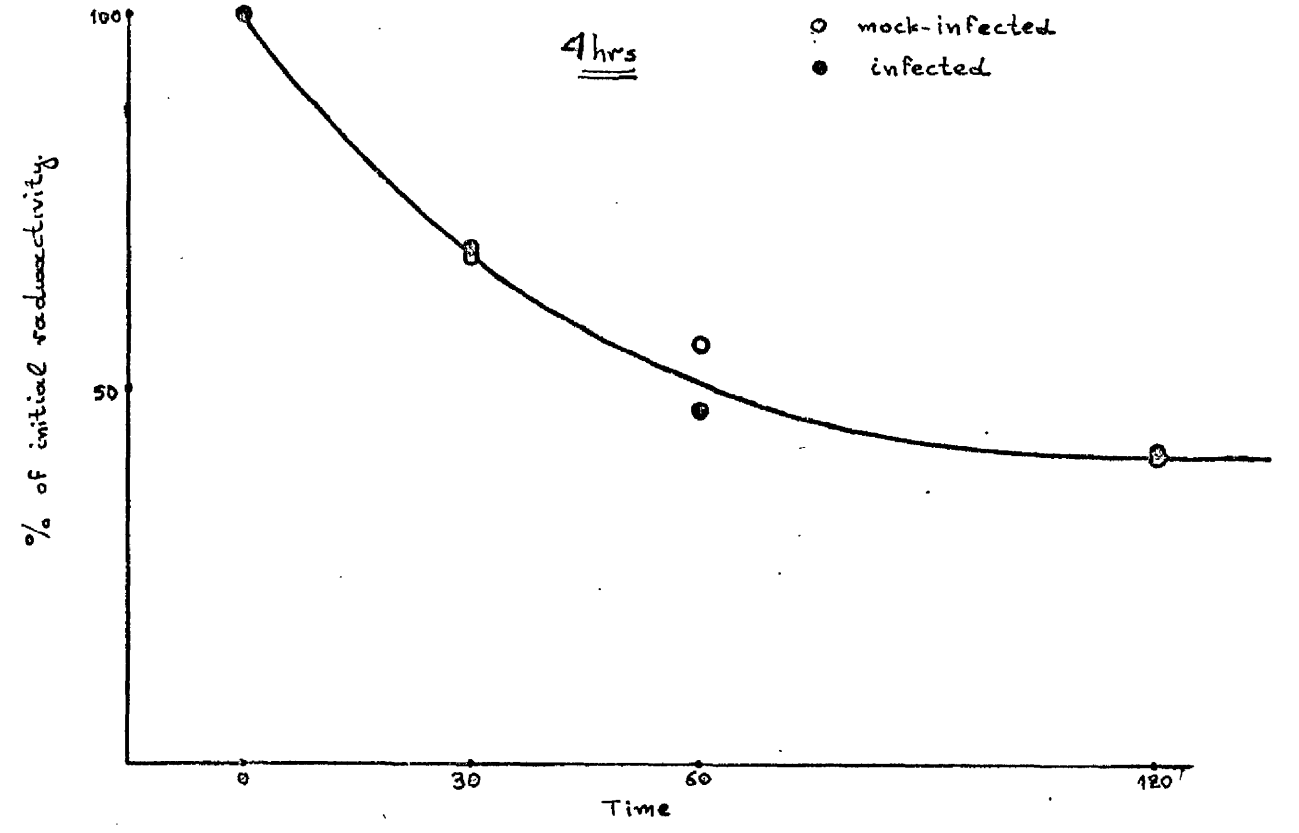

18S
a
b
c
CPM X10⁻³
O.D.
8
6
4
2
4.00
3.00
2.00
1.00
infected
Distance migrated

Fig. 5.3.3. Total radioactivity of individual ribosomal RNA species during the chase.

The sum of the radioactivities of the four main ribosomal RNA species was determined from the radioactivity profiles of figures 5.3.1. and 5.3.2.


4hrs
mock-infected
infected
100
50
% of initial radioactivity
0
30
60
120
Time

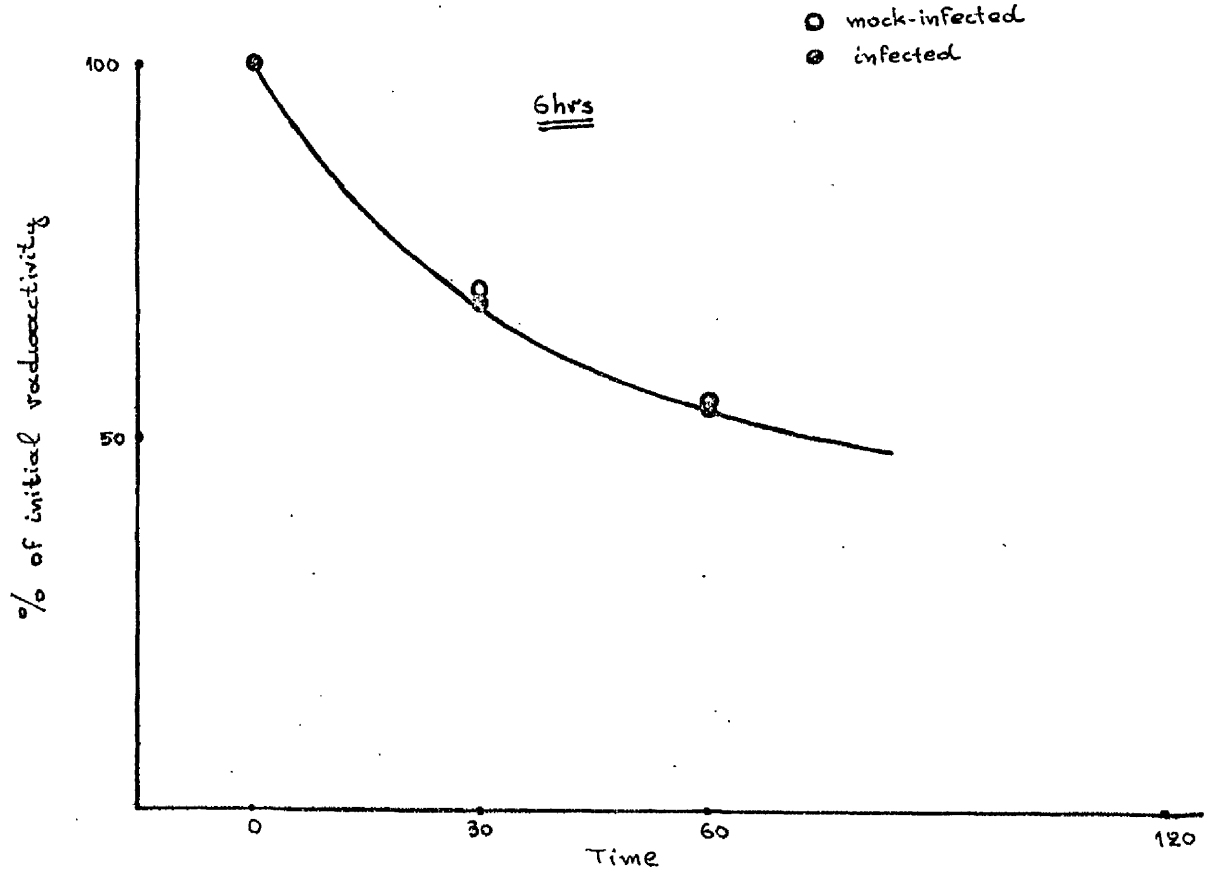
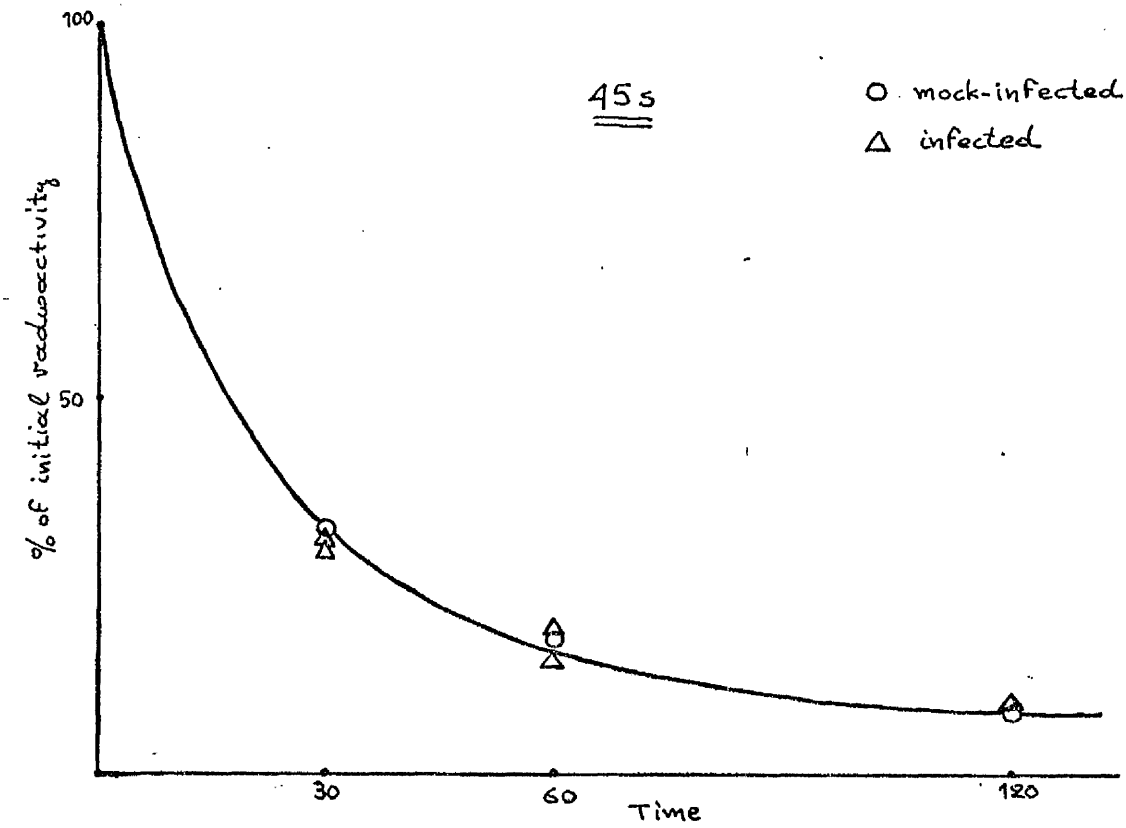

6hrs
mock-infected
infected
100
50
% of initial radioactivity
0
30
60
120
Time

Fig. 5.3.4. Radioactivity of 45s and 32s rRNA during the chase.

The radioactivities of 45s and 32s rRNAs were determined from Figs. 5.3.1 and 5.3.2.


45s
O mock-infected
Δ infected
100
50
% of initial radioactivity
30
60
120
Time

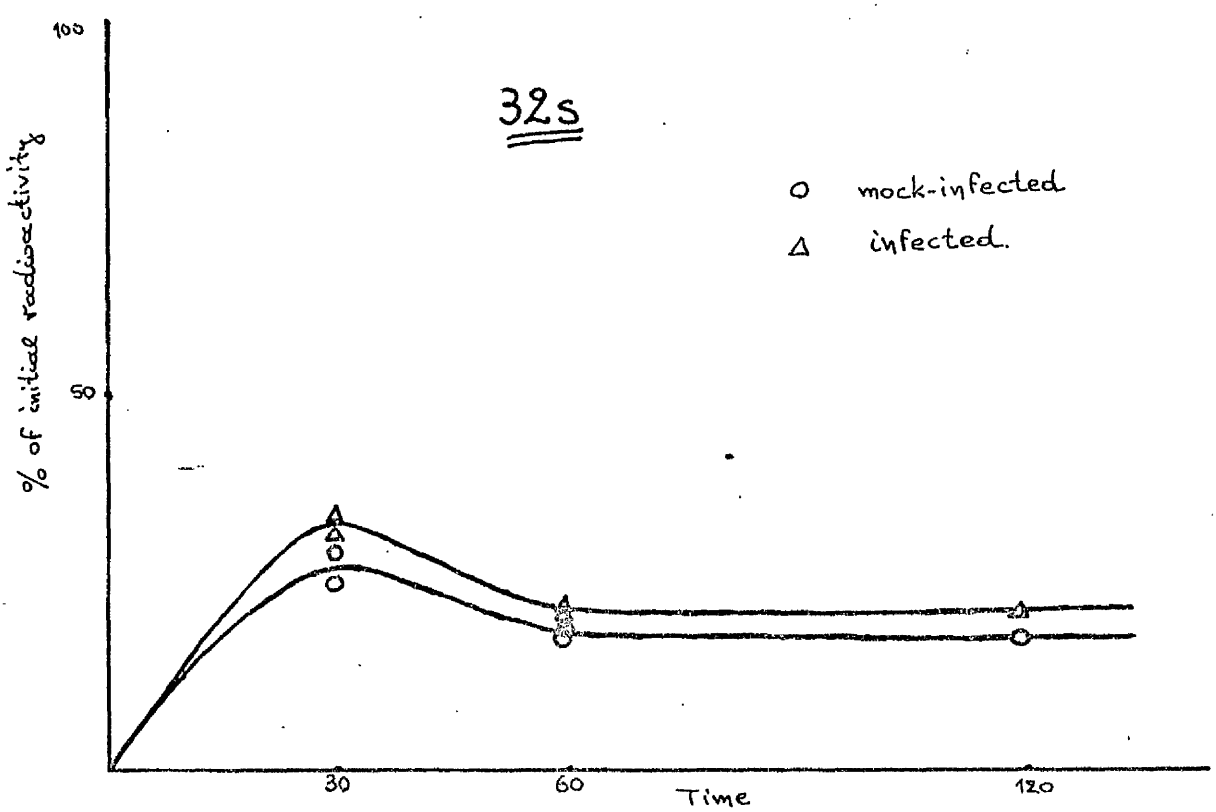
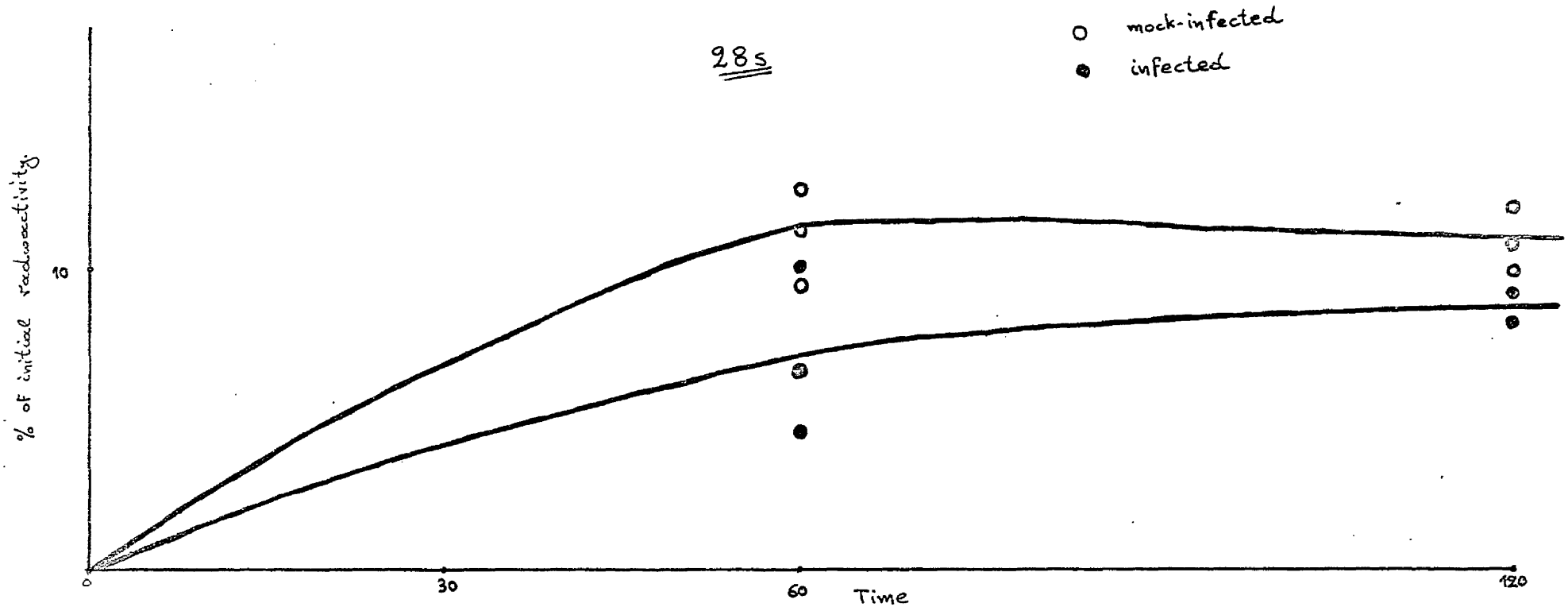

32s
O mock-infected
Δ infected.
100
50
% of initial radioactivity
30
60
120
Time

Fig. 5.3.5. Radioactivity of 28s and 18s rRNA during the chase.

The radioactivities of 28s and 18s rRNAs were determined from Figs. 5.3.1. and 5.3.2.


