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PSEUDORABIES VIRUS INFECTION AND RNA
METABOLISM IN HAMSTER KIDNEY CELLS

by

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A Dissertation Submitted to the
UNIVERSITY OF GLASGOW

for the Degree of

DOCTOR OF PHILOSOPHY

Institute of Biochemistry
University of Glasgow

September 1972

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TO ALAN

And as no chymique yet th'Elixar got,
But glorifies his pregnant pot,
If by the way to him befall
Some odoriferous thing, or medicinall.....

Donne, Loves Alchymie

Lines 7 - 10

ACKNOWLEDGMENTS

I thank Professors J.N. Davidson and R.M.S. Smellie for making the facilities of the Institute of Biochemistry available for this research.

I am deeply indebted to Dr. J. Hay for his encouraging supervision of this work, for many stimulating discussions, and for his generosity with both time and advice.

I wish to thank the many members of the Institute of Biochemistry who have assisted and advised me throughout this project; especial thanks are due to Dr. A.M. Campbell for many valuable discussions about problems relating to liquid scintillation spectrometry, to Dr. B.E.H. Maden for his assistance in the preparation of RNA "fingerprints" and to Dr. J.D. Pitts for help with cell autoradiography. The staff of the Wellcome Cell Culture unit have also been most helpful in their production of cells and virus.

I appreciate the encouragement and moral support I have been fortunate enough to have received from my family and friends, notably my husband, my parents, my siblings, my grandparents and my in-laws. I am also very grateful to my husband for proof-reading the text of this thesis.

I acknowledge with thanks the receipt of a research assistantship from the Cancer Research Campaign throughout the entire duration of this work.

ABBREVIATIONS

The nomenclature system of the "Biochemical Journal" (as described in its "Instructions to Authors", 1972) was generally followed. In addition, the following abbreviations were used :

P.I.	= post infection
p.f.u.	= plaque-forming units
PRV	= pseudorabies virus
HSV	= herpes simplex virus
EAV	= equine abortion virus
pre-tRNA	= precursor to tRNA
hnRNA	= heterogeneous nuclear RNA
TCA	= trichloroacetic acid
SDS	= sodium dodecyl sulphate
EDTA	= ethylenediaminetetraacetic acid
U.V.	= ultraviolet
d.p.m.	= disintegrations per minute
c.p.m.	= counts per minute
$^{32}_{4}\text{PO}_4$	= ^{32}P as inorganic phosphate

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INTRODUCTION

I. GENERAL INTRODUCTION

The study of RNA metabolism is complicated due not only to the large number of different RNA species but also to the fact that most (if not all) of these species are produced via a series of larger precursor molecules, which may appear in different compartments of the cell.

The infection of the mammalian cell, whilst introducing new factors in terms of the expression of the viral genome, may simplify the study of some aspects of host RNA metabolism by the viral suppression of some host functions; only a fraction of the host RNA, and viral RNA, needed for the less complex demands of the virus may be synthesised after infection.

In this thesis, some effects of pseudorabies virus on RNA metabolism are studied. The cells used are tissue culture cells, where the experimental conditions can be carefully controlled. Pseudorabies virus was chosen as the infecting virus because its biology and its effect on cellular protein and DNA synthesis have all been well characterised; also it replicates in the nucleus and the production of its RNA is in the nucleus, hence mimicking normal cell RNA production. This introduction outlines the current knowledge of these topics.

II. GENERAL NATURE OF RNA

1. Chemical Structure of RNA

II.1.(i) Basic Chemistry: RNA is a generic term indicating groups of non-branched polymers of the same basic structure, the units of which are ribonucleoside monophosphates. There are four commonly occurring purine or pyrimidine bases in RNA - adenine, guanine, cytosine and uracil (Fig. I). The bases occur in non-random sequence bound to the C-1 of ribose; the ribose molecules are joined to neighbouring ribose molecules via a 3'-5' phosphodiester linkage. The polymer thus formed has a sugar-phosphate backbone/ ...