28s
mock-infected
infected
% of initial radioactivity.
10
0
30
60
120
Time

% of initial reoviocentivity
10
8
0
30
60
120
Time
18s
o mock-infected
● infected

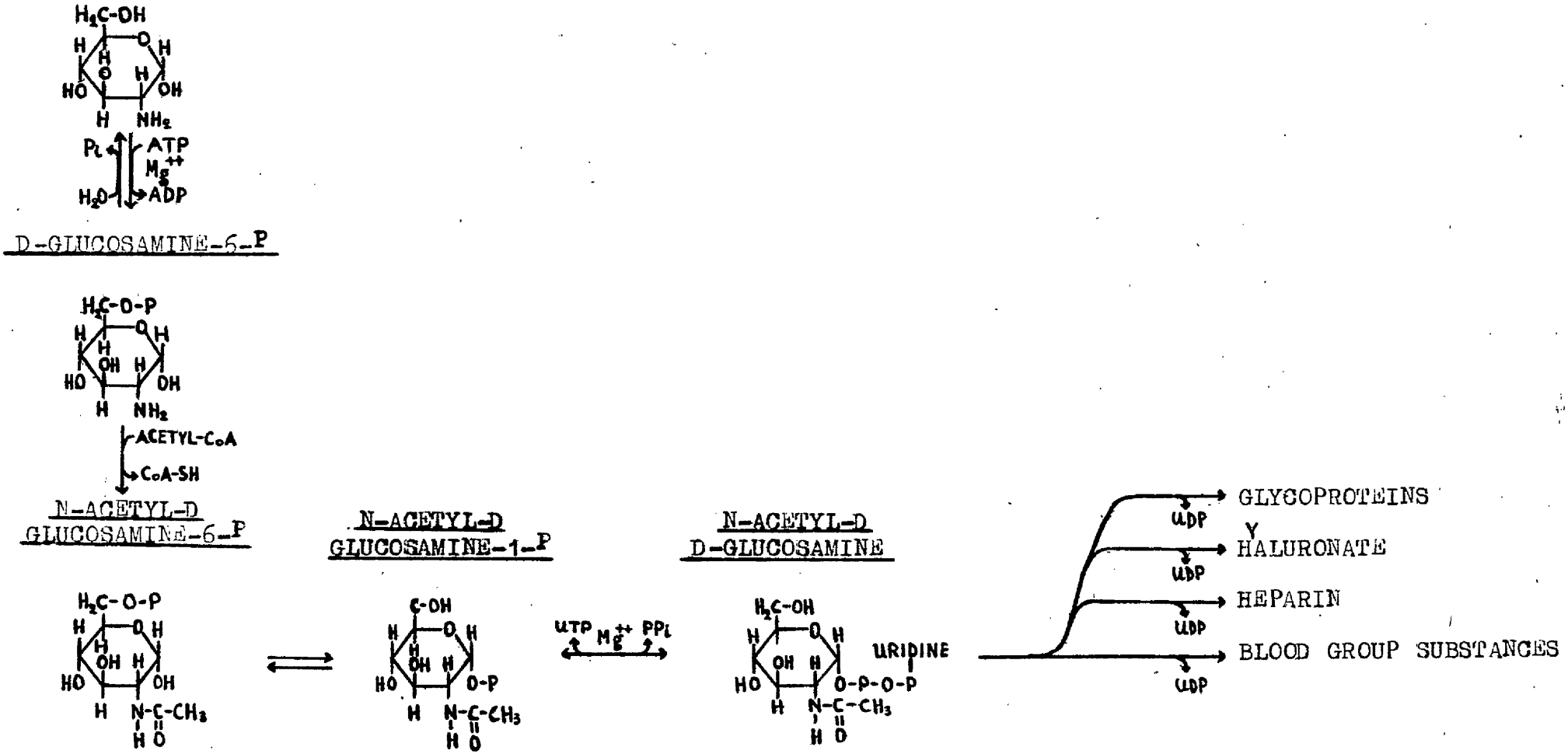


Figure 5.4.1 Schematic representation of D-glucosamine uridine interaction.

The decline of total radioactivity during the chase follows a similar pattern for both mock-infected and infected at 4 hrs and 6 hrs.

It has been found that the ratio of the optical density of the two stable ribosomal RNAs, 28s and 18s, appears to be the same in all of the cells the ratio being 28s: 18s= 1 : 0.5. In addition it has been shown in this laboratory that there is no degradation of cytoplasmic rRNA at least in the first six hours of infection.

The decline of radioactivity in 45s pre-rRNA and 32s pre-rRNA (Fig. 5.3.4) was expressed as a percentage of the initial amount of radioactivity under the 45s pre-rRNA peak at 15 min pulse. No difference could be found between mock-infected and infected Hela cells for 45s pre-rRNA. The 32 pre-rRNA radioactivity was slightly increased in the infected cells.

In a similar way 18s and 28s (Fig. 5.3.5) radioactivity was calculated during the chase. There is a decrease in the radioactivity accumulated into 28s and 18s rRNA's in the infected cells relative to the radioactivity in the mock-infected cells, i.e. whereas 18s accumulated 7% of initial radioactivity of 45s pre-rRNA in mock-infected Hela cells, it accumulated about 4% in the infected Hela cells. The corresponding figures for 28s rRNA were 11% and 8% respectively.

5.4. Discussion

From the results presented in this chapter the processing of the newly synthesized ribosomal RNA appears to follow a similar pattern in the pseudorabies virus infected Hela cells

to that of the mock-infected cells.

The glucosamine chase was employed in these experiments as an alternative to the actinomycin chase because it does not affect directly RNA synthesis as actinomycin does, but affects the UTP pool size by trapping UTP in the form of UDP- N-acetyl - D- glucosamine (Fig. 5.4.1) which is later released. The glucosamine method was used to minimize possible side effects which could hinder the assesment of the processing taking place during infection.

The degradation of the newly synthesized 45s pre-rRNA into oligonucleotides with s values less than 18s which was mentioned in the introduction to this chapter does not appear to occur in the pseudorabies virus infected cells. The decline of the initial pulse of radioactivity into the newly synthesized 45s pre-rRNA appears to be the same after the chase for both mock-infected and infected Hela cells, although there is an indication that processing is slowed down to some extent. This could be seen from the decrease in percentages of the initial radioactivity of 45s pre-rRNA which is later found in 18s and 28s of the infected cell.

CHAPTER 6. On the association of newly sunthesized proteins with the nuclei of Hela cells infected with PrV.

6.1. Introduction

Herpesviruses induce quantitative and qualitative changes in protein metabolism in the infected cell, as described in the Introduction. Thus the rate of protein synthesis drops soon after infection, rises for a short period and finally and irreversibly declines in late infection. Qualitatively the synthesis of most of the host identified polypeptides is abolished after infection and the synthesis of new polypeptides identified as viral-specific is induced. It has been suggested that about 50 new polypeptides are induced after infection with HSV-1 (Honess and Roizman, 1973; Powell & Courtney, 1975; Marsden et al., 1976). Whereas Haarr and Marsden (1981) detected about two hundred and thirty virus-induced polypeptides in BHK cells infected with HSV-1 by means of two dimensional gel electrophoresis. Some polypeptides, visualised as a single band on a one dimensional SDS-polyacrylamide gel, were resolved into several spots.

Most of the newly synthesized, viral-induced polypeptides migrate to the nucleus of the infected cells (Fujiwara & Kaplan 1967). Thus the major structural polypeptides have been shown to migrate to the nucleus (Courtney et al., 1971; Mark & Kaplan, 1971) where they are assembled into virus nucleocapsids (Olshevsky et al., 1967; Spear and Roizman, 1968; Ben-Porat et al., 1969) while other newly made polypeptides remain in the cytoplasm (Mc Combs, 1974; Fenwick et al., 1978). All of the immediate early polypeptides are transferred to the nucleus

(Pereira et al., 1977).

Furthermore, it has been suggested that viral induced nuclear polypeptides show affinities for various constituents of host cell nuclei which are likely to determine their nuclear accumulation (Fenwick et al., 1978) and therefore their possible function. Indeed in BHK 21 cells which were infected with pseudorabies virus, 2 viral acid-soluble polypeptides were found to be associated with isolated chromatin (Chantler and Stevely, 1973) and a number of HSV-1 and HSV-2 specific, DNA-binding proteins have been identified by affinity chromatography on columns containing cellular DNA (Bayliss al., 1975; Powell & Purifoy, 1976; Purifoy & Powell, 1976). Immediate early proteins of HSV-1 and HSV-2, bind with a range of affinities to native calf thymus DNA in vitro and to chromatin from infected cells (Hay & Hay, 1980).

A further demonstration of the affinity of a viral induced polypeptide for a nuclear component is in the report of the association of ICP 4 of HSV-1 with an intranuclear body resembling the nucleolus at late times after infection. This protein was not found in association with nucleocapsids or enveloped particles (Cabral et al., 1980).

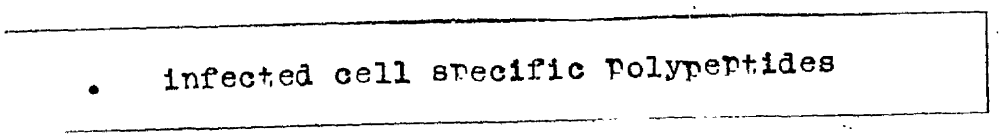
6.2. Association of newly synthesised proteins with the Nuclei of Hela cells infected with pseudorabies virus.

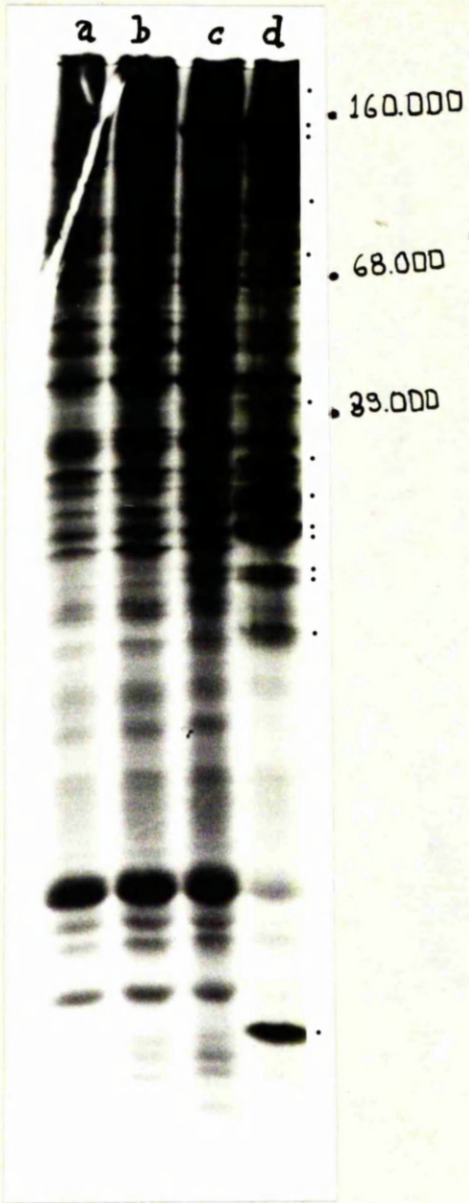
Hela cells grown in 80oz roller bottles, infected with pseudorabies virus at a multiplicity of 50 p.f.u. per cell and labelled with ^{35}S-methionine for 2hrs before being harvested at 2hrs, 4hrs and 6hrs after infection. Nuclei were isolated, treated with SDS, and subjected to electrophoresis in SDS-

Fig. 6.2.1. Polyacrylamide gel electrophoresis of mock-infected and infected Hela cell nuclear proteins.

Hela cells grown in 80oz roller bottles were mock-infected or infected with pseudorabies virus at 50 p.f.u. per cell and labelled with 10μCi/ml ^{35}S-methionine for 2 hours before harvesting at 2 hrs, 4 hrs and 6 hrs after infection. Nuclei were isolated, dissolved in SDS-buffer and the solution was subjected to electrophoresis in 12.5% acrylamide gels as described in Materials and Methods. Molecular weight markers were run in parallel lanes.

Track a	mock-infected
Track b	2 hrs infected
Track c	4 hrs infected
Track d	6 hrs infected




a b c d
160.000
68.000
39.000

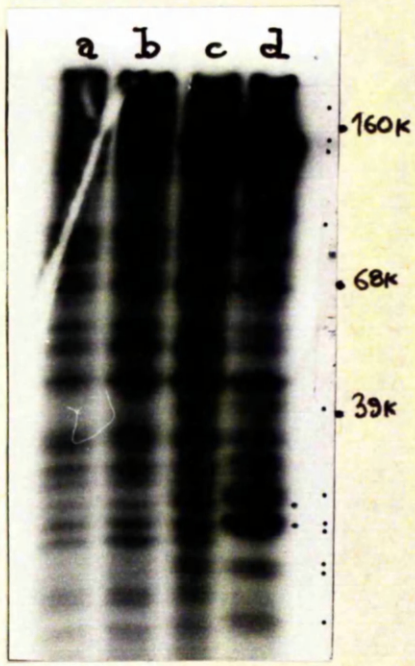

a b c d
160K
68K
39K

Table 6.2.1. Approximate molecular weights of nuclear viral induced polypeptides.

Approximate molecular weights of viral induced polypeptides at 2 hrs, 4 hrs and 6 hrs, were determined from Fig. 6.2.1.

Nuclear Proteins.

0-2h		2h-4h		4h-6h	
~200k	(1)	~200k	(1)	~200k	(1)
		135k	(2)	135k	(2)
		130k	(3)	130k	(3)
		87k	(4)	87k	(4)
				69k	(5)
42k	(6)	42k	(6)	42k	(6)
				36k	(7)
				33k	(8)
				31.5k	(9)
				30k	(10)
29k	(11)	29k	(11)	29k	(11)
28k	(12)	28k	(12)	28k	(12)
				26k	(13)
				13.5k	(14)

SDS-acrylamide thin layer slab gels, as described in Materials and Methods. Gels were dried and exposed to X-ray film.

About 14 new polypeptide bands appear in the nuclear fraction of infected cells, mostly at 4hrs to 6hrs after infection (Fig. 6.2.1, slot 4). About half of the bands appear at 2hrs to 4hrs (Fig. 6.2.1, slot 3) and 3 to 4 at 0hrs to 2hrs (Fig. 6.2.1, slot 2) after infection. The range of themolecular weights of the newly synthesized polypeptides is quite wide ranging from low molecular weight 13.5 K to the ~200 K high molecular weight band (Table 6.2.1). Moreover a number of host polypeptides cease to appear at late times after infection, particulary the histones and low molecular weight polypeptides whereas there are other host polypeptide bands which appear in all of the samples.

6.3. Association of newly synthesized proteins with the nucleoli of Hela cells infected with pseudorabies virus.

Hela cells grown in 80oz roller bottles were infected with PrV , labelled with ^{35}S-methionine and the nuclei isolated as described above. Nucleoli were further isolated from the nuclear fraction by sonication and after treatment with SDS were subjected to electrophoresis in 9% or 12.5% acrylamide thin layer slab gels in SDS. Gels were dried under vacuum and exposed to X-Ray film as described above.

The autoradiographs produced (Fig. 6.3.1) show at all times a similar distribution of polypeptides to these obtained for the nuclear fraction but the number of polypeptides (Fig. 6.3.1, slot 3) that appear at 6hrs after infection is fewer than for the corresponding nuclear fraction, being

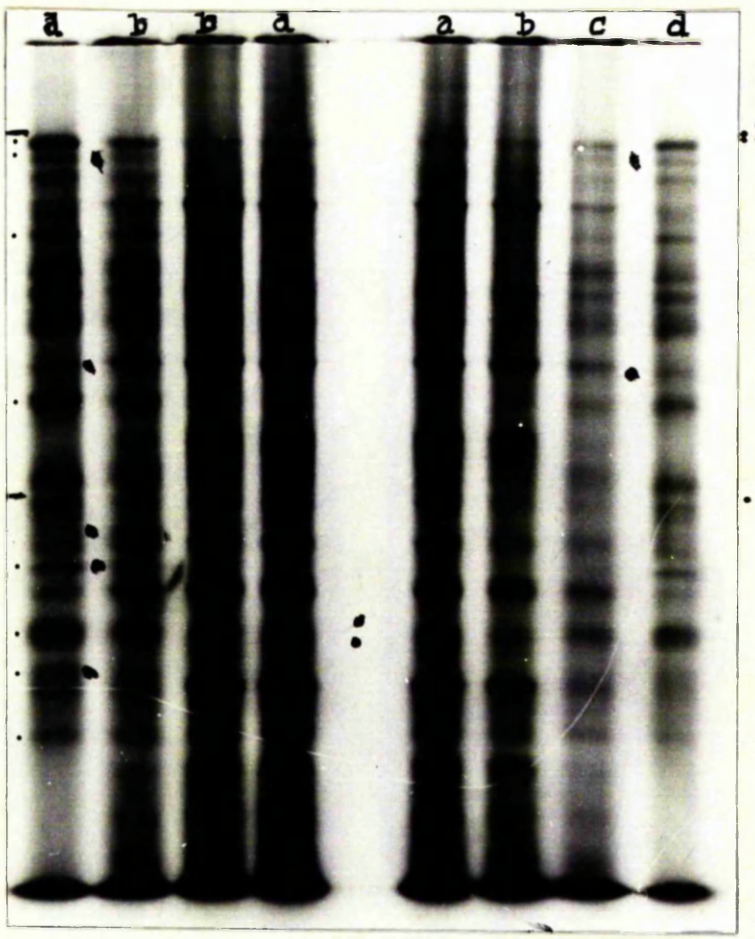
Fig. 6.3.1. Polyacrylamide gel electrophoresis of mock-infected and infected Hela cell nucleolar proteins.

Hela cells grown in 80oz roller bottles were mock-infected or infected with pseudorabies virus at 50 pfu per cell and labelled with 10μCi/ml ^{35}S-methionine for 2 hours before harvesting at 2 hrs, 4 hrs and 6 hrs after infection. Nucleoli were isolated by sonication as described in Materials and Methods and the proteins subjected to electrophoresis as for Fig. 6.2.1.

Gel A		Gel B	
Track a	mock-infected	Track a	mock-infected
Track b	2 hrs infected	Track b	6 hrs infecte
Track c	4 hrs infected		
Track d	6 hrs infected		

• infected cell specific polypeptides

Gel A



Gel B

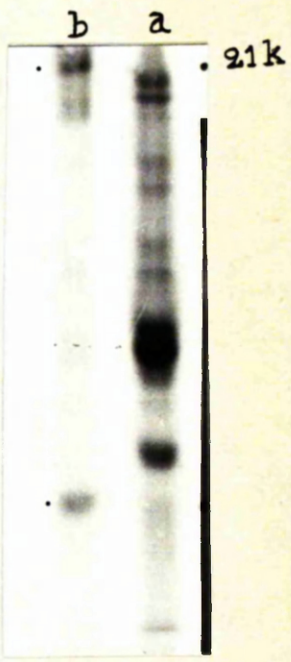


Table 6.3.1. Approximate molecular weights of nucleolar viral Induced polypeptides.

Approximate molecular weights of viral induced polypeptides at 2 hrs, 4 hrs and 6 hrs were determined from Fig. 6.3.1.

Nucleolar Proteins.

2h-4h		4h-6h		Cycloheximide treated	
				(÷200k)	(1)
135k	(2)	135k	(2)	135k	(2)
130k	(3)	130k	(3)	130k	(3)
87k	(4)	87k	(4)	87k	(4)
42k	(6)	42k	(6)	42k	(6)
36k	(7)	36k	(7)		
		33k	(8)	33k	(8)
29k	(11)	29k	(11)	29k	(11)
28k	(12)	28k	(12)	28k	(12)
		26k	(13)		
		13.5k	(14)		

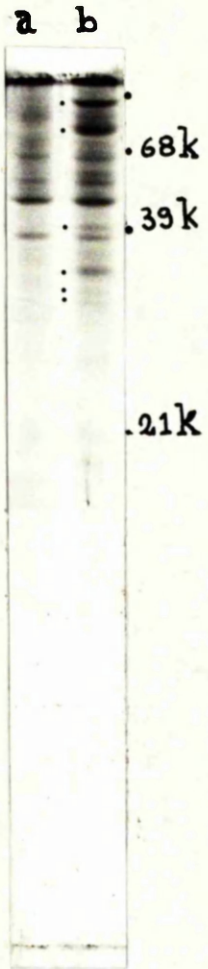
Fig. 6. 3. 2. Polyacrylamide gel electrophoresis of mock-infected and infected Hela cell nucleolar proteins after treatment with cycloheximide.

Hela cells grown in 80oz roller bottles were mock-infected or infected with pseudorabies virus at 50 p.f.u. per cell. Immediately after infection cycloheximide was added to the medium at a concentration of 50µg/ml for 4 hrs. Then medium was decanted and cells were labelled with 30µCi/ml ^{35}S-methionine for 1 hr. Nucleoli were isolated and their proteins subjected to electrophoresis in 9% and 15% acrylamide gels as described in Fig. 6.3.1.

Gel A		Gel B	
Track a	mock-infected	Track a	mock-infect
Track b	infected	Track b	infected

infected cell specific polypeptides

Gel A



Gel B

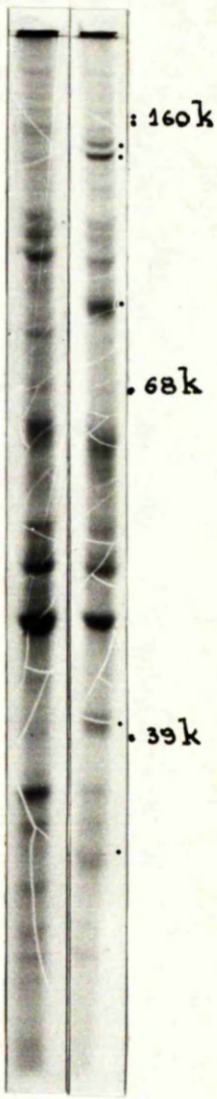


Fig. 6.3.3. Two-dimensional polyacrylamide gel electrophoresis of mock-infected and infected Hela cell nucleoli.

Hela cells grown in 80oz roller bottles, were mock-infected or infected with pseudorabies virus at 50 p.f.u. per cell and labelled with 50 μCi/ml ^{35}S-methionine, from 1 hr to 6 hrs postinfection. The nucleoli were isolated by sonication and their proteins subjected to two-dimensional electrophoresis as described in Materials and Methods.

Gel A — mock-infected

Gel B — infected.

/ major cell specific polypeptides

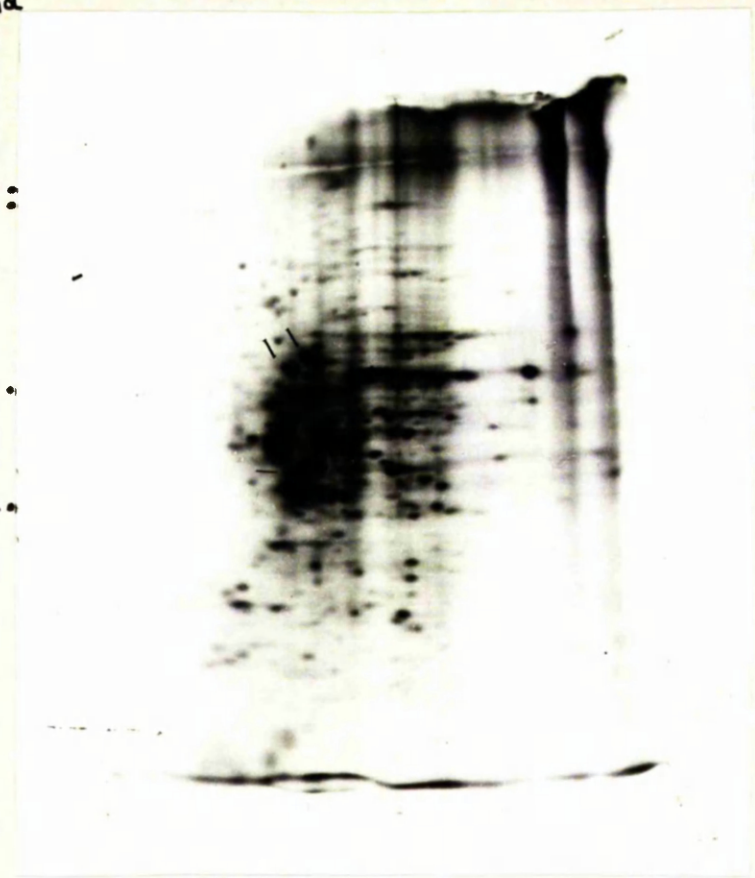
\ major infected cell specific polypeptides

\ medium infected cell specific polypeptides

Gel A

Acidic end

Basic end



Gel B

Acidic end

Basic end

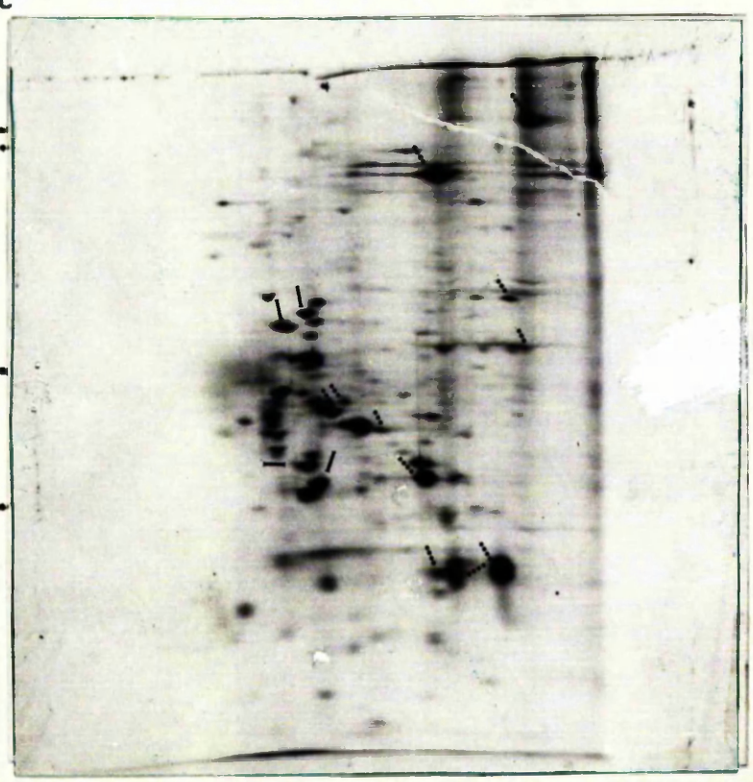


Table 6.3.2. Approximate molecular weights of nucleolar viral induced polypeptides as resolved by 2-D gel electrophoresis.

Approximate molecular weights were determined from Fig. 6.3.3.

Polypeptides in 2-D gels of nucleolar proteins.

o	~200k	(1)	●	59k	(15)
	135k	(2)	o	52k	(16)
●	130k	(3)		49k	(17)
	123k	(4)		42k	(18)
	120k	(5)	●	42k	(19)
	112k	(6)		40k	(20)
	112k	(7)		36k	(21)
o	87k	(8)	●	33k	(22)
o	87k	(9)	●	33k	(23)
	72k	(10)	●	31k	(24)
	72k	(11)	●	31k	(25)
o	72k	(12)		26k	(26)
	72k	(13)		23k	(27)
o	60k	(14)		20k	(28)

● intense

o medium

about 10 polypeptide bands. (Table 6.3.1).

In other experiments Hela cells grown in 80oz roller bottles infected and mock-infected with pseudorabies virus were incubated in the presence of cycloheximide for 4hrs and labelled with ^{35}S-methionine for 1hr after cycloheximide release. They were harvested as described in Materials and Methods. Samples were applied to wells in 9% and 12.5% acrylamide thin layer slab gels in SDS. After electrophoresis the gels were dried and autoradiographed (Fig. 6.3.2). About 8 new polypeptide bands appear in the infected cells after this treatment with cycloheximide. (Table 6.3.1).

Nucleolar extracts were further subjected to two-dimensional gel electrophoresis. Nucleoli were isolated from Hela cells mock-infected and infected with pseudorabies virus and further labelled with ^{35}S-methionine for a time period of 6hrs and then harvested.

Two-dimensional gel elecrophoresis was performed according to the method of O'Farrell as described in Materials and Methods. The autoradiographs produced (Fig. 6.3.3) show a number of new minor polypeptide spots in the infected cells and some of the major polypeptide spots which appear at the one dimensional acrylamide gels. (Table 6.3.2).

6.4. Association of newly synthesized proteins with the nucleolar chromatin fraction of Hela cells infected with pseudorabies virus.

Hela cells were mock-infected and infected with pseudorabies virus, labelled with ^{35}S-methionine for a 2hr time period from 4hrs to 6hrs postinfection. They were

Fig. 6.4.1. Polyacrylamide gel electrophoresis of mock-infect and infected Hela cell nucleolar chromosomal proteins.

Hela cells were grown in 80oz roller bottles and mock-infected or infected with pseudorabies virus at 50 p.f.u. per cell. Cells were labelled with 5μCi/ml ^{35}S-methionine for 2 hrs before harvesting at 6 hrs postinfection. Nucleolar chromatin fractions were isolated and their proteins subjected tc electrophoresis at 9% and 15% gels as described in Materials and Methods.

Gel A		Gel B	
Track a	mock-infected	Track a	mock-infecte
Track b	infected	Track b	infected.

infected cell specific polypeptides

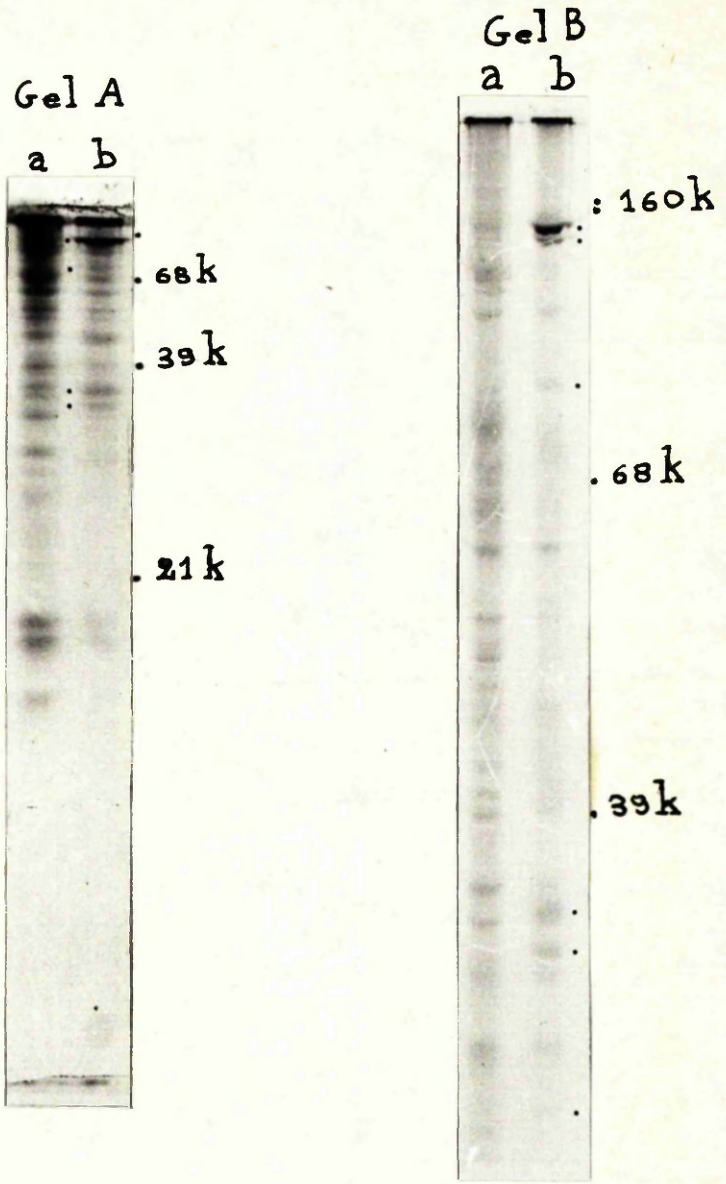

Gel A
a b
68k
39k
21k
Gel B
a b
160k
68k
39k

Table 6.4.1. Approximate molecular weights of nucleolar chromatin viral induced polypeptides.

Approximate molecular weights of viral induced polypeptides present in nucleolar chromatin were determined from Fig. 6.4.1.

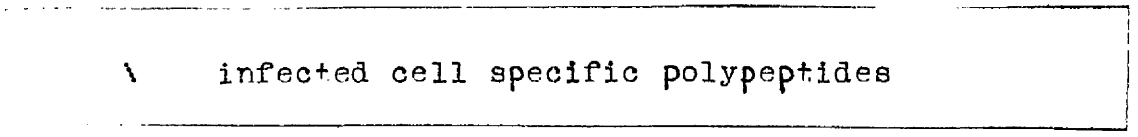
Nucleolar Chromatin Proteins
135k (2)
130k (3)
87k (4)
36k (7)
33k (8)
26k (13)

Fig. 6.4.2. Non-equilibrium polyacrylamide gel electrophoresis (NEPHGE) of nucleolar chromosomal proteins of mock-infected and infected Hela cells.

Nucleolar chromatin fractions from Hela cells prepared as for Fig. 6.4.1. were subjected to non-equilibrium isolelectring focusing as described in Materials and Methods.

Gel A — Mock-infected

Gel B — Infected.



Acidic end.

Basic end.

68K

21.5K

Gel B

Acidic end.

Basic end

68K

21.5K

harvested and the nucleolar chromatin fraction was extracted from isolated nucleoli.

Nucleolar chromatin extracts were subjected to one dimensional gel electrophoresis and two-dimensional non-equilibrium (NEPHGE) gel electrophoresis as described in Materials and Methods.

It can be seen from Fig. 6.4.1 that about 6 new polypeptides appear in the infected cells (Table 6.4.1). At least three of these appear to be basic proteins. (Fig. 6.4.2).

6.5. Uptake of mock-infected and PrV-infected polypeptides by isolated Hela cell nuclei in vitro.

Fenwick et al., (1978) showed that HSV-1 virus labelled polypeptides could be taken up by nuclei in vitro. A similar attempt was made to determine whether pseudorabies virus polypeptides could be taken into, by isolated nuclei in vitro. Thus unlabelled crude nuclei from both mock-infected and infected Hela cells were incubated in cytoplasmic preparations obtained from labelled infected or mock-infected Hela cells. The nuclei were then collected, washed with detergents, lysed and examined for the presence of radioactive virus polypeptides.. 15% to 20% of the total counts in the incubation mixture remained in the nuclei after treatment with detergent. Subsequent treatments with NaCl and SDS reduced the number of counts attached to the nuclei. (Table 6.5.1) Gel electrophoresis of the washed nuclei revealed that most of the polypeptide bands of infected cells were taken up by the isolated nuclei with a few exceptions (Fig. 6.5.1.). Nuclei incubated in cytoplasmic preparation from cycloheximide treated mock-infected and infected Hela cells show again all of the

Table 6.5.1. Uptake of radioactivity by unlabelled mock-infecte or infected Hela cell nuclei incubated with radioactively labelled mock-infected and infected Hela cell extracts.

Hela cells harvested at 5 hrs after infection or mock-infection were suspended in 1 mM PO_4 pH 7.4. and homogenised. Sucrose was added to give an 0.25 M concentration and the suspension wasspun at 1500 rpm for 10 min. The pellet is crude nuclei and the supernatant is "cytoplasm". 1 ml of appropiate cytoplasm labelled with ^{3}H- or ^{35}S-methionine was added to the pellet of nuclei, and mixed gently and incubated at 37°C for 30 min. The suspension was spun at 1500 rpm for 10 min and the ensuing pellet of nuclei was washed three times with 1% Tween 80, extracted with 0.35 M NaCl and the residue was dissolved in 2% SDS, 1% B-mercaptoethanol. Radioactivity was determined as described in Materials and Methods.

Incubation	Total Counts x 10^{-4} in incubation	Counts x 10^{-4} in supernatant after incubation	Counts x 10^{-4} in in Tween washes	Counts x 10^{-4} in NaCl	Counts x 10^{-4} in SDS	% in NaCl + SDS
Mock-infected nuclei + Mock-infected cytoplasm	653 (100)	411 (63)	97 (15)	40 (6)	25 (4)	10
Mock infected nuclei + Infected cytoplasm	395 (100)	288 (73)	59 (15)	28 (7)	27 (7)	14
Infected nuclei + Mock-infected cytoplasm	537 (100)	374 (70)	102 (19)	43 (8)	17 (3)	11
Infected nuclei + Mock-infected cytoplasm	419 (100)	292 (70)	86 (20)	26 (6)	22 (5)	11

Bracketed numbers are % of Total Counts in the incubations.

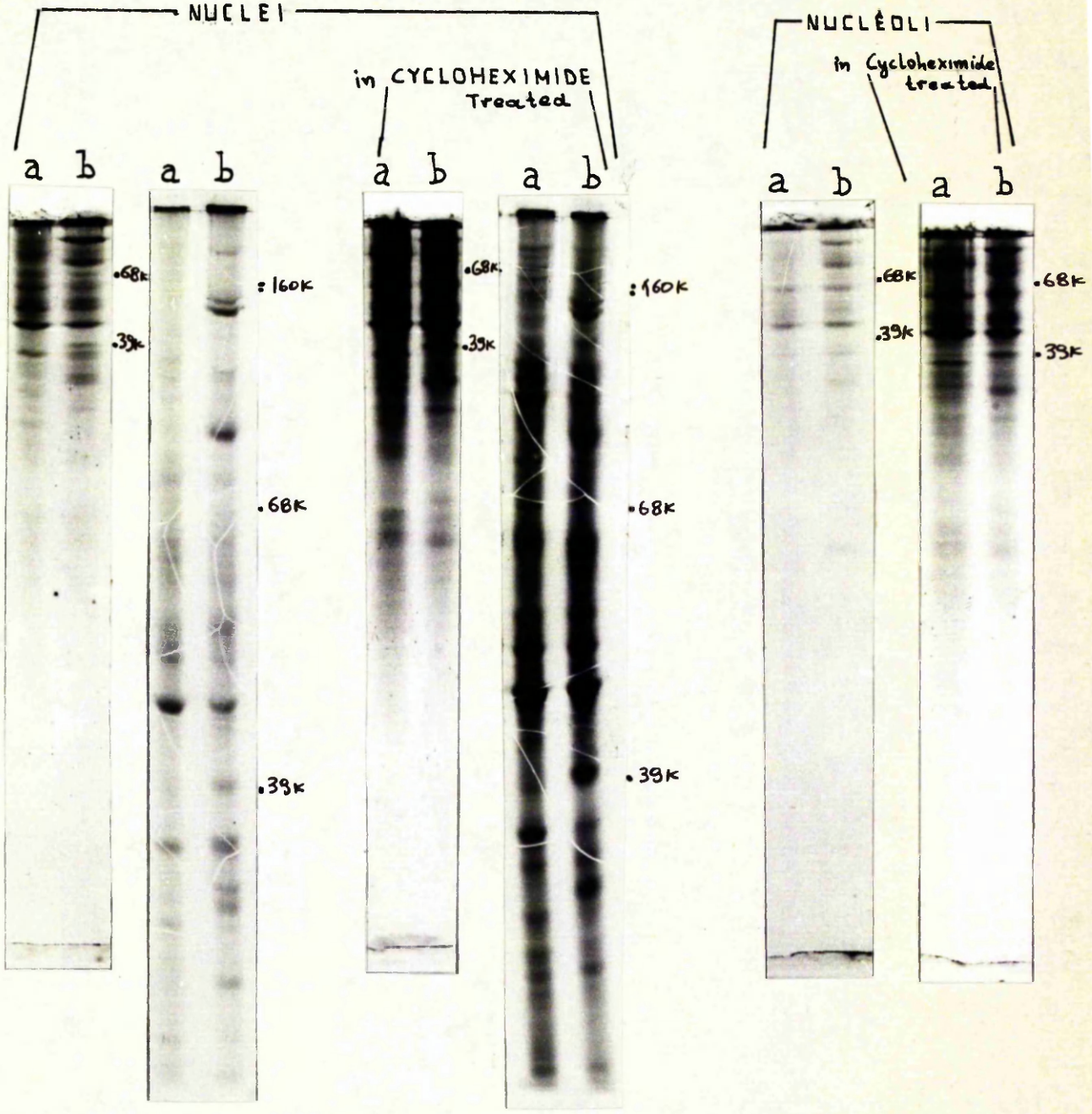
Fig. 6.5.1. Polyacrylamide gel electrophoresis of polypeptides from unlabelled uninfected nuclei and nucleoli incubated in the presence of radioactively labelled mock-infected and infected Hela cell Cytoplasmic extracts.