Figure 1

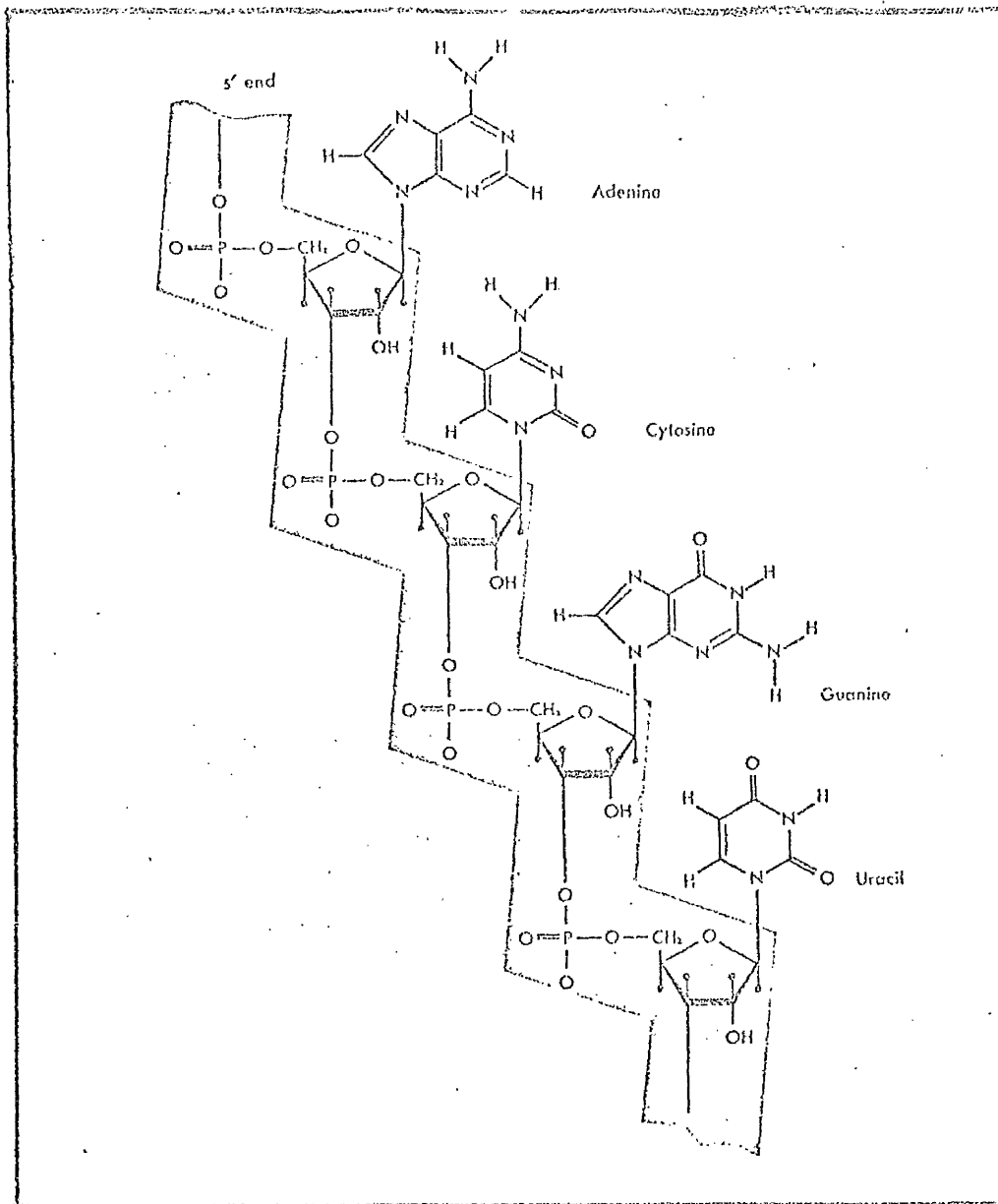


Figure 1. The chemical formula of a polyribonucleotide, showing
the structure of the four main bases (from Watson, 1970)