Hela cells were grown in 80oz roller bottles to confluency. Nuclei were isolated, after the cells were allowed to swell in RSB and homogenized in a stainless steel Dounce homogenizer. These crude nuclei were icubated with cytoplasmic extracts from mock-infected and infected Hela cells which had been labelled with ^{35}S-methionine.

Incubation was carried out at 30^{o} for a period of 30 min. Nuclei in the incubation mixture were pelleted and cleaned by treatment with mixed detergent as described in Materials and Methods. Nucleoli were prepared by sonication as described in Materials and Methods. The proteins of nuclei and nucleoli thus prepared were subjected to electrophoresis in 9% and 15% acrylamide gels as described in Materials and Methods.

Track a in mock-infected

b in infected


NUCLEI
in CYCLOHEXIMIDE Treated
NUCLÉOLI
in Cycloheximide treated
a b a b a b a b a b a b
160K
68K
39K
68K
39K
160K
68K
39K
68K
39K
68K
39K

polypeptides that appear in vivo. (Fig. 6.5.1).

Nucleoli were further isolated from the incubated nuclei and examined for radioactive virus polypeptides. Again most of the polypeptides found in vivo appear to have been taken up during the incubations in the cytoplasmic extracts. (Fig. 6.5.1).

6.6. Chase of ^{35}S-methionine in nuclear and nucleolar proteins of Hela cells after infection with pseudorabies virus.

Hela cells grown in 80oz roller bottles, were labelled with ^{35}S-methionine overnight and chased afterwards with a 400-fold excess cold methionine when the cells were infected or mock-infected with pseudorabies virus. At 2hrs, 4hrs and 6hrs after infection cells were harvested and nuclei were isolated and subjected to electrophoresis in acrylamide gels as described in Materials and Methods.

Figures 6.6.1 and 6.6.2 show the nuclear and nucleolar polypeptide pattern to be virtually identical for both mock-infected and infected and even the histones which cease to be synthesized after infection retain their label as late as 6hrs after infection at a time when synthesis of them practically ceases.

Fig. 6.6.1. Polyacrylamide gel electrophoresis of nuclei from mock-infected or infected Hela cells radioactively labelled before infection.

Hela cells grown in 80oz roller bottles were labelled with 10 μCi/ml of ^{35}S-methionine in $^{1}/_{5}$ the normal concentrati of methionine in the medium. The cells were subsequently mock-infected or infected with pseudorabies virus at 50 p.f.u. per cell and the label was chased with a 400-fold excess of cold methionine during infection. Nuclei were isolated and their proteins subjected to electrophoresis as for Fig. 6.2.1.

Track a mock-infected

b 2 hrs infected

c 4 hrs infected

d 6 hrs infected

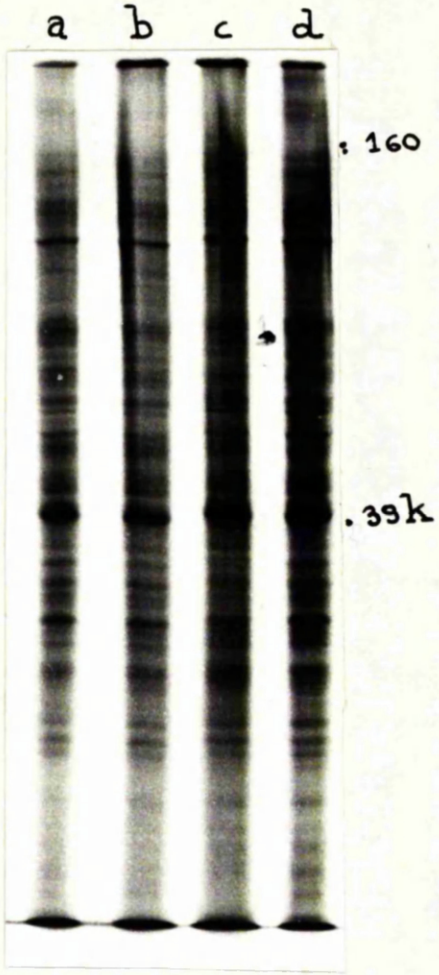
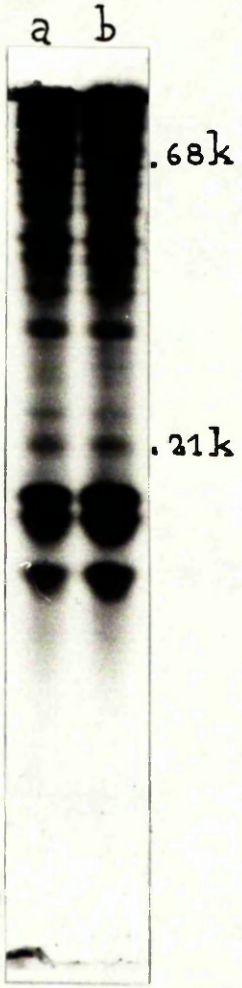

a
b
c
d
160
39k

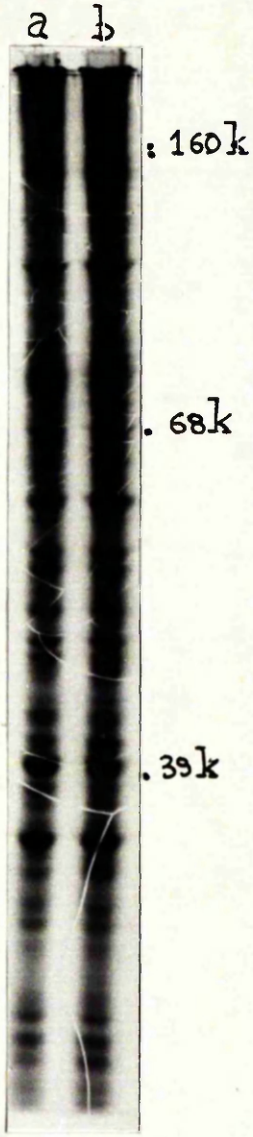
Fig. 6.6.2. Polyacrylamide gel electrophoresis of nucleoli from mock-infected or infected Hela cells radioactively labelled before infection.

Nucleoli were isolated by sonication from Hela cells prepared as for Fig. 6.6.1. and their proteins were subjected to electrophoresis in 9% and 12.5% acrylamide gels as described in Materials and Methods.

Track a mock-infected

b infected


a b
.68k
.21k


a b
.160k
.68k
.39k

6.7. Discussion.

From the results presented in this chapter it is clear that viral-induced polypeptides migrate into the nucleus of Hela cells as early as 2hrs after infection, and the number of new polypeptides that appear into the nucleus increases as the infection proceeds.

Fewer polypeptides are isolated with the nucleoli, however the nucleolus does not appear to contain any viral-induced polypeptides which are absent from the rest of the nucleus.

About 28 viral-induced polypeptides were detected in nucleoli when two-dimensional gel electrophoresis was employed, as characterized by both relative mobility following iso electric focusing and apparent molecular weight in SDS-polyacrylamide gels.

The (relatively) large number of polypeptides detected is due to the additional resolution afforded by two-dimensional gel electrophoresis which separates viral-induced polypeptides that co-migrate in one-dimensional SDS-polyacrylamide gels with more predominant host cell polypeptides and also separates into several spots viral-induced polypeptides which are visualized as a single band in one-dimensional gels. These multiple spots at a single molecular weight may be related to modifications of single species such as phosphorylation, glycosylation (Haarr and Marsden, 1981) rather than distinct species.

Viral-induced polypeptides associated with the nucleolar chromatin fraction of Hela cells infected with pseudorabies virus number about six.

Almost all of these six viral-induced polypeptides are taken up by isolated nuclei of uninfected cells in vitro when incubated with cytoplasmic extracts of Hela cells infected with pseudorabies virus. Furthermore most of them appear to be basic as is illustrated by non-equilibrium two-dimensional SDS-polyacrylamide gel electrophoresis (NEPHGE).

Nucleoli isolated from cycloheximide treated Hela cells contain about eight viral-induced polypeptides which might include more than the immediate early species because the labelling time employed was relatively long. Polypeptides from the above cycloheximide treated nucleoli comprise four of the six viral-induced polypeptides isolated with the nucleolar chromatin.

CHAPTER 7. DNA-dependent RNA polymerase activity in isolated nuclei of mock-infected and pseudorabies virus infected Hela cells.

7.1. Introduction.

That there is a decrease in ribosomal RNA synthesis after infection with herpesviruses has been suggested by the inhibition of incorporation of exogenous uridine into cellular RNA (Wagner and Roizman, 1969; Flanagan, 1964; Hay et al., 1966).

A detailed analysis of this process in whole cells is hindered by the complexity of the uptake and metabolism of nucleoside triphosphates in cultured cells (Plageman, 1971 a, b), the possibility that there is a seperate nucleotide pool responsible for ribosomal RNA synthesis (Wiegers et al., 1976) and also by the effects of herpesviruses on other aspects of cellular metabolism, for example in protein synthesis.

The use of isolated nuclei to study transcription in infected cells provides a system in which RNA polymerase synthesis is almost completely dependent upon the addition of nucleoside triphosphates.

At least three DNA-dependent RNA polymerase activities (Blatti et al., 1970) have been detected by means of chromatography of nuclear extracts of eukaryotic cells. These are, RNA polymerase I located mainly in the nucleolus and RNA polymerases II and III which are mainly nucleoplasmic (Roeder and Rutter, 1970). The fungal toxin α-amanitin inhibits both RNA polymerase II and III at appropriate concentrations, whereas RNA-polymerase I is resistant to

inhibition by a-amanitin.

It has been demonstrated that isolated nuclei from HSV-infected cells show decreased endogenous activity of RNA polymerase (Preston and Newton, 1976) which might be mediated by an inhibitor found in the herpesvirus-infected cells. This inhibitor was suggested to preferentially inhibit the nucleolar enzyme and to act by stopping chain elongation (Sasaki et al., 1974.). Preston and Newton, 1976 further suggested that there was no transferable inhibitor of RNA synthesis detected when mixed nuclei of mock-infected and infected cells are assayed for RNA polymerase activity. However these conclusions are open to criticism (see General Discussion).

7.2. a-Amanitin resistant RNA polymerase activity in the nuclei from mock-infected and infected Hela cells.

It has been suggested in Chapter 4 that ribosomal RNA synthesis in Hela cells declines after infection with pseudorabies virus. However, the processing of 45s pre-rRNA is not greatly altered (Chapter 5). Because of the many problems involved in using the incorporation of ^{3}H-uridine as a measure of RNA synthesis it was decided to employ an assay of RNA polymerase activity in isolated nuclei to examine further the decline of RNA synthesis during infection.

Thus, Hela cells grown in 80oz roller bottles were mock-infected or infected with pseudorabies virus and harvested at 6 hours postinfection. Cells were allowed to swell in hypotonic buffer at 4^{o}C, homogenized with a stainless steel D ounce homogenizer and nuclei were isolated by differential centrifugation and stored in aliquots at - 70^{o}. Nuclei were

Fig. 7.2.1. Total and a-amanitin resistant RNA polymerase activities in isolated mock-infected and infected Hela cell nuclei at 6 hrs postinfection.

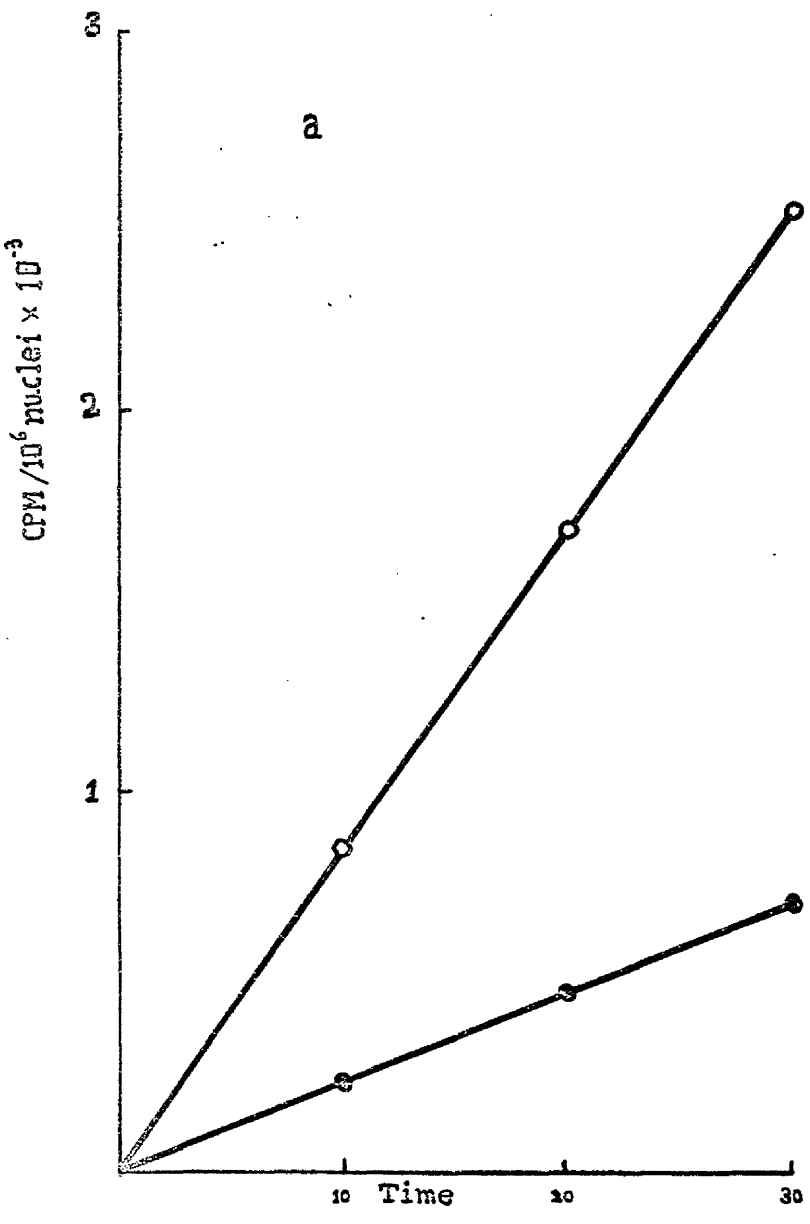
Hela cells were grown in 80oz roller bottles and mock-infected or infected with pseudorabies virus at 50 pfu. per cell. Nuclei were isolated as described in Materials and Methods. RNA polymerase activity was determined in a 200-µl reaction mixture at 25°C. a-amanitin resistant RNA polymerase activity was determined in the presence of 200µg/ml a-amanitin. At intervals, 10 µl samples were spotted onto Whatman No 1 paper discs, dried, washed in 10% cold TCA containing 1% pyrophosphate, washed in absolute alcohol and finaly in acetone. They were dried and radioactivity was determined in the presence of 10 ml toluene-PPO in a Beckman scintilation counter

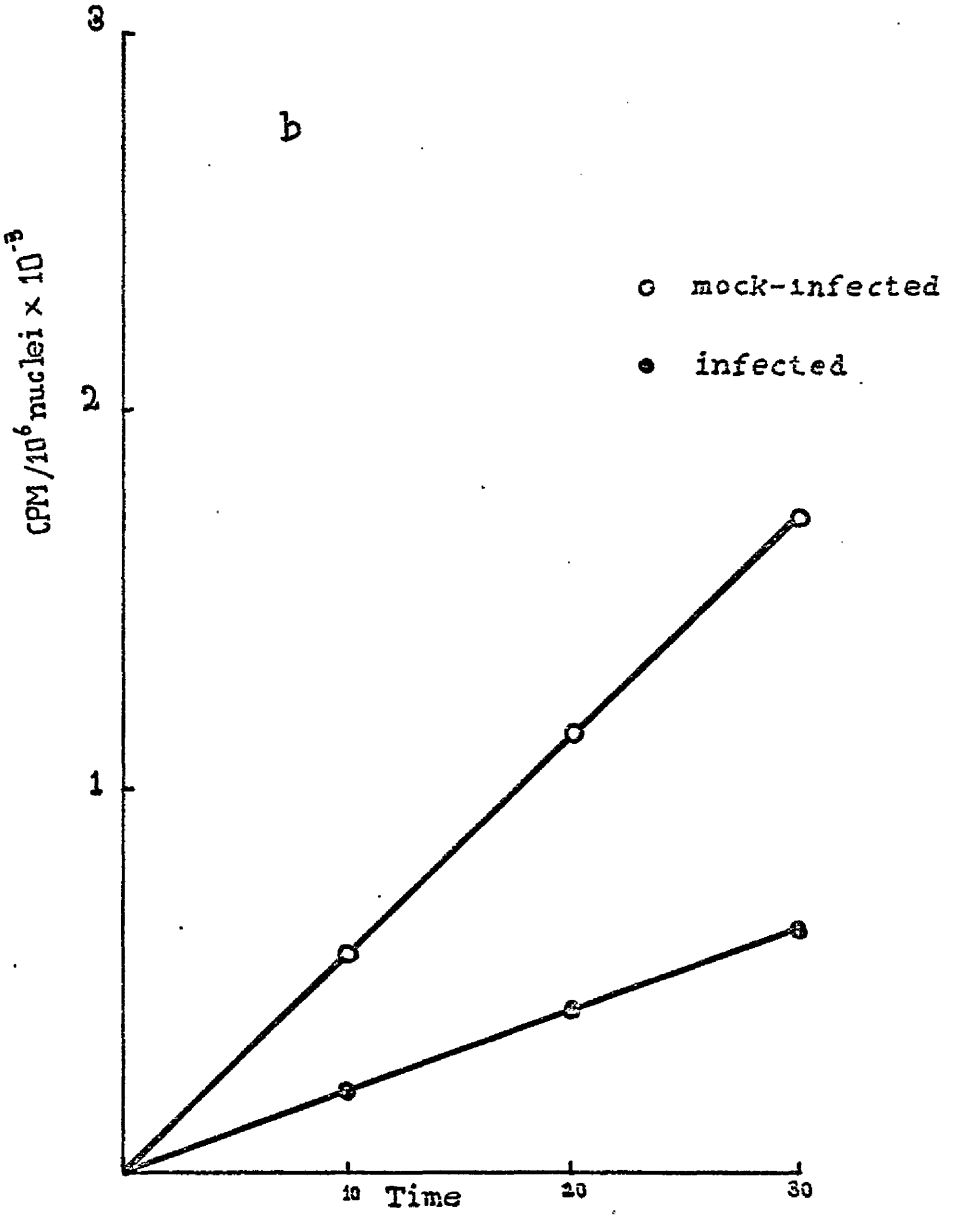
Assay Mixture

25 mM	Hepes pH 7.6
12.5%	glycerol
75 mM	K Cl
5mM	Mg acetate
0.4mM	ATP
0.4mM	GTP
0.4mM	CTP
0.05 mM	UTP
10 µCi/ml	^{3}H-UTP

a. without amanitin

b. with amanitin


a
CPM/10^6 nuclei x 10^-3
3
2
1
10
Time
20
30


b
CPM/10^6 nuclei x 10^-3
mock-infected
infected
3
2
1
10
Time
20
30

assayed for RNA polymerase activity in a 200µl incubation mixture with or without a-amanitin as described in Materials and Methods.

Fig. 7.2.1 shows that there is a drop in RNA polymerase activity both in the absence and in the presence of a-amanitin for the nuclei from the infected cells compared with the nuclei from the mock-infected cells. Total RNA polymerase activity in the infected nuclei appears to be about 30% of that of the of the mock-infected nuclei whereas the a-amanitin resistant RNA polymerase activity in the nuclei from the infected cells is about 40% of that of the mock-infected nuclei. Under the conditions of assay the a-amanitin resistant activity is due entirely to RNA polymerase I.

7.3. RNA polymerase activity in the presence of cytoplasmic extracts from mock-infected and infected Hela cells.

In the experiments described above it was shown that RNA polymerase activity in nuclei from infected cells is reduced compared with that in the nuclei from mock-infected cells. It has been shown in Chapter 6 that a number of newly synthesized viral induced polypeptides migrate into the nucleus and certain of them were found to be associated with the nucleolus and nucleolar chromatin. These polypeptides were shown to be taken up by isolated nuclei in vitro.

In view of these results, experiments were carried out to examine whether incubation of cytoplasmic or whole cell extracts would in any way affect the RNA polymerase activity of the mock-infected or infected nuclei.

Thus nuclei from mock-infected or infected Hela cells were assayed for RNA polymerase activity after co-incubation

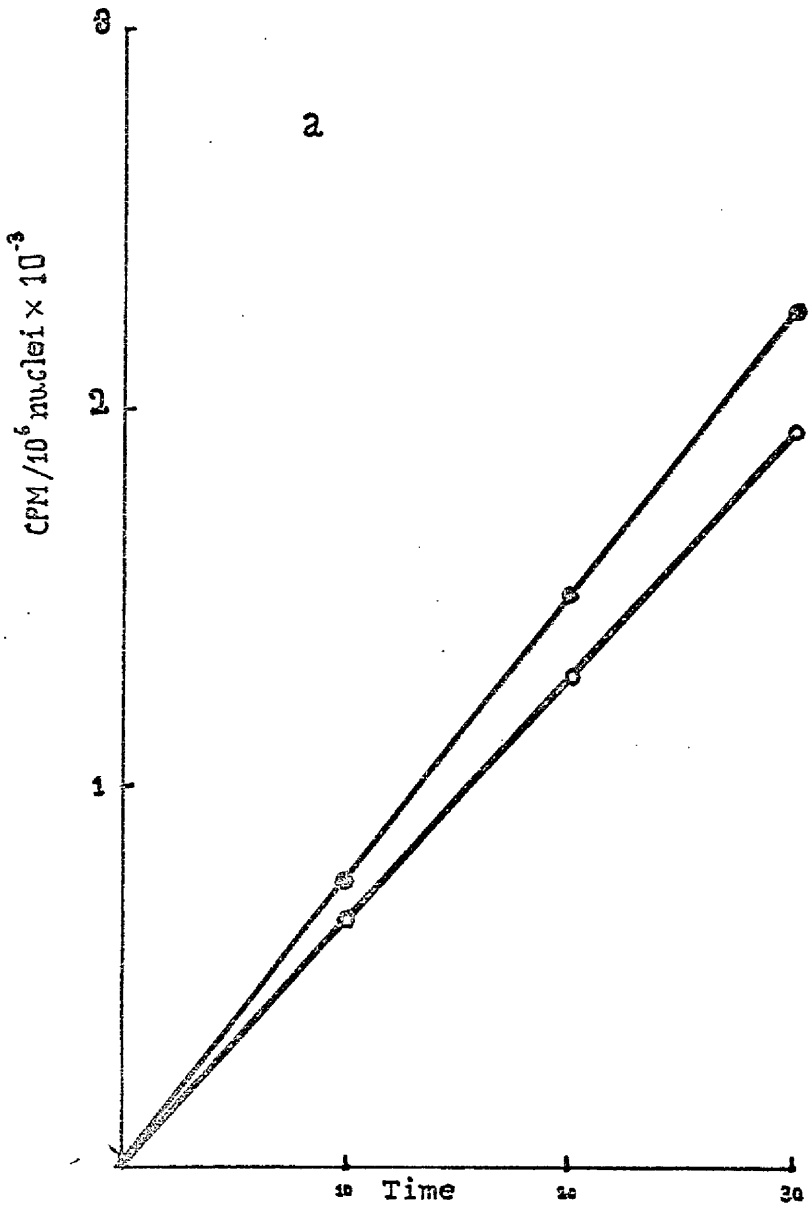
Fig. 7.3.1. RNA polymerase activity of isolated mock-infected and infected Hela cell nuclei co-incubated with cytoplasmic extracts from mock-infected and infected Hela cells.

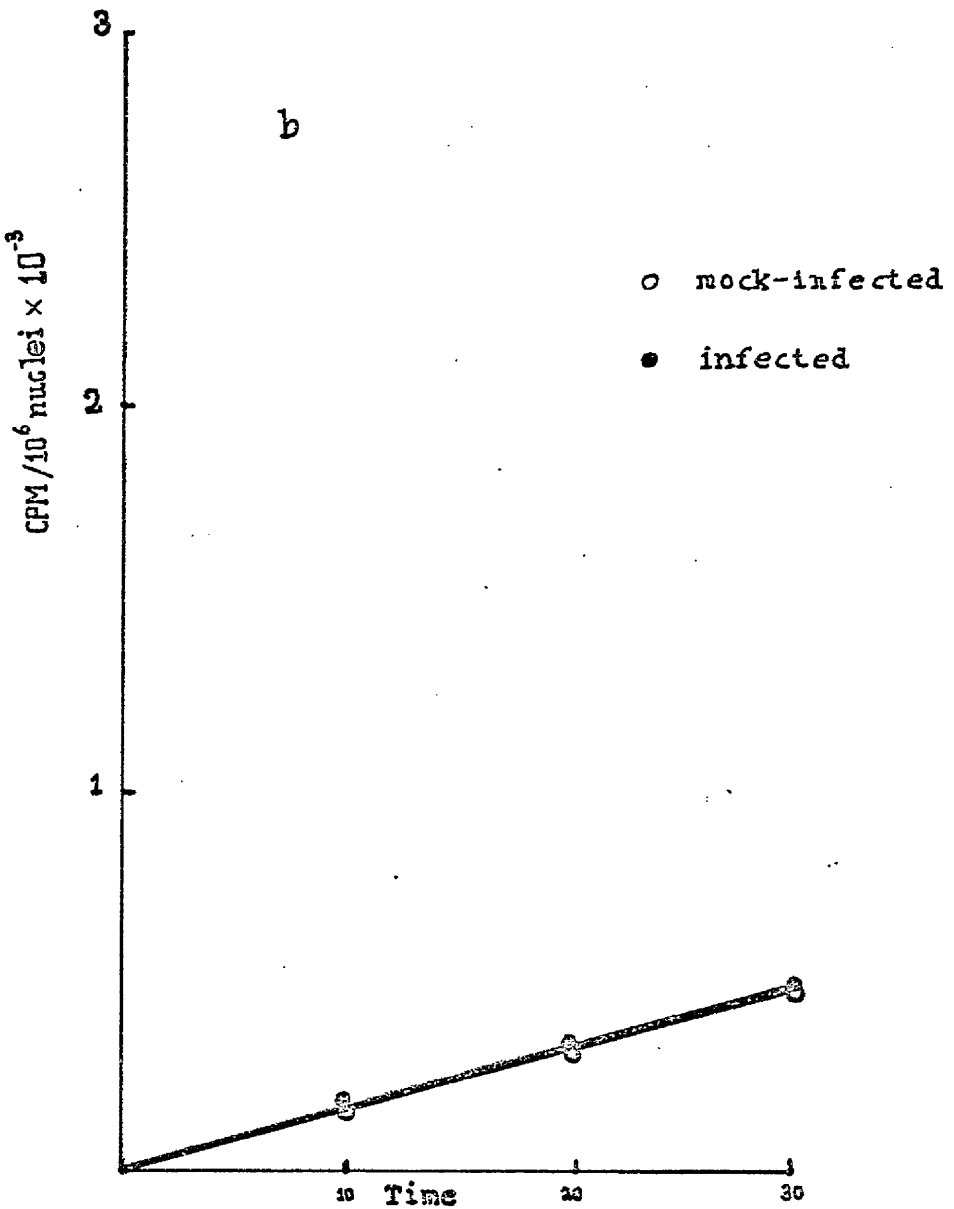
The RNA polymerase activity of nuclei from mock-infected and infected Hela cell nuclei prepared as for Fig. 7.2.1. was determined in a 200-µl incubation mixture at 25°C, in the presence of cell extracts prepared from mock-infected or infected Hela cells by sonication in salt solution for 5 min in an MSE sonifier, centrifugation for 15 min at 17k in a Sorvall rotor. 10 µl aliquots were spotted onto Whatman No 1 papers and radioactivity was determined as for Fig. 7.2.1.

○ in mock infected cell extract, ● in infected cell extracts.

a. mock-infected nuclei

b. infected nuclei.


a
3
2
1
CPM/10^6 nuclei × 10^-3
10
Time
20
30


b
3
2
1
CPM/10^6 nuclei × 10^-3
o mock-infected
● infected
10
Time
20
30

with cytoplasmic extracts from mock-infected and infected Hela cells prepared as described in Materials and Methods. Assays were carried out in a 200µl incubation mixture at 25°C and 10µl aliquots were spotted onto Whatman No 1 filter paper and radioactivity was determined as described in Materials and Methods.

Fig. 7.3.1 shows that there is no drop in RNA polymerase activity in the mock-infected nuclei when they were co-incubated with the infected Hela cell extract, nor was there a rise in the infected cell nuclei co-incubated in the mock-infected Hela cell cytoplasmic extract.

Results were similar whether the RNA polymerase assays were carried out while the nuclei were still in the presence of the cytoplasmic extracts or after the nuclei had been reisolated.

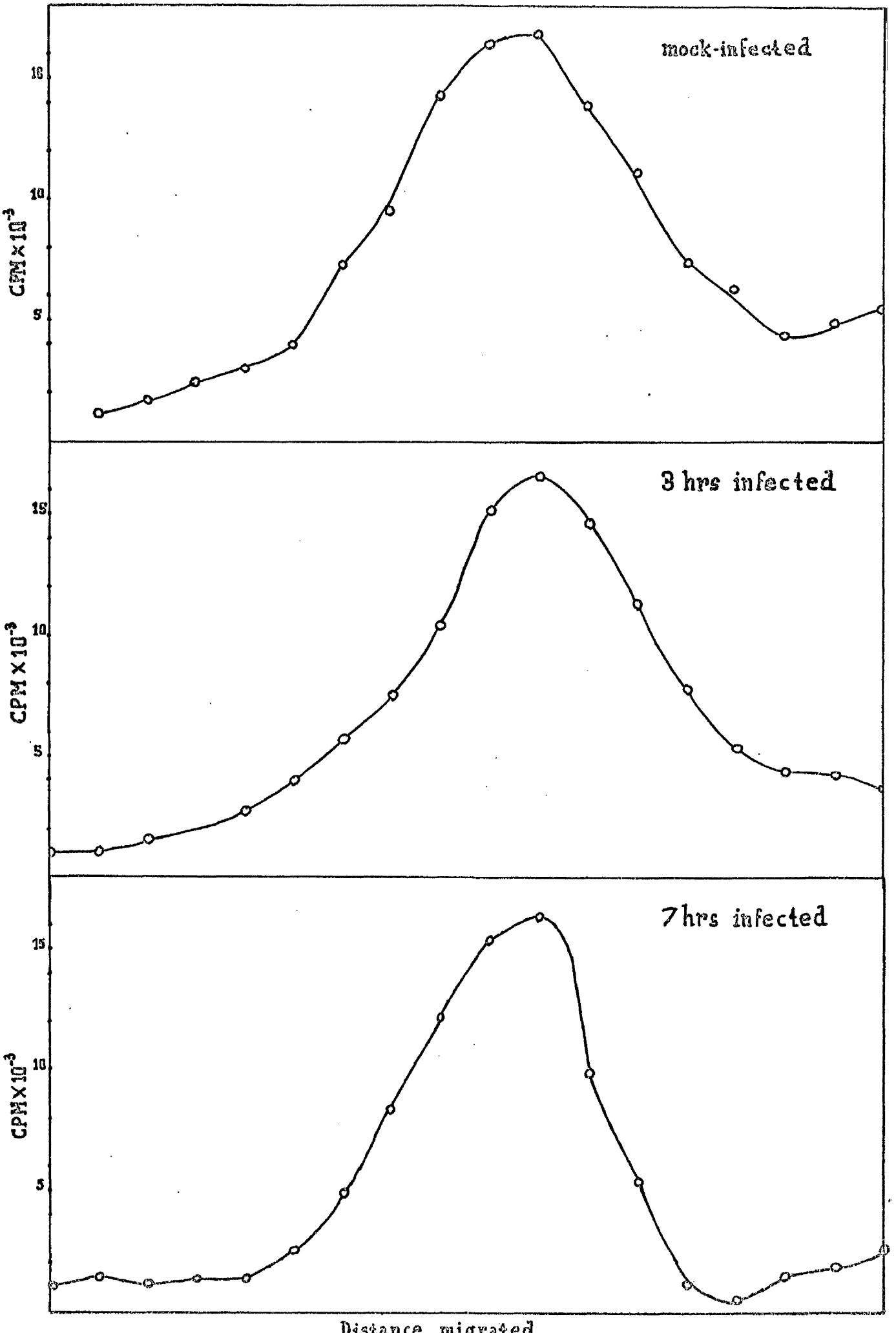
7.4. Integrity of DNA in mock-infected and infected Hela cells.

A possible reason for the decrease in RNA synthesis after infection is that general or specific degradation of cell specific DNA might be induced by the virus. In an attempt to examine this possibility, DNA from mock-infected and infected Hela cells was analysed on alkaline sucrose density gradients.

Hela cells grown in Roux bottles, labelled with ^{3}H-thymidine overnight, were mock-infected or infected with pseudorabies virus, the label was chased during infection with excess cold thymidine. The cells were harvested at 6 hours postinfection, lysed and applied onto 15% to 30% sucrose gradients. Gradients were centrifuged at 26k for 6 hours in a

Figure 7.4.1 Radioactivity profiles of cellularDNA from mock-infected and infected Hela cells after centrifugation on alkaline sucrose density gradients.

Hela cells were grown in Roux bottles, and labelled with 5 μCi/ml of ^{3}H-thymidine overnight. Cells were then mock-infected or infected with pseudorabies virus at 50 p.f.u. per cell and the label was chased with excess cold thymidine for 6 hrs. Cells were then harvested and suspende in SSC. The suspension in SSC was pipetted into 0.3N NaOH, 0,001M EDTA and 0.5% SDS, then layered onto 15% to 30% (w/v sucrose gradients in 0.5M NaCl, 0.25N NaOH, 0.001M EDTA, 0.01% SDS. The gradients were kept at room temperature for 8 hrs, and then centrifuged at 26k for 6 hrs at 25° in a Bechman SW27 rotor. Radioactivity was determined in 1 ml fractions as described in Materials and Methods.


mock-infected
3 hrs infected
7 hrs infected
CPM X 10⁻³
15
10
5
Distance migrated

Beckman SW27 motor. They were harvested and radioactivity was determined after precipitation of the DNA onto glass fibre filter discs as described in Material and Methods.

The profile of cellular DNA (Fig. 7.4.1) appears to be similar both for mock-infected and infected Hela cells. No radioactivity accumulated at the top of the gradient in the case of the sample from infected cells which would have indicated degradation of the DNA.

7.5. RNA polymerase assay in the presence of sarkosyl.

It has been suggested that the detergent sarkosyl removes most of the proteins attached to the DNA template leaving only RNA polymerase- DNA template complexes and inactivates free RNA polymerase (Chesterton et al., 1975). It was shown above (Chapter 6) that viral induced polypeptides are associated with the nucleolar chromatin in infected cells. These viral induced polypeptides might be the cause of the decrease in RNA synthesis, thus experiments were carried out to examine whether treatment with sarkosyl could restore the RNA polymerase activity in the infected cells.

Nuclei from mock-infected and infected Hela cells were assayed for RNA polymerase activity in the presence of 0.8% sarkosyl in an 80µl incubation mixture, as described in Materials and Methods.

Fig 7.5.1 shows that RNA polymerase activity in infected cell nuclei remained well below the level of the RNA polymerase activity of mock-infected cell nuclei. This could mean that there are fewer RNA polymerase molecules bound to the DNA template in infected cell nuclei than in the mock-infected

Fig. 7.5.1. RNA polymerase activity of isolated mock-infected and infected Hela cell nuclei, in the presence of sarkosyl.

Nuclei from mock-infected and from infected cells were prepared as for Fig. 7.2.1. RNA polymerase activity was determined in an 80 µl incubation mixture in the presence of 0.8% sarkosyl. The reaction was stopped with 1% SDS, 1mM EDTA and TCA precipitable material was collected onto GF/C glass fibre discs. Radioactivity was determined as for Fig. 7.2.1.