Figure 2

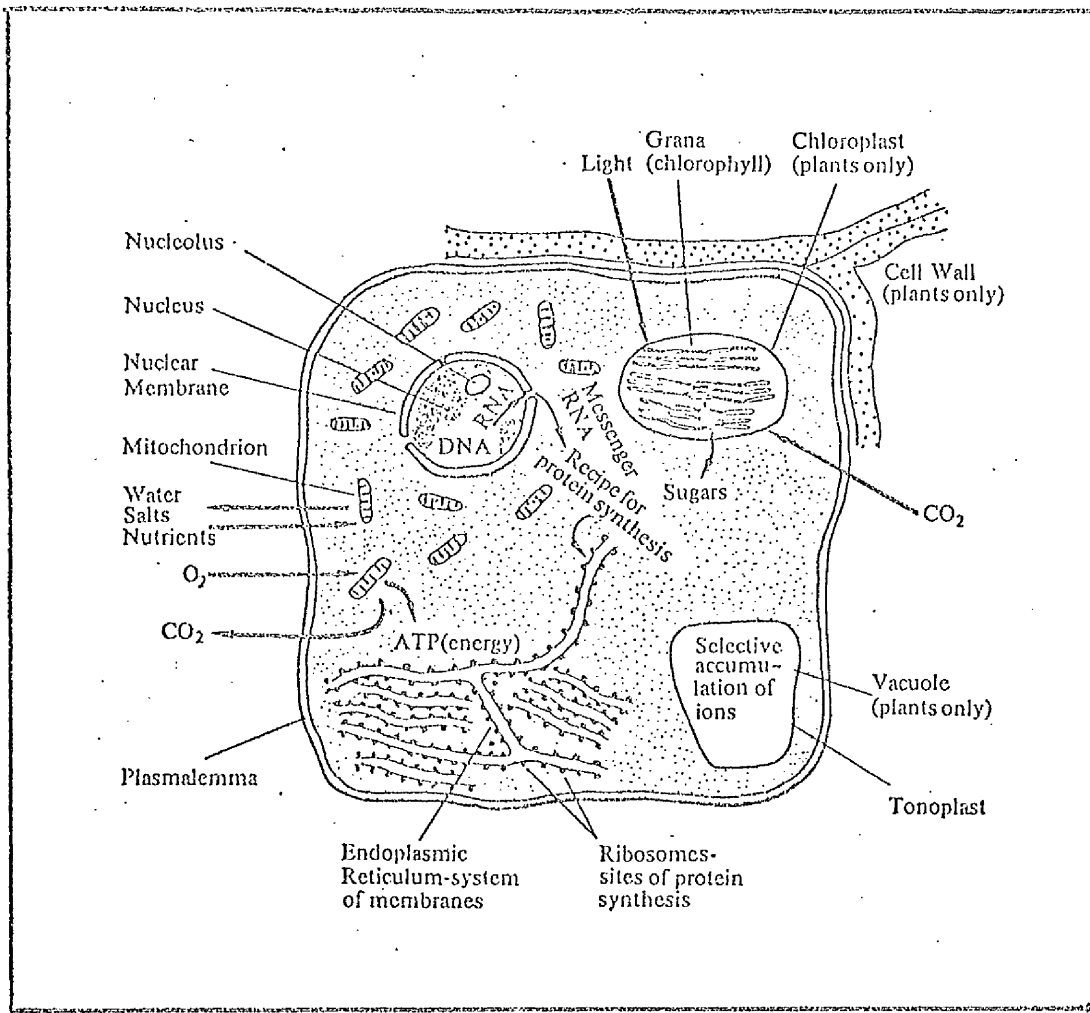


Figure 2. Highly schematic structure of a generalised eukaryotic cell (from Edwards & Hassall, 1971)

backbone (Fig.I.). It is an important feature of RNA chemistry that the 2' -OH group on the ribose is not esterified. Thus RNA is susceptible to alkali digestion, forming a mixture of 2' and 3' ribonucleoside monophosphates, via the intermediate cyclic 2', 3' ribonucleoside monophosphates (Brown & Todd, 1955).

II.1.(ii) Minor bases and nucleosides: Apart from the four main bases, small amounts of other bases occur in RNA. Many of these bases are methylated derivatives of the four major bases; the methyl group is attached either to the base or the 2'-OH position of the ribose moiety (Table I). The presence of a methyl group on the 2'-O-ribose position confers alkali-resistance on the phosphodiester linkage from the 3'-O-ribose position.

II.2. Ubiquity and Diversity of RNA species.

All living matter (with the possible exception of the DNA-containing viruses) contains at least one species of RNA. RNA is the genetic material in the case of the RNA viruses.

Probably all cellular RNA is transcribed from DNA by the enzyme DNA-dependent RNA polymerase. The main species of RNA, occurring in all cell types, are the following: ribosomal RNA, messenger RNA and transfer RNA. In mammalian cells the following RNA species also occur: heterogeneous nuclear RNA, mitochondrial RNA, chromosomal RNA and small nuclear RNA.

III. RNA IN PROKARYOTIC AND EUKARYOTIC CELLS - WITH EMPHASIS ON MAMMALIAN CELLS.

III.1. General Biology

The distribution of the various RNA species in eukaryotic cells is diagrammatically shown in Fig. 2. The main difference between bacterial and eukaryotic cell types is the compartmentalisation of the eukaryotic cell.

III. 2. Ribosomal RNA. /...

Table 1.

Unusual bases and nucleosides in RNA (from Low, 1970)

Dihydrouracil	1-methyluracil
5-hydroxyuracil	3-methyluracil
2-thiouracil	5-methyluracil (thymine)
4-thiouracil	5-hydroxymethyluracil
1,5-diribosyluracil	5-methylaminomethyl-2-thiouracil
5-ribosyluracil	3-methylcytosine
2-thiocytosine	N ⁴ -methylcytosine
N ⁶ -acetylcytosine	5-methylcytosine
N ⁶ -aminoacyladenine	5-hydroxymethylcytosine
N ² -ribosylguanine	6-amino-N ⁵ -methylformamido-isocytosine
N ³ -ribosylguanine	N ¹ -methylcytosine
Orotic acid	1-methyladenine
Hypoxanthine	2-methyladenine
Xanthine	7-methyladenine
Pseudouridine	6-methyladenine
2 ^l (-3 ^l)-O-ribosyl-adenine	6-dimethyladenine
2 ^l -O-methyladenosine	N ⁶ ,N ⁶ -dimethyladenine
2 ^l -O-methylcytidine	1-methyl-N ⁶ -methyladenine
2 ^l -O-methylguanosine	1-Δ ² -isopentenyladenine
2 ^l -O-methyluridine	N ⁶ -Δ ² -isopentenyladenine
2 ^l -O-methylpseudouridine	2-methylthio-N ⁶ -isopentenyladenine
7-methylxanthine	1-methylguanine
1-methylhypoxanthine	7-methylguanine
	N ² -methylguanine
	N ² ,N ² -dimethylguanine

III. 2. Ribosomal RNA.

Ribosomal RNA molecules are the species of RNA which complex with specific proteins to form the ribonucleoprotein units called ribosomes. There are three classes of ribosomes: 80S ribosomes in eukaryotes, 70S ribosomes in bacteria and 55-60S ribosomes in organelles; the latter two types have many similarities (see section III.7). Ribosomes from all species are composed of two dissimilar ribonucleoprotein entities. Each subunit has one main species of RNA; the larger subunit has also one molecule of 5S RNA and, in some eukaryotes, also one molecule of 7S RNA (see section III.2.(iii)). A difference between prokaryotes and eukaryotes is in the production of their ribosomal RNAs; in the animal cell there is a complex sequence of larger ephemeral precursor molecules, which are specifically trimmed into shape. This will be discussed in Section III.2.(ii).

III.2.(i) The structure of high molecular weight ribosomal RNA.

a) Size.

The two high molecular weight rRNA components in the bacterial ribosome are 23S and 16S RNAs, corresponding to a molecular weight of about 1.1 and 0.55 million daltons respectively. All eukaryotic organisms have a similar low molecular weight rRNA of 18S (0.7 million daltons); the larger rRNA increases in size in relation to the evolutionary position of the organism from 25-28S (1.4-1.9 million daltons). The molecular weights of the two major rRNA components from many species have been determined by Loening, 1968.