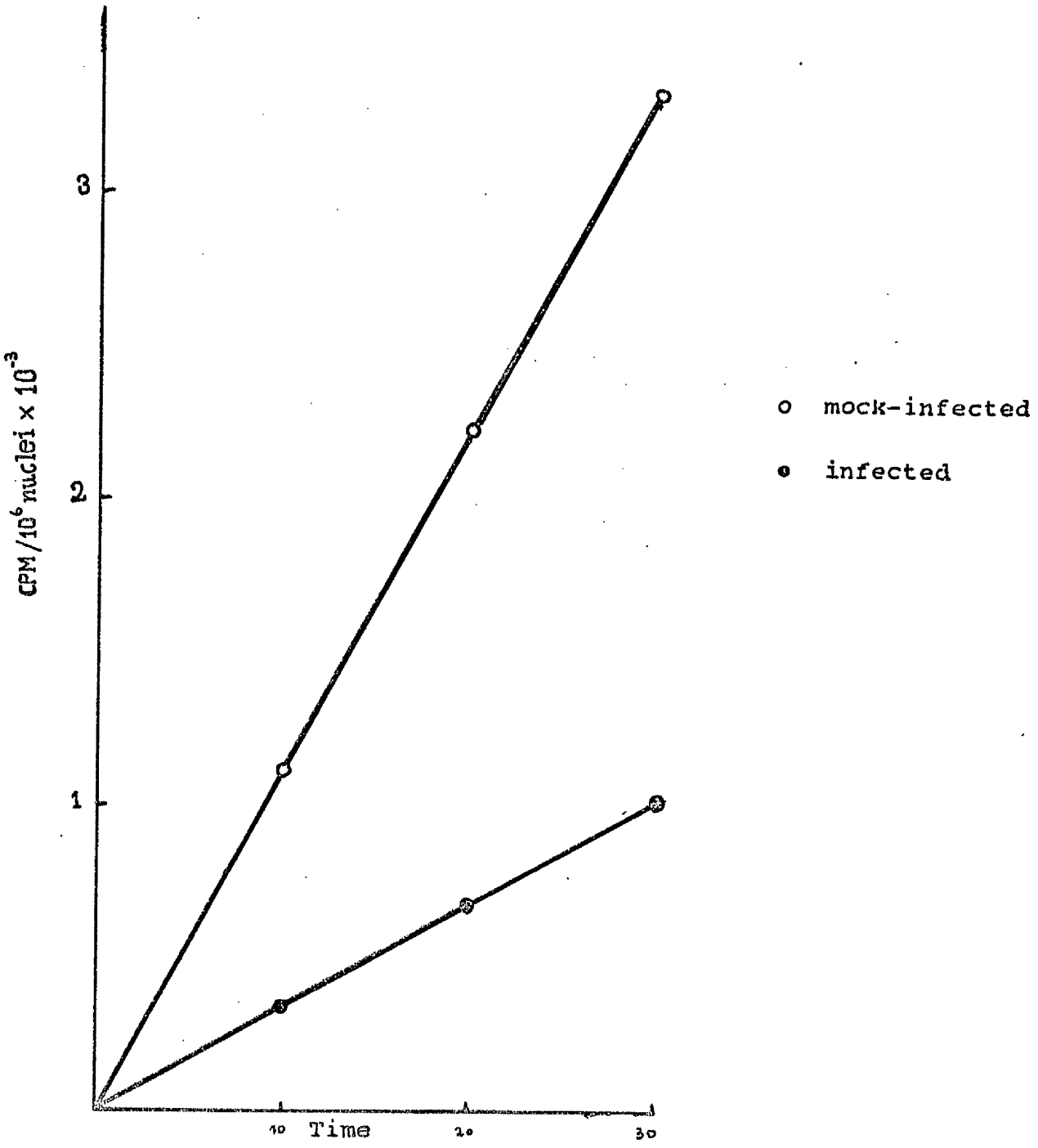

CPM/10⁶ nuclei × 10⁻³
3
2
1
10
Time
20
30
o mock-infected
• infected

Table 7.5.1. RNA polymerase activity of isolated mock-infected Hela cell nuclei co-incubated with cytoplasmic extracts of mock-infected and infected Hela cells and treated with sarkosyl.

Nuclei from mock-infected Hela cells were prepared as for Fig. 7.2.1. Cell extracts of mock-infected and infected Hela cells were prepared as for Fig. 7.3.1. Nuclei were incubated with cell extracts in a 200 µl reaction mixture without ^{3}H-UTP for 15 min, at 25°C. Then 50 µl were transferred into 50 µl of an incubation mixture containing 0.8% sarkosyl and ^{3}H-UTP. The incubation was continued for a further 15 min. Reaction was stopped with 1% SDS, 1mM EDTA and radioactivity was determined as described in Materials and Methods.

	In mock-infected cell extracts	In infected cell extracts
Percentage of radioactivity	100%	45%

cell nuclei.

Next, a combination of co-incubation with cytoplasmic extracts for 15 minutes followed by treatment with sarkosyl for 10 minutes was carried out. This experiment showed a significant decrease in the RNA polymerase activity of mock-infected Hela cell nuclei when incubated in an infected cell extract compared to the activity when the mock-infected nuclei were incubated in the mock-infected cell extract(Table 7.5.1)

7.6. Discussion.

As described above, RNA polymerase activity in nuclei isolated from pseudorabies virus infected Hela cells is much lower than that of the mock-infected Hela cells, both for total and a-amantin resistant enzymes, which agrees with previous work (Preston and Newton, 1976) and the suggested decrease in ribosomal RNA synthesis described in Chapter 4.

In further attempts to investigate possible cause(s) of the decline in RNA polymerase activity after infection, assays were carried out in which the nuclei were co-incubated with cytoplasmic extracts and/or treated with sarkosyl, an agent known to strip off proteins from the DNA template.

Thus, isolated nuclei from mock-infected and infected Hela cells showed no difference when assayed for RNA polymerase activity both in the presence of mock-infected or infected cytoplasmic extracts, their RNA polymerase activity levels being similar to the non co-incubated nuclei.

RNA polymerase activity levels of infected Hela cell nuclei assayed in the presence of sarkosyl was not restored to normal, presenting a similar decline in RNA polymerase activity as with the non-treated with sarkosyl mock-infected

and infected Hela cell nuclei.

However, mock-infected nuclei co-incubated with mock-infected or infected cytoplasmic extracts and assayed in the presence of sarkosyl showed RNA polymerase activity levels when co-incubated with infected cytoplasmic extract, of less than half the RNA polymerase activity when co-incubated with mock-infected cytoplasmic extract. This is fully discussed in the General Discussion.

In another line of experiments designed to examine the integrity of the DNA template, alkaline linear density sucrose gradients of mock-infected and infected Hela cell DNA which had been radioactively labelled before infection did not show any evidence of wholescale degradation after infection.

CHAPTER 8. General Discussion.

It is now well established that infection of a number of cell lines with various animal viruses results in a marked inhibition in cytoplasmic ribosomal RNA production (see Nayak, 1977). In studies of this inhibition by many investigators it has been suggested that either the synthesis or the processing of ribosomal RNA or both may be affected by the virus.

This study has examined the effect of pseudorabies virus on the nucleolar metabolism of Hela cells. The data of Chapter 6 suggest that pseudorabies virus inhibits the synthesis of ribosomal RNA. This is further supported by the decline in the total nucleolar area presented in Chapter 3, as well as by the decline in RNA polymerase I activity of nuclei from pseudorabies virus infected Hela cells, described in Chapter 7.

In addition, the data on processing of ribosomal RNA, presented in Chapter 5, suggest that the processing of the newly synthesized ribosomal RNA of infected Hela cells, though similar in pattern to that of the mock-infected Hela cells, is slower. On balance it appears that synthesis is more affected than processing.

Similar results have been reported for other viruses, though it appears that the level of inhibition of synthesis or processing varies, depending on the particular virus and possibly the cell line employed. Thus herpes simplex virus infection of HEp-2 cells results in the inhibition of both

synthesis and processing of ribosomal RNA with the major effect on processing, which has been suggested to be abortive (Roizman et al, 1971).

In vaccinia virus infection of L cells, where both synthesis and processing are also inhibited, it has been reported that the processing of precursor rRNA and the rate of maturation of ribonucleoprotein particles was slowed (Jefferts and Holowczak, 1971).

Similarly poliovirus and foot-and-mouth disease virus infection cause inhibition of the synthesis of 45s precursor RNA and a delay in the cleavage of this precursor (Darnell et al., 1967; Brown et al., 1966; Vande Wounde and Ascione, 1969).

It has also been reported that the effect of vaccinia virus infection on synthesis and maturation of ribosomal RNA in mouse fibroblasts is more rapid than that for Hela cells (Becker and Joklik, 1964; Salzman et al., 1964; Oda and Joklik, 1967). The multiplicity of infection has also been shown to affect the rate of inhibition of synthesis and processing of ribosomal RNA (Roizman et al; 1971).

However, in discussing the effects of viruses on ribosomal RNA synthesis and processing, it must be borne in mind that they resemble those caused by inhibition of protein synthesis.

For example, it has been reported that inhibition of protein synthesis results in a reduced rate of 45s precursor rRNA processing and a reduction in the formation of ribosomes (Willems et al., 1968). Further it has been reported that cycloheximide, which inhibits protein synthesis by 99%, results within 60 seconds in a complete block of precursor

rRNA processing (Udem and Warner, 1972) whereas when protein synthesis inhibition was only partial the inhibition of processing was less complete (Pederson and Kumar, 1971).

In view of the well established inhibition of protein synthesis by many viruses one cannot exclude the possibility that the effect on inhibition of processing by viruses might be a result of protein synthesis inhibition.

This seems a reasonable explanation in view of the significance of proteins in the maturation of ribosomal RNA. Thus cleavage enzymes, methylases and ribosomal proteins are all at various stages associated with the ribosomal RNA. The possibility exists then that a depletion in the amount of any of these proteins might affect the normal processing of the pre-rRNA in virus-infected cells.

Therefore it might be expected that a decrease in the rate of processing of ribosomal RNA would vary between viruses, depending upon their efficiency in the shut-off of host protein synthesis. It has been reported that HsV-2 shuts-off host protein synthesis extensively at 90 minutes after infection and HSV-1 has a similar effect at 3.5 to 4 hours post-infection (Pereira et al., 1977). It remains to be seen whether HSV-2 reduces processing more rapidly than HSV-1.

When considering general effects of reduced protein synthesis it is of interest, that there has been a suggestion, that the reported concomitant inhibition in ribosomal RNA synthesis is due to failure to synthesize a rapidly turning over transcription factor (Muramatsu et al., 1970; Franze Fernantez and Fontanive-Sanguesa, 1975). However it has been

proposed that levels of cellular ATP and GTP are critical for ribosomal RNA synthesis and that there is no need to postulate a special transcription factor (Grummt and Grummt, 1976). Clear evidence for such (a) short lived protein(s) has not yet been provided.

Despite these remarks on the effect of protein synthesis inhibition, the data of Chapter 6 open the possibility that there is virus-mediated inhibition of ribosomal RNA synthesis, by viral-induced proteins which are found to be associated with the nucleolus and the nucleolar chromatin of pseudorabies virus infected Hela cells. A role for viral-induced inhibition of ribosomal RNA is quite attractive, and has been suggested by other investigators (Sasaki et al., 1974; Preston and Newton, 1976).

As far as the mechanism of inhibition of ribosomal RNA synthesis is concerned, it has been reported that RNA polymerase I levels in HSV-1 infected BHK-21 cells remain virtually the same up to 8 hours after infection (Lowe, 1978) however a decrease has been observed in RNA polymerase I activity after infection of RK cells with the same virus. (Müller et al., 1978). It is possible that this discrepancy might be a result of the different extraction and assay procedures and cell lines employed.

However inhibition of rRNA synthesis might occur by interference with the template, possibly by viral induced degradation of the template or by the interference in the normal events of transcription by viral induced factors. The data in Chapter 7 suggest that host DNA in infected cells is not affected or at least that the host DNA is not nonspecifically degraded. Similar findings have been reported

by other investigators (Francke, 1977).

As far as interference with the DNA template is concerned, electron microscopic studies on nucleolar morphology in herpesvirus infected cells (Sirtori and Bossisio-Bestetti, 1967) indicated that changes in the nucleolar ultrastructure i.e. segregation of nucleolar components, resemble those of a suggested pattern of ultrastructural changes induced when ribosomal RNA synthesis is affected by agents which interact with the DNA template (Shinozuka, 1973).

Possible candidate(s) for this inhibitor could be the viral-induced polypeptides which migrate into the nucleus. Many herpes induced proteins are known to have DNA-binding properties. Proteins associated with the nucleoli and nucleolar chromatin in pseudorabies-virus infected Hela cells are described in Chapter 6.

It has been assumed that the putative inhibitor is not transferable (Preston and Newton, 1976) which partly agrees with the results in Chapter 7 where the RNA polymerase activity of mock-infected cells appeared not to be affected when nuclei were incubated in infected cell extracts. That viral-induced polypeptides migrate into the incubated nuclei in vitro was shown in Chapter 6.

However, the relatively short time period and the conditions of assay employed were such that it would not be certain that the effect of an inhibitor could be registered and thus whether it is transferable or not cannot be conclusively assumed.

An additional complication is that in most systems,

the RNA polymerase activity registered is thought generally to represent completion of already initiated polynucleotide chains.

Thus, it could only be concluded that the inhibitor does not interfere with transcription at the elongation level of the polynucleotide chain, which is in contrast with a previously reported action of this inhibitor (Sasaki et al., 1974). However, this suggestion is open to criticism on account of the template used in their study. The calf thymus DNA employed, due to methods of preparation, is likely to have present nicks and single strand regions which would favour the initiation of new RNA polynucleotide chains. Thus the effect of the inhibitor could be either on initiation or elongation.

The finding that indicates that the effect of the inhibitor might be at the initiation level rather than that at elongation, comes from the data on sarkosyl treatment of mock-infected and infected nuclei described in Chapter 7.

It has been shown that although transcription is blocked in eukaryotic mitotic chromosomes, RNA polymerase remains bound. Transcription can be reactivated by removing chromosomal proteins with the detergent sarkosyl which does not affect bound RNA polymerase (Carriglio, 1975).

RNA polymerase activity in mock-infected and infected cells was tested to find out whether or not transcriptional activity can be restored to normal with sarkosyl treatment. A positive result would mean that enzyme remained bound to

the template at a time that rRNA synthesis was inhibited. A negative result must indicate that synthesis is inhibited by a mechanism which prevents the enzyme from initiating.

In these experiments the difference in RNA polymerase activity in isolated nuclei is not changed from similar experiments without sarkosyl. This suggests that in infected cells fewer RNA polymerase molecules are in the form of the sarkosyl-resistant RNA polymerase-DNA template transcriptional complexes.

Interference in the initiation of new stable transcriptional complexes could be concluded from the data presented in Chapter 7 where RNA polymerase activity was examined after an initial incubation in cytoplasmic extracts in vitro. These activities were determined in the presence of sarkosyl.

The rationale behind this experiment is that if an effect of the incubation of mock-infected nuclei in infected cell cytoplasmic extracts cannot be detected, due to the problem of the assay conditions, the combined assay with sarkosyl would enable an examination of transcription, a step earlier.

The possible formation of transcription complexes between RNA polymerase and DNA template cannot be excluded in an assay of RNA polymerase activity of isolated nuclei even though it would be difficult to detect, but when sarkosyl is used it would be expected that any differences in that level (due here to the incubation of nuclei in mock-infected or infected cytoplasmic extracts) could now be detected, due to the removal of the chromosomal proteins, which would allow bound but not actively transcribing RNA polymerase molecules to transcribe.

The RNA polymerase activity in mock-infected nuclei incubated in infected cell cytoplasmic extracts was shown to

be lower than that of the mock-infected nuclei pre-incubated in mock-infected cytoplasmic extracts.

This suggests the presence in the cytoplasmic extract of infected cells of a factor(s) which affects the amount of RNA polymerases bound to the template and further that inhibition at the initiation level could be the cause of the decrease in RNA synthesis. This could be due to one of the viral polypeptides shown to be associated with the nucleolar chromatin. Confirmation of this requires experiments using purified viral-induced proteins and more stringent conditions of assay.

This suggestion would not fit with the model for a single promoter being responsible for the transcription of multiple ribosomal genes linked in tandem (Perry et al., 1970). However recent findings about the existence of promoter sites for each individual ribosomal gene at the spacer regions (Scheer et al., 1976; Hackett and Sauerbier, 1975) seem more attractive and would support the above proposed mechanism.

Thus, according to these results we could form the following hypothesis. Viral-induced proteins in infected cells are attached to/or compete for the same sites in the DNA template which are used by RNA polymerase molecules for initiation.

Evidence for the validity of this hypothesis would come by defining further the viral induced proteins in cell extracts. Purification and identification of individual proteins would allow a check on their effect on the RNA polymerase activity of isolated nuclei or nucleoli with or without sarkosyl.

Further evidence for their function as DNA binding proteins could be provided by incubation in vitro with nucleolar chromatin or naked cell DNA. Possible binding

specificity could be provided by co-incubation with plasmids, containing rDNA and employing the filter retention technique. Restriction endonuclease fragments of rDNA might be further used.

Another line of experiments would be to examine possible virus proteins effects on RNA synthesis using plasmids containing rDNA and employing purified RNA polymerase I.

REFERENCES

ABODEELY, R.A., PALMER, E., LAWSON, L.A. and RANDALL, C.C. (1971) Virology, 44, 146 - 152.

ALLEN, G.P., O'CALLAGHEN, D. J. AND RANDALL, C.C. (1977). Virology, 76, 395 - 408.

AMES, B.N. and DUBIN, D.T. (1960) J. Biological Chem., 235, 769 - 775.

ATTARDI, G. and AMMALDI, F. (1970) A. Rev. Biochem., 39, 183 - 226.

ATTARDI, G., HUANG, P.C. and KABAT, S. (1965) Proc. Natl Acad. Sci. USA, 54, 185 - 192.

AUGER-BUENDIA, M-A. and LONGUET, M. (1978) Eur. J. Biochem., 85, 105 - 114.

BACHENHEIMER, S. and ROIZMAN B. (1972) J. Virol., 10, 875 - 879.

BARR, H.J. and ESPER, H. (1963) Exptl. Cell Res., 31, 211 - 214.

BARTKOSKI,M. and ROIZMAN, B. (1976) J. Virol., 20, 583 - 588.

BARTKOSKI,M. and ROIZMAN, B. (1978) Virology, 85, 146 - 156.

BAYLISS, G.J., MARSDEN, H.S. and HAY, J (1975) Virology, 68, 124 - 134.

BECKER, Y. and JOKLIK, W.K. (1964) Proc. Natn. Acas. Sci. USA, 51, 577 - 585.

BECKER, Y., PYM, H. and SAROV, I. (1968) Virology, 36, 184 - 192.

BEN-PORAT, T., JEAN, J - H. and KAPLAN, A.S. (1974) Virology 59, 524 - 531.

BEN - PORAT, T. and KAPLAN, A.S. (1962) Virology, 16, 261 - 266.

BEN-PORAT, T., RAKUSANOVA, T. and KAPLAN, A.S. (1971) Virology, 46, 890 - 899.

BEN-PORAT, T., RIXON, F.J. and BLAKENSHIP, M.L. (1979) Virology, 95, 285 - 294.

BEN-PORAT, T., SHIMONO, H. and KAPLAN, A.S. (1969) Virology, 37, 56 - 61.

BEN-PORAT, T. and VEACH, R.A. (1980) Proc. Natl. Acad. Sci USA, 77, 172 - 175.

BEN-ZEAV., A. and BECKER, Y. (1977) Virology, 76, 246 - 253.

BERNHARD, W. (1966) Proc Natl. Cancer Inst. Mon., 23, 13 - 38 .

BERNHARD, W. and GRANBOULAN, N. (1968). In "The Nucleus" (A.J. Dalton and F.Hagenau eds), pp. 81 - 149. Academic Press, New York.

BIRNSTEELL, M.L., CHIPCHASE, M and SPEIRS, J. (1971) Prog. Nucleic Acid Res. Molec. Biol., 11, 351 - 389.

BLATTI, S.P. INGLES, C.J., LINDELL, T.J., MORRIS, P.W., WEAVER, R.F., WEINBURG, F. and RUTTER, W.J. (1970) Cold Spring Harbor Symp. Quant. Biol., 35, 649 - 657.

BOLLUM, F.J. (1975) In "Progress in Nucleic Acid Research and Molecular Biology" (W.E. COHN, ed.), Vol. 15, pp. 109 - 144. Academic Press, New York.

BONNER, W.M. and LASKEY, R.A. (1974) Eur.J. Biochem, 46, 83 - 88.

BOOKOUT, J.B. and LEVY, C.C. (1980) Virology, 101, 198 - 216

BORNKAMM, G.W., DELIUS, H., ZIMBER, U., HUDEWENTZ, J and EPSTEIN, M.A. (1980) J. Virol., 35, 603 - 618.

BOUTEILLE, M., LAVAL, M. and DUPUI - COIN, A.M. (1974). In "The Cell Nucleus" (H. Busch, ed.), Vol.1, pp. 3 - 71. Academic Press, New York.

BRADFORD, M.M. (1976) Anal. Biochem., 72, 248 - 254.

BROWN, F., MARTIN S.J. and UNDERWOOD, B. (1966) Biochem. Biophys Acta, 129, 166 - 177.

BUCHER, N.L. and SWAFFIELD (1969) Biochim. Biophys. Acta, 174, 491 - 502.

BUCHMAN, T. and ROIZMAN, B. (1978) J. Virol., 27, 239 - 254.

BUHLER, J.M., IBORA, F., JENTENAC, A. and FROMAGEOT, P. (1976) J. Biol. Chem., 251, 1712-1717.

BUSCH, H, and SMETANA, K. (1970) "The Nucleolus" Academic Press, New York.

CABRAL, G.A., COURTNEY, R.J., SCHAFFER, P.A. and MARLIANO-CABRAL, F. (1980) J.Virol, 33,1192-1198.

CASTON, J.P. and JONES, P.H. (1972) J.molec. Biol.,69, 19-38.

CHAMBON,P. (1975) Ann.Rev. Biochem.,44,613-638.

CHANDLER, J.K. and STEVELY, W.S. (1973) J.Virology, 11,815-822.

CHENG,Y., GOZ,B. abd PRUSSEL, W.H. (1975) Biochim. Biophys. Acta 390, 253-263.

CHESTERTON, C.J. and BUTTERWORTH, P.H.W. (1971) Eur. J. Biochem., 19, 232-241.

CHESTERTON, C.J., COUPAR, B.E.H., BUTTERWORTH, P.H.W., BUSS, J. and GREEN, M.H. (1975) Eur. J. Biochem., 57, 79-83.

CHESTERTON,C.J.,HUMPHREY, S. and BUTTERWORTH, P.H.W. (1972) Biochem. J., 126, 676-683.

CHOUINARD, L.A. (1975) J. Cell Science, 19, 85-101.

CHOUSTERMAN, S., LACASA, M.and SHELDRICK. P.(1979)J. Virol., 31, 73-85.

CLEMENTS, J.B., CORTINI,R. and WILKIE, N.M. (1976) J. Gen. Virol., 30, 243-256.

CORTES, P., LEVIN, N.W. and MARTIN, P.R. (1976) Biochem. J., 158, 457-470.

COSTANZO, F., CAMPADELLI-FIUME, G., FOA-TOMASI, L. and CASSI, E. (1977) J. Virology, 21, 996-1001.

COURTNEY, R.J., McCOMBS, R.M. and BENYESH-MELNICK, M. (1971) Virology, 43, 356-365.

COX, R.F. (1976) Cell, 7, 455-465.

CRAIG, N.C. and PERRY, R.P. (1970) J. Cell Biol., 45, 554-564.

CRAIG, N.C. and PERRY, R.P. (1971) Nature New Biol., 229, 79-80.

DALES, S. and SILVERBERG, H. (1969), Virology, 37, 475-480.

DARLINGTON, R.W. and GRANOFF, A. (1973). Replication-biological aspects in the Herpesviruses, pp. 93-132. Edited byA.S. Kaplan, Academic Press, New York.

DARLINGTON, R.W. and MOSS, L.H. (1968) J. Virology, 2, 48-55.

DARNEL, J.E.. Jr., GIRARD, M., BALTIMORE, D., SUMMERS, D.F., and MAIZEL, J.V. (1967). In "The Molecular Biology of Viruses" (J.S. Colter and W. Paranchych, eds.), pp.376-401, Academic Press, New York.

DASKAL, Y., BALLAL, N.R. and BUSCH, H. (1978) Exp. Cell Res., 111, 153-165.

DAWID, I.B., BROWN, P.D.,and REEDER, R.H. (1970) J.Mol. Biol., 51, 341-360.

DELIUS, H. and CLEMENTS, J.B. (1976) J.Gen. Virol., 33, 125-133.

DOLYNIUK, M., PRITCHETT, R. and KIEFF, E. (1976) J. Virol., 17, 935 949.

DUPOY-COIN,A.M., EGE,T.,BUTTEILLE,M. and RINGERTZ,N.R.(1976)Exp. Cell Res.,101,355-369.

EJERCITO,P.M.,KIEFF,E.D.and ROIZMAN,B.(1968)J.Gen.Virol.,2,357-364.

ELSEVIER, S.M. and RUDDLE, F.H. (1975) Chromosoma, 52, 219 - 228.

EMERSON, C.P.,Jr and HUMPHREYS, T. (1971) Anal. Biochem., 40, 254 - 266

EPSTEIN, M.A. (1962) J. Exp. Med., 115, 1-11.

EPSTEIN, M.A. (1962a) J. Cell Biology, 12, 589-597.

EVANS, H.K., BUCKLAND, R.A. and PARDUE, M.L. (1974) Chromosoma, 48, 405- 426.

FAKAN, S. (1971) J. Ultrastruct. Res., 34, 586-596.

FAKAN S. (1978). In " The Cell Nucleus" (H. Busch, ed.), Vol.5, pp. 3-53. Academic Press. New York.

FENNER, F. (1976) Intervirology, 7, 1-115.

FENWICK, M.L. and WALKER, M.J. (1978) J. gen. Virol., 41, 37-51.

FENWICK, M.L., WALKER, M.J. and PETKEVICH, J.M. (1978) J. Gen Virol., 39, 519-529.

FERENCZ, A. and SEIFART, K.H. (1975) Eur. J. Biochem., 53, 605-613.

FLANAGAN, J.F. (1967) J. Virol., 1, 583-590.

FONG, C.K.Y., TENSER, R.B., HSIUNG, G.D. and GROSS, P.A. (1973) Virology, 52, 468-477.

FRANCKE, B. (1977) Biochemistry, 16, 5664-5670.

FRANZE - FERNANTEZ, M.T. and FONTANIVE - SANGUESA, A.V. (1975) FEBS Lett., 54, 26-29.

FUJIWARA, S. and KAPLAN, A.S. (1967) Virology, 32, 60-68.

FURLONG, D., SWIFT, H. and ROIZMAN, B. (1972) J. Virol., 10, 1071- 1074.

GARIGLIO, P., BUSS, J. and GREEN, M.H. (1974) FEBS Lett., 44, 330-333.

GERGELY, L., KLEIN, G. and ERNBERG, I. (1971). Virology. 45, 22-29.

GEY, G.O., COFFMAN, W.D. and KUBICEK, M.T. (1952) Cancer Res., 12, 264-265.

GIBSON, W. and ROIZMAN, B. (1971) Proc. Natl. Acad. Sci. USA, 68, 2818-2821.

GIBSON, W. and ROIZMAN, B. (1973) in Polyamines in Normal and Neoplastic growth (D.H. Russel, ed.) Raven, New York, p. 123.

GISSINGER, F. and CHAMBON, P. (1975) FEBS Lett., 58, 53-56.

GOESSENS, G. (1976) Exp. Cell Res., 100, 88-94.

GOESSENS, G. (1979) Cell Biol. Int. Rep., 3, 337-343.

GOESSENS, G. and LEPOINT, A. (1974) Exp. Cell Res., 87, 63-72.

GOESSENS, G. and LEPOINT, A. (1979) Biol. Cell., 35, 211-220.

GOTOH, S., NIKOLAEV, N., BATTANER, E., BIRGE, L.H. and SCH LESSINGER, D. (1974) Biochem. Biophys. Res. Commun., 59, 972-978.

GOODY, H.E. and ELLEM, K.A.O. (1975) Biochim. Biophys. Acta., 383, 30-39.

GRAFSTROM, R.H., ALWINE, J.C. STEINHART, W.L. and HILL, C.W. (1974) Cold Spring Harbour Symposium on Quantitative Biology, 39, 679-681.

GRAFSTROM, R.H. ALWINE, J.C., STEINHART, W.L., HILL, C.W., and HYMAN, R.W. (1975) Virology, 67, 144-157.

GRANBOULAN, N. and GRANBOULAN, P. (1965) Expl. Cell Res., 38, 604-619.

GRUMMT, I., HALL, S.H. and CROUCH, R.J. (1979) Eur. j. Biochem., 94, 437-443.

GRUMMT, I. and GRUMMT, F. (1976) Cell, 7, 447-453.

GRUMMT, I., LOENING, U.E. and SLACK, J.M.W. (1975) Eur. J. Biochem. 59, 313-318.

HARRIS,H.(1967) J.Cell Sci.,2,23-32.

GRUMMT, I., SMITH, V.A. and GRUMMT, F. (1976) Cell,7, 439-446.

HAAR, L. and MARSDEN, H.S. (1981) J. Gen. Virol., 52, 77-92.

HACKETT, P.B. and SAUERBIER, W. (1975) J. Mol.Biol., 91, 235-256.

HADJOLOV, A.A. and NIKOLAEV, N. (1976) Prog. Biophys. Molec. Biol., 31, 95-144.

HALL, S.H. and CROUCH, R.J. (1977) J. Biol. Chem., 252, 4092-4097.

HARBERS, E., CHAUDHURI, N.K. and HEIDELBERGER, C. (1959) J. Biol. Chem., 234, 1255-1262.

HAUSCHKA, P.V.(1973) Methods Cell Biol., 7, 361-462.

HAYWARD,G.S., FRENKEL, N. and ROIZMAN, B. (1975) Proc. Natl. Acad. Sci. USA, 72, 1768-1772.

HAY, J., KOTELES, G.J., KEIR, H.M. and SUBAK-SHARPE, H. (1966) Nature, 210, 387-390.

HAY, R.T. and HAY. J. (1980) Virology, 104, 230-234.

HEITZ , E. (1931) Planta, 12, 774-844.

HEITZ, E. and BAUER, H. (1933) Z. Zellforsch. mikrosk. Anat., 17, 67-82.

HENDERSON, A.S., EIGHER, E.M., YU,M. and ATWOOD, K.C. (1976) Cytogen. Cell Genet., 17, 307-316.

HENDERSON, A.S., WARBURTON, P., ATWOOD, K.C.(1972) Proc. Natl. Acad. Sci. USA, 69, 3394-3398.

HERNANTEZ-VERDUN, D. and BUTEILLE, M. (1979) J.Ultrastruct. Res., 69, 164-179.

HERNANTEZ-VERDUN, D., BOUTEILLE,M., EGE,T. and RINGERTZ, N.R. (1979) Exp. Cell Res., 124, 223-235.

HIDVEGI, E.J., PRESTAYAKO, A.W. and BUSCH, H. (1971) Physiol. Chem. Phys., 3, 17 - 35.

HILDEBRANDT, A., SEBASTIAN, J. and HALVORSON, H.O. (1973) Nature (london) New biolog., 246, 73 - 74.

HOCHBERG and BECKER Y. (1968) J. Gen. Virol., 2, 231 - 241.

HOLMES, I,H. and WATSON, D.H. (1963) Virology, 21, 112 - 123

HONESS, R.W. and ROIZMAN, B. (1973) J. Virol., 12, 1347 - 1365.

HONESS, R.W. and ROIZMAN, B. (1974). J. Virol., 14, 8 - 19.

HONESS, R.W. and ROIZMAN, B. (1975) Proc Natl. Acad. Sci. USA, 72, 1276 - 1280.

HONESS, R.W. and WATSON, D.H. (1977) J. gen. Virol., 37, 15 - 37.

HOUSE, W. and WILDY, P. (1965) Lab. Pract., 14, 594 - 595.

HSU, T.C., BRINKLEY, R.B. and ARRIGHI, F.E. (1967) Chromosoma, 23, 39 - 52.

HSU, T.C. SPIRITO, S.E. and PARDUE, M.L. (1975) Chromosoma 53, 25 - 36.

HYMAN, R.W. (1980) Virology, 105, 254 - 255.

JACKOWSKI, G., SURIA, D. and LIEW, C.C. (1976) Can. J. Biochem., 54, 9 - 14.

JACQUERMONT, B. and ROIZMAN, B. (1975) J. Gen. Virol., 29, 155 - 165.

JEAN, J.H. and BEN-PORAT, T. (1976) Proc. Natl, Acad. Sci. USA, 73, 2674 - 2678.

JEFFERTS, E.R., and HOLLOWCZAK, J.A. (1971) Virology, 46, 730 - 744.

JORDAN, E.G. (1971) The nucleolus, London Oxford U.P.

JORDAN, E.G. (1977) Nature, 269, 376 - 378.

JORDAN, E.G. and LUCK, B,T. (1976) J. Cell. Sci., 22, 75 - 86.

KALRA, J. and WHELDRAKE, J.F. (1972) FEBS lett., 25, 298 - 300.

KANG, Y.-J., OLSON, M.O.J. and BUSCH, H. (1974) J. Biol. Chem. 249, 5580 - 5585.

KAPLAN, A.S. and BEN-PORAT, T. (1963), Virology, 19, 205 - 214.

KAPLAN, A.S. and BEN-PORAT, T. (1976) Virology, 70, 561 - 563.

KAPLAN, A.S. and VATTER, A.E. (1959) Virology, 7, 394 - 407.

KATSUMOTO, T., HIRANO, A., KURIMURA, T. and TAKAGI, A. (1981) J. Gen. Virol., 52, 267 - 278.

KAUKEL, E., FUHRMANN, U. and HILZ, H. (1972) Biochem. Biophys. Res. Commun., 48, 1516 - 1524.

KEDINGER, C., GISSINGER, F. and CHAMBON, P. (1974) Eur. J. Biochem., 44, 421 - 436.

KELLAS, B.L., AUSTOKER, J.L., BEEBEE, T.J.C. and BUTTERWORTH P.H.W. (1977) Eur. J. Biochem, 72, 583 - 594.

KIEFF, E.D., BACHENHEIMER, S.L. and ROIZMAN, B. (1971) J. Virol., 8, 125 - 132.

KNIBIEHLER, B., NAVARO, A., MIRRE, C. and STAHL, A. (1977) Exp. Cell Res., 110, 153 - 157.

KNOWLER, J.T. and SMELLIE, R.M.S. (1971) Biochem. J., 125, 605 - 614.

KOSHIBA, K., THIRUMALACHARY, C., DASKAL, Y. and BUSCH, H. (1971) Exp. Cell Res., 68, 235 - 246.

KOZAK, M. and ROIZMAN, B. (1974) Proc. Natl. Acad. Sci. USA, 71, 4322 - 4326.

KUMAR, A. and SUBRAMANIAN, A.R. (1975) J. molec. Biol., 94, 409 - 423.

KUTTER, D.J. and RODGERS, A. (1976) Exp. Cell Res., 102, 205 - 212.

KWAN, C.N. (1976) J. Biol. Chem., 22, 7132 - 7136.

LADIN, B.F., BLANKENSHIP, M.L. and BEN-PORAT, T. (1980) J. Virol., 33, 1151 - 1164.

LAEMMLI, U.K. (1970) Nature (London), 227, 680 - 685.

LAFONTAINE, J.G. and LORD, A. (1973) J. Cell Sci., 12, 369 - 382.

LAMPERT, A. and FEIGELSON, P. (1974) Biochem. Biophys. Res. Commun., 58, 1030 - 1038.

LEE, L.F., KIEFF, E.D., BACHENHEIMER, S.L., ROIZMAN, B., SPEAR, P.G., BURMESTER, B.R. and NAZERIAN, K. (1971) J. Virol., 7, 289 - 294.

LEPOINT, A. and GOESSENS, G. (1978) Exp. Cell Res., 117, 89 - 94.

LESTOURGEON, W.M. and BEYER, A.L. (1977) Methods in cell Biology, 16, 387 - 406.

LEVIS, R. and PENMAN, S. (1977) Cell, 11, 105 - 113.

LEWIN, B. (1975) Cell, 4, 11 - 20.

LEWIS, A.M. Jr., LEVIN, M.J., WIESE, W.H., CRUMPACKER, C.S. and HENRY, P.H. (1969) Microbiology, 63, 1128 - 1135..

LINDELL, T.J., WEINBERG, F., MORRIS, P.W., ROEDER, R.G. and RUTTER, W.J. (1970) Science, 170, 447 - 451.

LOENING, U.E., JONES, K.W. and BIRNSTIEL, M.L. (1969) J. Mol Biol., 45, 353 - 366.

LONG, E.O. and DAWID, I.B. (1980) Ann. Rev. Biochem., 49, 727 - 764.

LOVE, R and WILDY, P (1963) J. Cell Biol., 17, 237 - 254.

LOWE, P.A. (1978) Virology, 86, 577 - 580.