III.2.(i)b) Nucleotide composition.

Major species:- The nucleotide composition of the two major rRNA species has been summarised and analysed by Amaldi, 1969. The G+C content, especially of the larger rRNA species, increases on ascending the/...

the evolutionary scale (as does size), the range being from 40% G+C in certain invertebrates to 67% G+C in mammalian rRNA. In mammalian cells the 28S RNA has a higher G+C content than the 18S RNA eg. 67% and 59% respectively in HeLa cells (Amaldi & Attardi, 1968); it should be noted that, as the G+C content of mammalian DNA is 40-44%, ribosomal RNA is not "DNA-like". Another change in ascending the evolutionary scale is an increase in secondary structure (Amaldi, 1969).

Pseudouridine:- The major rRNA components contain a low proportion of unusual nucleotides. Pseudouridine is present in small amounts in both bacterial and eukaryotic cell rRNA (Dubin & Gunlap, 1967; Amaldi & Attardi, 1968); there is more pseudouridine in animal cells than in E.Coli. eg., in HeLa cells there is 1.1 and 1.5 moles% pseudouridine in 28S and 18S RNA respectively.

Methylated nucleotides:- All known major rRNA species have methylated components. Bacteria have most of their methyl groups on various bases, the remaining few being 2'-O-methyl ribose substituents (Dubin & Gunlap, 1967; Fellner & Sanger, 1968). Animal and plant cells have about 12 and 18 methyl groups per 1000 nucleotides in 28S and 18S RNA respectively, which is a rather higher level of methylation than occurs in bacterial rRNA (Vaughan et al., 1967; Wagner, Penman & Ingram, 1967; Lane & Tamaoki, 1967). The figures for distribution of the methyl label in mammalian cells vary from about 50 - 80% being 2'-O-methyl ribose (Brown & Attardi, 1965; Iwanami & Brown, 1968), the rest being methylated bases (Table I). The addition of methyl groups is an integral part of the maturation of rRNA (see next section).

III.2.(ii). Maturation and metabolism of high molecular weight ribosomal RNA

The production of rRNA differs considerably in bacterial and mammalian cells. In bacteria, the two major rRNA species are synthesised separately, and/...

and the primary transcription product is believed to be only slightly larger than the mature species (Hecht & Woese, 1968; Attardi & Analdi, 1970); in mammalian cells both major rRNA species have a much larger, common precursor; also maturation takes place in a specialised organelle.

III.2.(ii).a). The maturation sequence in mammalian cells.

The maturation process of ribosomal RNA can be summed-up as follows: 45S RNA is the common rRNA precursor and it is formed in the nucleolus; it is subsequently methylated and cleaved to form 28S RNA and 18S RNA via a series of larger, ephemeral intermediates; 45S RNA and all the other intermediates exist in ribonucleoprotein complexes. The sequence of events producing mature 28S and 18S RNA, and some of the evidence for the scheme, are detailed below.

Kinetic evidence obtained by various workers using HeLa cells (see summary and discussion by Darnell, 1968) showed that a short pulse-label of radioactive RNA precursor does not label the stable 28S and 18S RNA species but labels two other main species. One of these is a very large, heterodisperse nucleoplasmic species (see section III.4); the other is a discrete species, 45S RNA, located in the nucleolus (Scherzer, Latham & Darnell, 1963; Penman, Smith & Holtzman, 1966). On introducing a longer pulse-label (of about 20min) a 32S RNA peak of radioactivity also appears, at the same time as the 18S RNA peak; only later does the 28S RNA peak appear. The precursor-product relationship between 45S RNA and 28S and 18S RNAs suggested by this is confirmed by the use of actinomycin D (Girard, Penman & Darnell, 1964; Perry & Kelley, 1970). The development of polyacrylamide gel electrophoresis (Loening, 1967) permitted the resolution of other rRNA precursors apart from 45S and 32S; these species are 41S and 20S RNAs, which are normally present in very small amounts but accumulate in poliovirus-infected cells (Weinberg et al., 1967); the 36S and 24S species which are also found are probably artefacts due to aberrant cleavage - see Fig.3.

Figure 3

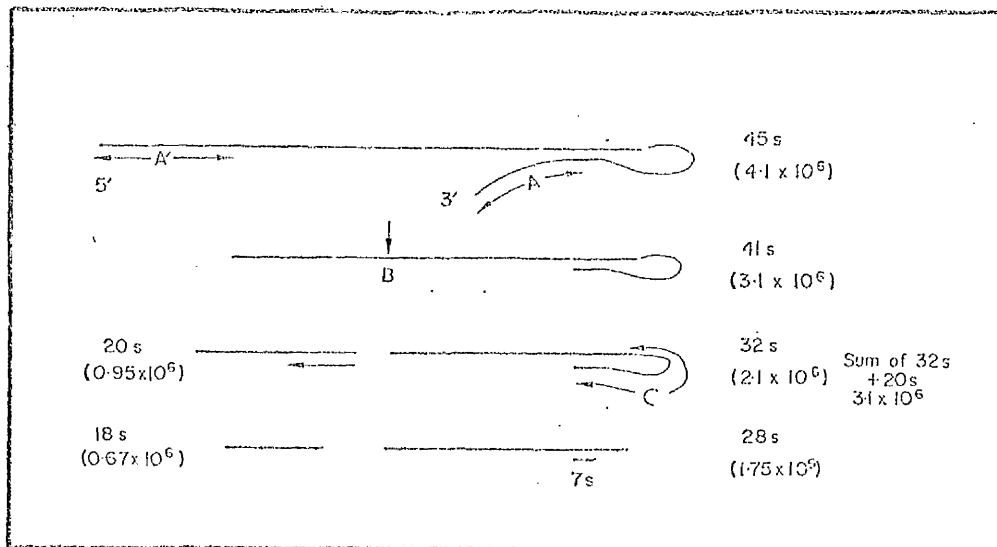


Figure 3. The maturation of ribosomal RNA (from Maden, 1970)

This is a possible arrangement for ribosomal and non-ribosomal sequences in mammalian (HeLa) cell ribosomal precursor RNAs, where the heavy lines represent the conserved ribosomal sequences. The sequence of events is as follows :-

1. Removal of A and A' \longrightarrow 41S (containing 28S and 18S RNA sequences).
2. Cleavage at B \longrightarrow 32S RNA + 20S RNA .

Aberrant cleavage: Cleavage at B before removal of A and A' \longrightarrow 36S + 24S RNAs.

In the scheme for production of 28S and 18S RNAs, two other RNA species also appear, 7S RNA and 5S RNA (Fig.3). 7S originates during the development of 28S RNA, from the same precursor, while 5S RNA has a completely separate origin (see section III.2.(iii)).

The precursors of rRNA have been studied with regard to molecular weight (McConkey & Hopkins, 1969), base composition (Scherrer *et al.*, 1963; Willems *et al.*, 1968), pseudouridine formation (Amaldi & Attardi, 1968; Jeanteur, Amaldi & Attardi, 1968) and methylation (Burdon, 1966; Wagner, Penman & Ingram, 1967; Vaughan *et al.*, 1967; Weinberg *et al.*, 1967; Weinberg & Penman, 1970; Maden, Salim & Summers, 1972). Such studies have not only characterised these RNA species but have also confirmed the precursor-product relationship detailed above and in Fig. 3. These studies have shown that 45S RNA is the substrate for methylation and pseudouridine formation; the regions excised during the non-conservative transition of 45S RNA to the mature 28S and 18S ribosomal RNAs are methyl-poor, pseudouridine-poor and G+C rich.

III.2.(iii) Ribosomal RNA - low molecular weight species.

There are two low molecular weight ribosomal RNA species, 5S RNA and 7S RNA. Their origin is completely different, and so they will be discussed separately in the next two sections.

III.2.(iii).a). 7S RNA.

This species of RNA, which is 130 nucleotides long and has a nucleotide composition of 56-58% G+C, has been described by Pène, Knight & Darnell, 1968, Weinberg & Penman, 1968 and Prestayko, Tonato & Busch, 1970. It is not found in bacteria, and occurs in mammalian cells hydrogen-bonded to 28S RNA. 7S RNA has been shown to derive from 45S RNA, but it is not released from 45S RNA by techniques which disrupt hydrogen bonds; 7S RNA is believed to arise in the transition from 32S to 28S RNA as shown in Fig.3.

III.2.(iii). b). 5S RNA.

The major ribosomal subunit from almost all bacterial and eukaryotic systems studied contains in a 1:1 molar ratio per subunit a low molecular weight RNA species with a sedimentation coefficient of 5S (Galibert et al., 1965). A large amount of work has been carried out on the structure and synthesis of 5S RNA; because of its particular relevance to this thesis, various aspects of this work will be discussed in some detail.

Structure of 5S RNA: 5S RNA - from all sources - contains no methylated bases or pseudouridine (Galibert et al., 1965; Brownlee, Sanger & Barrell, 1967). The G+C content has been reported to be 58-60% in mammalian cells (Forget & Weissman, 1967a; Hatlen, Amaldi & Attardi, 1969) and 64% in E.Coli (Brownlee et al., 1967). 5S RNA has been completely sequenced in E.Coli (Brownlee, Sanger & Barrell, 1967 and 1968) and in KB cells (Forget & Weissman, 1967b). The sequence from two mouse cell lines is probably identical to the human (KB) 5S RNA (Williamson & Brownlee, 1969). The 5S RNA from KB cells is 120 or 121 nucleotides long (there is sometimes an extra uridylic acid residue at the 3' - terminus); if, in addition to Watson-Crick base-pairing one accepts occasional base-pairing of guanylic acid to uridylic acid, then long sequences of complementary nucleotides can be identified within the molecule, and Rasko, 1968 has proposed a cloverleaf conformation for 5S RNA similar to that generally accepted for 4S RNA (Fig.4).

It is of interest that, even although the bacterial and mammalian 5S RNA molecules are different, all the 5S RNAs sequenced so far have two sequences present (UGAAC and GAAU) which are complementary in an antiparallel fashion to the tetra-nucleotide GTTC found in all known tRNAs; this implicates 5S RNA in the binding of the tRNA to the ribosome (as has already been suggested from other evidence for E.Coli by Siddiqui & ...