LOWRY, D.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951) J. Biol. Chem., 193, 265 - 275.

MADEN, B.E.H. (1971) Prog. Biophys. molec. Biol., 22, 127 - 177.

MADEN, B.E.H., SALIM, M., ROBERTSON, J.S. (1974) in RIBOSOMES ed. M. Nomura, A. Tissieres, P. Lengyell pp. 829 - 839. Cold Spring Harbor, NY. Cold Spring Harbor Lab.

McCOMBS, R.M. (1974) Virology, 57, 448 - 458.

Mc KNIGHT and MILLER, (1976) Cell, 8, 305 - 319.

MARINOZZI, V. (1964) J. Ultrastruct. Res., 10, 433 - 456.

MARINOZZI, V. and BERNHARD, W. (1963) Exp Cell Res., 32, 595 - 598.

MARK, G.E. and KAPLAN, A.S. (1971) Virology, 45, 53 - 60.

MARSDEN, H.S., CROMBIE, I.K. and SUBAK-SHARPE, J.H. (1976) J. Gen. Virol., 31, 347 - 372.

MARSDEN, H.S., STOW, N.D., PRESTON, V.G., TIMBURY, M.C. and WILKIE, N.M. (1978) J. Virol., 28, 624 - 634.

MATSUI, T., ONISHI, T. and MURAMATSU, M. (1976a) Eur. J. Biochem., 71, 351 - 360.

MATSUI, T., ONISHI, T. and MURAMATSU, M. (1976b) Eur. J. Biochem., 71, 361 - 368.

MATSUURA, S., MORIMOT, T., TASHIRO, Y., HIGASHINAKAGAWA, and MURAMATSU, M. (1974) J. Cell Biol., 63, 629 - 640.

MILLER, R.L. and RAPP, R. (1977) J. Gen. Virol., 36, 515 - 524.

MIRAULT, M.E. and SCHERRER, K. (1972) FEBS Lett., 20, 233 - 238.

MIRRE, C., and STAHL, A. (1976) J. Ultrastruct. Res., 56, 186 - 201.

MIRRE, C., and STAHL, A. (1978a) J. Cell Sci., 31, 79 - 100.

MIRRE, C., and STAHL, A. (1978b) J. Ultrastruct. Res., 64, 377 - 387.

MIRRE, C., and STAHL, A. (1978c) Biol. Cell., 32, 9a.

MORGAN, G., ROSE, H.M., HOLDEN, M. and JONES, E.D. (1954) J. Exp. Med., 110, 643 - 656.

MORGAN, C. ROSE, H.M., HOLDEN, M. and JONES, E.P. (1959) J. Experim. Medicine, 110, 643 - 656.

MORGAN, C., ROSE, H.M. and MEDNIS, B. (1968) J. Virol., 2, 507 - 516.

MOSS, B., GERSHOWITZ, A., STRINGER, J.R., HOLLAND, L.E. and WAGNER, E.K. (1977) J. Virol., 23, 234 - 239.

MÜLLER, W.E.G., FALKE, D., ZAHN, R.K. and ARENDES, J. (1980) Arch. Virol., 64, 269 - 275.

MULLER, W.E.G., ZAHN, R.K., BEYER, R. and FALKE, D. (1977) Virology, 76, 787 - 796.

MULLER, W.E.G., ZAHN, R.K. and FALKE, D. (1978) Virology, 84 320 - 330.

MURAMATSU, M., HAYASHI, Y., ONISHI, T., SAKAI, M., TAKAI, K. and KASHIYAMA, T. (1974) Exp. Cell Res., 88, 345 - 351.

MURAMATSU, M., ONISHI, T., MATSUI, T., KAWABATA, C. and TOKUGAWA, S. (1975) FEBS Symp., 33, 325 - 330.

MURAMATSU, M., SHIMADA, N. and HIGASHINAKAGAWA, T. (1970) J. Mol. Biol., 53, 91 - 106.

MURAMATSU, M., SMETANA, K. and BUSCH, H. (1963) Cancer Res., 23, 510 - 518.

NAHMIAS, A.J. (1972) in Pathobiol Annual, (H. Ioachim ed.) Vol. 2, p. 153 - 182.

NAYAK (1977) In "Molecular Biology of Animal Viruses" pub. Mercel Dekker Inc., New York and Basel.

NII, S., MORGAN, C. and ROSE, H.M. (1968) J. Virol., 2, 517 - 536.

NONOYAMA, M. and PAGANO, J.S. (1972) Nature New Biology, 238, 169 - 171.

O'CALLAGHAN, D.T., CHEEVERS, W.P., GENTRY, G.A. and RANDALL, C.C. (1968) Virology, 36, 104 - 114.

ODA, K.I. and JOKLIK, W.K. (1967) J. Mol. Biol., 27, 395 - 419.

O'FARRELL, P.H. (1975) J. Biol. Chem., 250, 4007 - 4021.

O'FARRELL, P.Z, GOODMAN, H.M. and O'FARRELL, P.H. (1977) Cell, 12, 1133 - 1142.

OLSHEVSKY, U., LEVITT, J. and BECKER, Y. (1967) Virology, 33, 323 - 334.

ORRICK, L.R., OLSON, M.C.J. and BUSCH, H. (1973) Proc. Natl. Acad. Sci. USA. 70. 1316 - 1320.

PEDERSON, T. and KUMAR, A. (1971) J. Mol Biol., 61, 655 - 668.

PENMAN, S. (1966) J. Mol. Biol., 17, 117 - 130.

PERDUE, M.L. (1974) Cold Spring Harbor Sump. Quant. Biol., 38, 475 - 482.

PERDUE, M.L., COHEN, J.C., RANDALL, C.C. and O'CALLAGHAN, D.J. (1976) Virology, 74, 194 - 208.

PERDUE, M.L. and HSU, T.C. (1975) J. Cell Biol., 64, 251 - 254.

PERDUE, M.L., KEMP, M.C., RANDALL, C.C. and)'CALLAGHAN, D.J. (1974) Virology, 59, 201 - 216.

PEREIRA, L., WOLFE, H.M., FENWICK, M.L. and ROIZMAN, B. (1977) Virology, 77, 733 - 749.

PERRY, R.P. (1976) Ann. Rev. Biochem., 45, 605 - 629.

PERRY, R.P., GREENBERG, J.R. and TARTOFF, K.D. (1970) Cold Spring Harbor Symp. Quant. Biol., 35, 577 - 587.

PLAGEMAN, P.G.W. (1971a) J. Cell. Physiol., 77, 213 - 240.

PLAGEMAN, P.G.W. (1971b) J. Cell. Physiol., 77, 241 - 258.

PLANTA, R. and MEYERINK, J. (1979) In ribosomes, Structure, Function and Genetics.p. 871-887 ed. CHAMBLISS et al., pub. Academic Press, Baltimore.

POWELL, K.L. and COURTNEY, R.J. (1975) Virology, 66, 217 - 228.

POWELL, K.L. and PURIFOY, D.J.M. (1976) Intervirology, 7, 225 - 239.

PRESTAYAKO, A.W., LEWIS, B.C. and BUSCH, H. (1973) Biochim. Biophys. Acta, 319, 323 - 335.

PRESTAYAKO, A.W., TONATO, M. and BUSCH, H. (1970) J. Mol. Biol., 47, 505 - 515.

PRESTAYAKO, A.W., KLOMP, G.R., SCHMOLL, D.J. and BUSCH, H. (1974) Biochemistry, 13, 1945 - 1951.

PRESTON, C.M. and NEWTON, A.A. (1976) J. Gen. Virol., 33, 471 - 482.

PURIFOY, D.J.M and Powell, K.L. (1976) J. Virol., 19, 717 - 731

RAKUSANOVA, T., BEN-PORAT, T., HIMENO, T. and KAPLAN, A.S. (1971) Virology, 46, 877-889.

RAKUSANOVA, T., BEN-PORAT, T.and KAPLAN, A.S. (1972) Virology, 49, 537-548.

RECHER, L., WHITESCARVER, J. and BRIGGS, L.(1969) J. Ultrastuct. Res., 29, 1-14.

REYNOLDS, R.C., MONTGOMERY, P.O'B. and HUGHES, B. (1964) Cancer Res., 24, 1268-1272.

RO-CHOI, T.S. and BUSCH, H. (1974) In The Cell Nucleus III, 151-208, (BUSCH, H., ed.,), Academic Press, New York and London.

ROEDER, R.G. and RUTTER, W.J. (1969) Nature (London) 224, 234-237.

ROEDER, R.G. and RUTTER, W.J. (1970) Proc. Natl. Acad. Sci. USA, 65, 675-682.

ROIZMAN, B. (1962) Proc. Natl. Acad. Sci. USA, 48, 228-234.

ROIZMAN, B. (1972) in Oncogenesis and Herpesviruses p.1 (P.M. Biggs, G. de The, and L.N. Payne, eds), IARC, Lyon.

ROIZMAN, B. (1977) The Herpesviruses. In "Molecular Biology of Animal Viruses" vol.2, 770-848 (ed. Nayak, D.P.),pub. Mercel Dekker Inc. New York and Basel.

ROIZMAN, B., BACHENHEIMER, S.L., WAGNER, E.K. and SAVAGE, T. (1970) Cold Spring Harbour Symp. Quant. Biol., 35, 753-771.

ROIZMAN, B., BORMAN, G. and KAMALI-ROUSTA, M.(1965) Nature 206, 1374-1375.

ROIZMAN,B. and ROANE, P.R. Jr., (1964) J. Virol., 22, 262-269.

ROIZMAN, B. and FURLONG, D. (1974) in Comprehensive Virology, Vol.3, p.299 (H. Fraenkel Conrat and Wagner R.R. eds.) Plenum, New York.

ROUNDS, D.E., NAKANASHI, Y.H. and POMERAT, C.M. (1960) Antibiotics and Chemotherapy, 10, 597-603.

ROYAL, A. and SIMARD, R. (1975) J. Cell Biol., 66, 577-585.

RUBENSTEIN, A.S., CRAVELL, M. and DARLINGTON, R. (1972) Virology, 50, 287-290.

RUBENSTEIN, A.S. and KAPLAN, A.S. (1975) Virology, 66, 385-392.

RUSSELL, W.C. and CRAWFORD, L.V. (1963) Virology, 21, 353-361.

RYSER, U., FAKAN, S. and BRAUN, R. (1973) Exp. Cell Res., 78, 89-97.

SALTZMAN, N.P., SHATKIN, A.J. and SEBRING, E.D. (1964) J. Mol. Biol., 8, 405-416.

SAROV, I. and ABADY, I. (1975) Virology, 66, 464-473.

SASAKI, Y., SASAKI,R., COHEN, G.H., PIZER, L.T.(1974) Intervirology, 3, 147-161.

SCHEER, U., TRENDELENBERG, M.F. and FRANKE, W.W. (1976) J. Cell Biol., 69, 465-489.

SCHERRER, K. and DARNELL, J.E. (1962) Biochem. Biophys. Res. Commun., 7, 486-490.

SCHLEHOFER, J.R., HABERMEHL, K.-O., DIEFENTHAL, W. and HAMPL, H. (1979) Intervirology, 11, 158-166.

SCHLEHOFER, J.R., HAMPL, H. and HABERMEHL, K.-O. (1979) J. Gen. Virol., 44, 433-442.

SCHOEFL, G.I. (1964) J. Ulrastuct. Res., 10, 224-243.

SCHOLTISSEK, C. (1971) Uur. J. Biochem., 24, 358-365.

SCHMID, W. and SEKERIS, C.E. (1975) Biochim. Biophys. Acta, 402, 244-252.

SCHWARTZ, J. and ROIZMAN,B. (1969) J. Virol., 4, 879-889.

SCHWARTZ, J. and ROIZMAN, B. (1969a) Virology, 38, 42-49.

SHELDRICK, P. and BERTHELOT, N. (1974) Cold Spring Harbour Sump. Quant. Biol., 39, 667-678.

SHELDRICK, P., LAITHER, M., LANDO, D. and RYHINER, M-L. (1973) Proc. Natl. Acad. SCI. USA, 70, 3621-3625.

SHEPERD, J. and MADEN, B.E.H. (1972) Nature, 236, 211-214.

SHINOZUKA, H. (1972) in the Pathology of Transcription and Translation, (E. Farber ed.), Marcel Dekker Inc., New York.

SILVERSTEIN, S. and ENGELHARDT, D.L. (1979) Virology, 95, 334-342.

SIMARD, and BERNHARD, (1967) J. Cell Biol., 34, 61-76.

SIRTORI, C. and BOSISIO-BESTETTI, M. (1967) Cancer Res., 27, 367-376.

SMETANA, K. and BUSCH, H. (1974) In The Cell Nucleus (ed. H. Busch) pp.73-147, Academic Press, New York.

SMETANA, K., FREIRICH, E.J. and BUSCH, H. (1968) Exp. Cell Res., 2,112-128.

SMETANA,K.,UNUMA,T.and BUSCH,H.(1968)Exp.Cell Res.,51,105-122.

SMITH, J.D. and de HARVEN, E. (1973) J. Virol., 12, 919-930.

SNYDER, A.L., KAHN, H.E. and KOHN, K.W. (1971) J. Mol. Biol., 58, 555-565.

SOEIRO, R. (1968), Fed. Proc., 27, Abs., 460.

SPEAR, P.G. and ROIZMAN, B. (1968) Virology, 36, 545-555.

SPEAR, P.G. and ROIZMAN, B. (1972) J. Virol., 9, 143-159.

SPRING, S.B. and ROIZMAN, B. (1968) J. Virol., 2, 979-985.

STAHL, A., HARTUNG, M., CAPODANO, A.M. and FOUET, C. (1976) Humagenetik, 35, 27-34.

STAHL, A., MIRRE, C., HARTUNG, M., KNIBIELER, B. and NAVARRO, A. (1977). IN Chromosomes Today, A. de la Chapelle. M. Sorsa (eds.), Vol.6, pp.255-264. Amsterdam, Elsevier/North Holland.

STAHL, A., MIRRE, C., HARTUNG, M., KNIBIEHLER, B. and NAVARRO, A. (1978) Ann. Biol. Anim., Biochem. Biophys., 18, 399-408.

STANBROOK, P. (1974) J. Mol. Biol., 82, 303-313.

STEIN, S., TODD, P. and MAHONEY (1970) Can, J. Microbiol., 16, 851-854.

STEVELY, W.S. (1975) J. Virol., 16, 944-950.

STEVELY, W.S. (1977) J. Virol., 22, 232-234.

STRINGER, J.F., HOLLAND, L.E., SWANSTROM, R.I., PIVO, K. and WAGNER, E.K. (1977) J. Virol., 21, 889-901.

SWANSTROM, R.I., PIVO, K. and WAGNER. E.K. (1975) Virology, 66, 140-150.

SWANSTROM, R.I. and WAGNER, E.K. (1974) Virology, 60, 522-533.

SWIFT, H. (1959) Symp. Mol. Biol., 266.

TANTRAVAHI, R., MILLER, D.A., DEV, V.G. and MILLER, O.J. (1976) Chromosoma, 56, 15-27.

TEVETHIA, S.S., LOWRY, S., RAWLS, W.E., MELNICK, J.L. and McMILLAN, V. (1972) J. Gen, Virol., 15, 93-97.

TRENDELENBURG, M.F. (1974) Chromosoma, 48, 119-135.

TRENDELENBURG, M.F., SHEER, U. and FRANKE, W.W. (1973) Nature New Biol., 245, 167-170.

UDEM, S.A. and WARNER, J.R. (1972) J. Molec. Biol., 65, 227-242.

UNUMA, T., FLOYD, L.R. and BUSCH, H. (1968) Exp. Cell Res., 52, 101-111-242.

VAGNER-CAPODANO, A.M., DINNA-DELGROSSI, M.H. and STAHL, A. (1977) Biol. Cell., 29, 2-3.

VAGNER-CAPODANO, A.M. and STAHL, A. (1980) Biol. Cell., 37, 293-296.

VANDE WOUDE, G,F, and ASCIONE, R. (1969) J. Virol., 4, 727-737.

VAUGHAN, M.H., SOEIRO, R., WARNER, J.R. and DARNELL, J.E. (1967) Proc. Natn. Acad. Sci. USA., 58, 1527-1534.

WADSWORTH, S., JACOB, R.J. and ROIZMAN, B. (1975) J. Virol., 15, 1487-1497.

WAGNER, E.K. and ROIZMAN, B. (1969) J. Virol., 4, 36-46.

WAGNER, E.K. and ROIZMAN B. (1969à) Proc. Natl. Acad. Sci. USA, 64, 626-633.

WARBURTON, D., HENDERSON, A.S. and ATWOOD, K.C. (1975) Chromosoma, 51, 35-40.

WARNER, J.R. (1974) in Ribosomes (Eds. Nomura, M., Tissieres, A. and Lengyel, P.) p.461-488, Cold Spring Harbour Lab., New York.

WARNER, J.R. and SOEIRO, R. (1967) Proc. Natn Acad. Sci. USA, 58, 1984-1990.

WEINMANN, R. and ROEDER, R.G. (1974) Proc. Natn. Acad. Sci. USA. 71, 1790-1794.

WELLAUER, P.K. and DAWID, I.B. (1973) Proc. Natm. Acad. Sci. USA, 70, 2827-2831.

WELLAUER, P.K., DAWID, I.B., KELLEY, D.E. and PERRY, R.P. (1974) J. Molec. Biol., 89, 397-407.

WESTTRATE, M.W., GEELEN, J.L.M.C. and VAN DER NOORDAA, J. (1980) J. Gen. Virol., 49, 1-21.

WIEGERS, U., KRAMER, G., KLAPPROTH, K. and HILZ, H. (1976) Eur. J. Biochem., 64, 530-540.

WILDY, P. (1972) In Monographs In Virology, vol.5, Basel:Karger.

WILDY, P., RUSSELL, W.C. and HORNE, R.W. (1960) Virology, 12, 204-222.

WILKIE, N.M. and CORTINI, R. (1976) J. Virol., 20, 211-221.

WILLEMS, M., PENMAN, M. and PENMAN, S. (1969). J. Cell Biol., 41, 177-187.

WU, R.S. and WARNER, J.R. (1971) J. Cell Biol., 51, 643-652.

YEO, J., KILLINGHTON, R.A., WATSON, D.H. and POWELL, K.L. (1981) Virology, 108, 256-266.

YEOMAN, L.C., TAYLOR, C.N. and BUSCH, H. (1973) Biochem. Biophys. Res. Comm., 51, 956-966.

YU, F.-L. (1974) Nature (London), 251, 344-346.

YU, F.L. and FEIGELSON, P. (1970) Arch. Biochem. Biophys., 141, 662-667.

YUNIS, J.J. and YASMINEH, W.G. (1971) Science, 174, 1200-1209.

ZEE, Y.C. and TALENS, L. (1972) J. Gen. Virol., 17, 333-336.