Figure 4

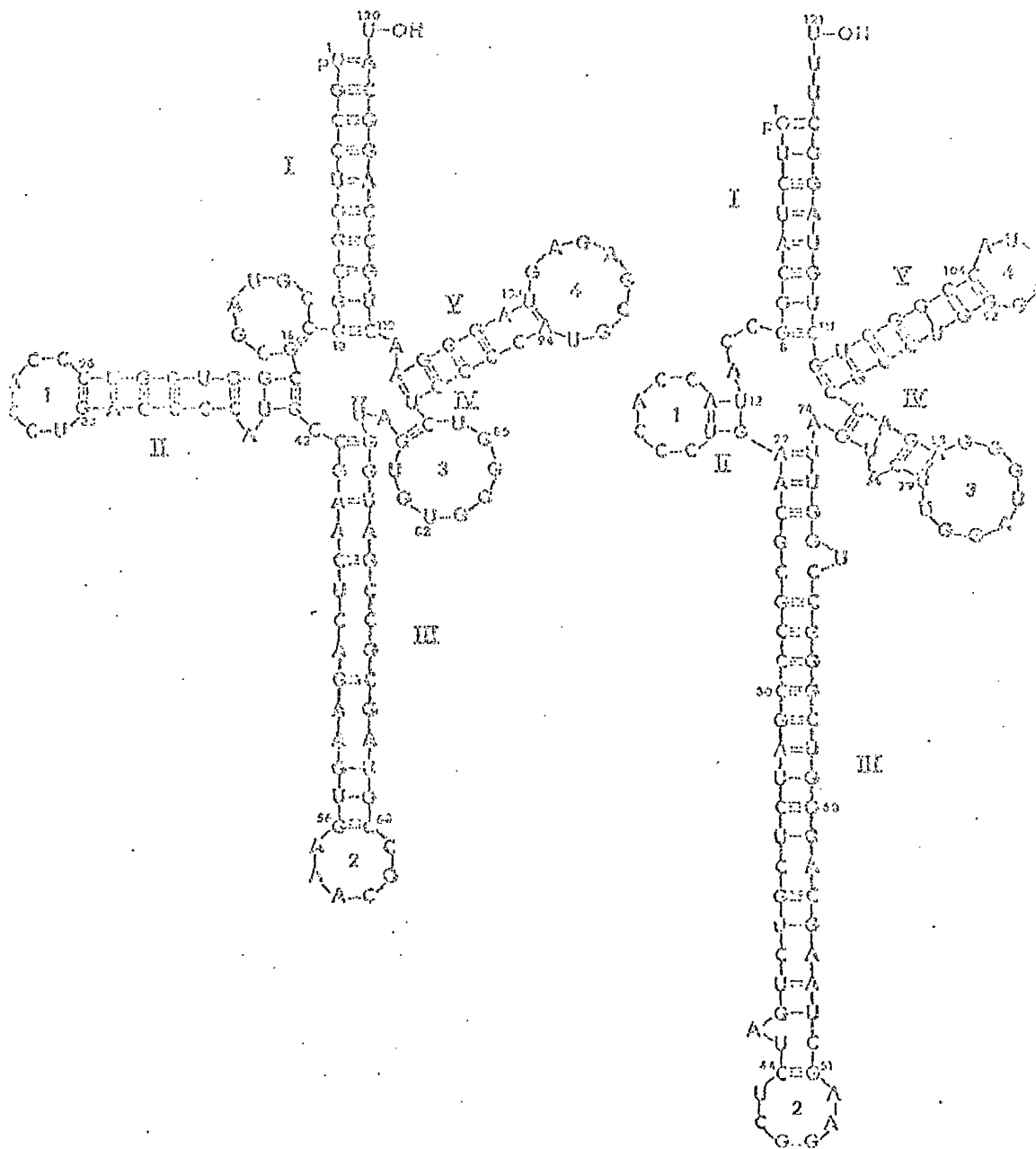


Figure 4. Proposed cloverleaf structures for 5S RNA (from Rasacke, 1968)

& Hosokawa, 1969). The fact that the sequences of 5S RNA from man and mouse are probably identical (Williamson & Brownlee, 1969) implies a strong selective pressure in favour of the conserved sequence and again suggests a functional significance for 5S RNA.

Another interesting feature of 5S RNA is that HeLa cell 5S RNA has alternative 5'-termini: pG, ppG or pppG (Hatlen, Amaldi & Attardi, 1969).

Release of 5S RNA from the ribosome: The release of 5S RNA from the bacterial ribosome can be achieved by mild treatment with EDTA (Siddiqui & Hosokawa, 1969) but stronger methods are required to release 5S RNA from the mammalian ribosome (Knight & Darnell, 1967; Zehavi-Willner, 1970). Although it was formerly believed that 5S RNA is released as the free ribonucleic acid (Zehavi-Willner, 1970) recent evidence suggests that 5S RNA is removed as a ribonucleoprotein sedimenting at about 7S, the protein component having a molecular weight of about 35,000 daltons (Blobel, 1971). There is a concomitant drop in S value of the larger subunit, which is too big to be accounted for by the loss in mass, and is therefore probably the result of conformational change; this resulting subunit is also biologically inactive.

Synthesis of 5S RNA in bacterial cells: 5S RNA in *E. Coli* is believed to be first transcribed in the form of a longer polynucleotide (Monier et al., 1969; Doolittle & Pace, 1970), and it is probably cleaved to form mature 5S RNA after the entry of precursor into the 50S subunit (Monier et al., 1969).

Synthesis and distribution of 5S RNA in mammalian cells: This is the one species of ribosomal RNA that does not derive from 45S RNA, and it is synthesised in the nucleoplasm. This conclusion has been based on evidence from various sources; these have included the use of anucleolar mutants of/...

of Xenopus laevis (Brown & Weber, 1968), gene amplification studies in Xenopus laevis (Brown & Dawid, 1968) and studies on the differential effects of actinomycin D on various RNA species (Burdon, Martin & Lal, 1967; Perry & Kelley, 1968, 1970).

Although 5S RNA is synthesised in the nucleoplasm, it becomes rapidly associated with 45S RNA in the nascent ribosomal particle (Warner & Soeiro, 1967). The molar ratio of 5S RNA to 28S RNA is 1:1 in polyribosomes, cytoplasmic ribosomes, the larger ribosomal subunits and in nuclear 50S subunits, and there is no 5S RNA associated with the small ribosomal subunit (Knight & Darnell, 1967); the nuclear 5S RNA is chemically indistinguishable from cytoplasmic 5S RNA (Knight & Darnell, 1967).

Kinetic data (Knight & Darnell, 1967) and direct measurement (Weinberg & Penman, 1968; Perry & Kelley, 1968) have established that in the nucleus there is a large "pool" of 5S RNA.

III.3. Transfer RNA

The existence of this group of RNA molecules was first postulated by Crick, 1955 who proposed the existence of "adaptor" molecules to interact specifically with both aminoacids and the messenger RNA. About 15% of the cell RNA occurs as tRNA molecules, which sediment at around 4S and which function in protein biosynthesis as specific transporters of the amino acids. In all cell types, tRNA is a mixture of RNA molecules of molecular weight 25,000 - 30,000 corresponding to chain lengths of 75 - 85 nucleotides (Phillips, 1969). Transfer RNA is found free in the cytoplasm and also attached to the ribosomes (eg. Holder & Lingrel, 1970; Fig. 8).

III.3.(i) Structure of tRNA

tRNA contains a high proportion of atypical nucleotides such as methylated nucleotides, dihydrouridylic acid, thiouridylic acid, pseudouridylic acid and others which will be discussed in section

III.3.(ii) /...

Figure 5

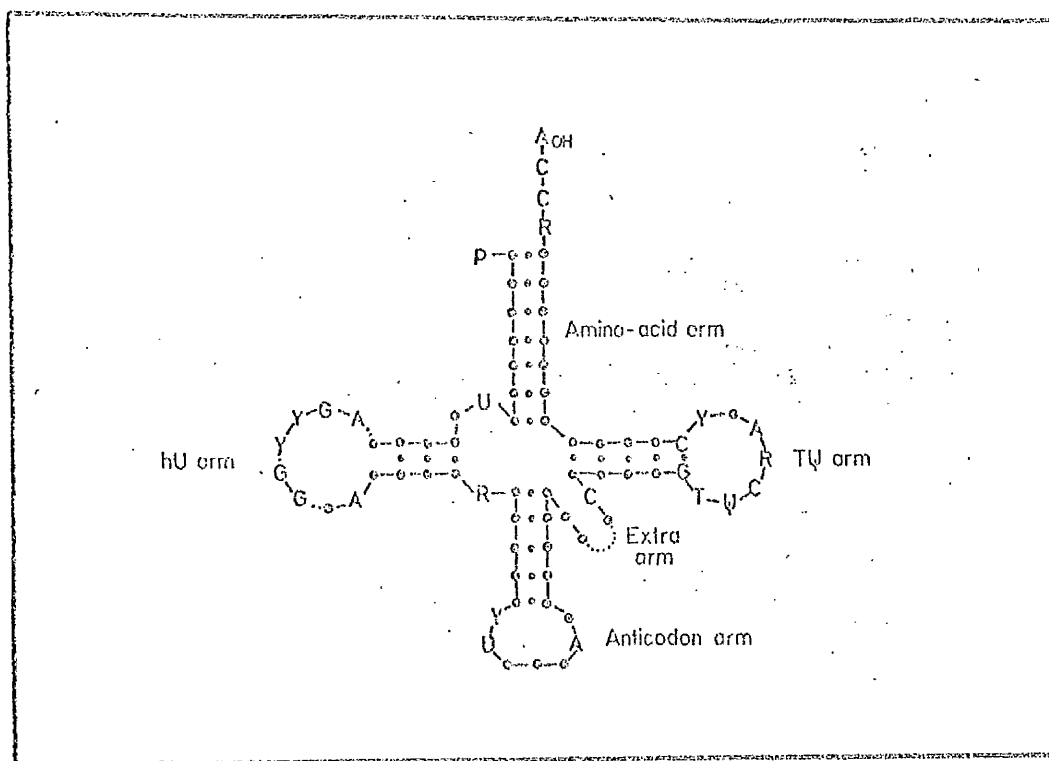


Figure 5. General cloverleaf structure for tRNA (from Cramer, 1971)

Y = pyrimidine nucleoside

R = purine nucleoside

III.3.(ii) (maturation of tRNA). The complete primary structure of several tRNAs from bacteria, yeast, plant and mammalian cells has been obtained (Zachau, 1969; Phillips, 1969). The first complete sequence of a transfer RNA was obtained by Holley et al., 1965 for tRNA^{ALA} from yeast. The cloverleaf structure which he proposed for it has proved amenable to the primary sequence of all known tRNAs, and this alone suggests that the cloverleaf model is substantially correct. A generalised structure from Cramer, 1971 is given in Fig. 5.

Note that in this cloverleaf structure all the unusual bases occur in the unpaired regions; this is especially obvious on comparison of a few complete structures (eg. Zachau, 1969). This may have structural significance since it is the non-paired regions which can move or bend when the molecule is in solution giving the tertiary structure of tRNA. Many methods have been used to obtain a knowledge of the tertiary structure of tRNA, eg. physical methods (Henley et al., 1966; Pilz et al., 1970) and chemical modification methods (Zachau, 1969 for several examples). Using such evidence several models for the tertiary structure of tRNA have been proposed; six of them have been detailed and critically discussed by Cramer, 1971. Fig. 6 is a diagram of one of the proposed structures of tRNA.

Only recently have crystals of tRNA been available (Hampel & Bock, 1970) and this, especially with the development of isomorphous derivatives, should yield useful information about the tertiary structure of tRNA.

III.3(ii) Maturation of tRNA

a) Maturation of tRNA in mammalian cells

There is now a substantial amount of evidence to show that tRNA has a longer precursor RNA molecule; the conversion of this molecule to the mature/...

Figure 6

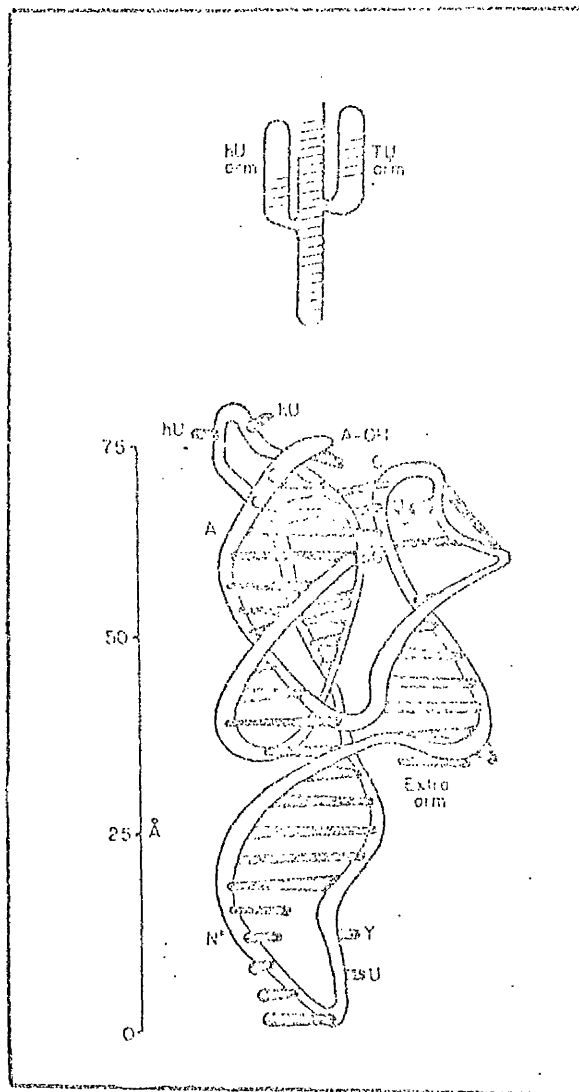


Figure 6. A proposed tertiary structure for tRNA (from Cramer, 1971)

- a) Schematic drawing
- b) Detailed structural model

mature tRNA involves scission of the molecule and also modification of certain of the nucleosides.

The first evidence for a longer precursor to tRNA (pre-tRNA) was obtained in Krebs 2 ascites tumour cells (Lal & Burdon, 1967; Burdon, Martin & Lal, 1967; Burdon, 1967a) and pre-tRNA has since been demonstrated in HeLa cells (Bernhardt & Darnell, 1969), in human lymphocytes (Kay & Cooper, 1969) and also in insects (Egyházi et al., 1969); pre-tRNA also occurs in bacteria - see section III.3.(ii) b). When mammalian tissue culture cells are labelled for very short periods (5 - 10 min) with radioactive nucleosides and the cytoplasmic RNA is extracted and fractionated, the labelling pattern does not correspond to the absorbancy pattern; in the low molecular weight region only 5S RNA and a rather heterogeneous new peak, termed " $4\frac{1}{2}$ S", eluting between 5S and 4S RNAs appear. When longer labelling times (say 30 min) are used, this " $4\frac{1}{2}$ S" RNA peak shifts and coelutes with 4S RNA (Lal & Burdon, 1967; Bernhardt & Darnell, 1969). This suggests a precursor-product relationship between " $4\frac{1}{2}$ S" RNA and 4S RNA, and such a relationship is confirmed by a "pulse-chase" experiment using actinomycin D (Lal & Burdon, 1967).

It was possible that the " $4\frac{1}{2}$ S" RNA has merely a different conformation than 4S RNA, but is not longer. That this is partly true is shown by the fact that heating cytoplasmic RNA at 60° for 5min in the presence of 0.02M MgCl₂ causes the " $4\frac{1}{2}$ S" RNA to elute in a position somewhat closer to 4S RNA, but not exactly in the 4S RNA position (Burdon, 1967a). The suggestion that the maturing process of " $4\frac{1}{2}$ S" → 4S RNA involves scission of a larger molecule was confirmed by the ability of a cell-free cytoplasmic extract, in the absence of cofactors, to convert in vitro partially-purified pre-tRNA to a species that co-elutes with 4S RNA, releasing about 31 nucleotide residues (Smillie & Burdon, 1970); the product is undermethylated and deficient in pseudouridine. Similar results have been reported by Nowshowitz, 1970.

Figure 7

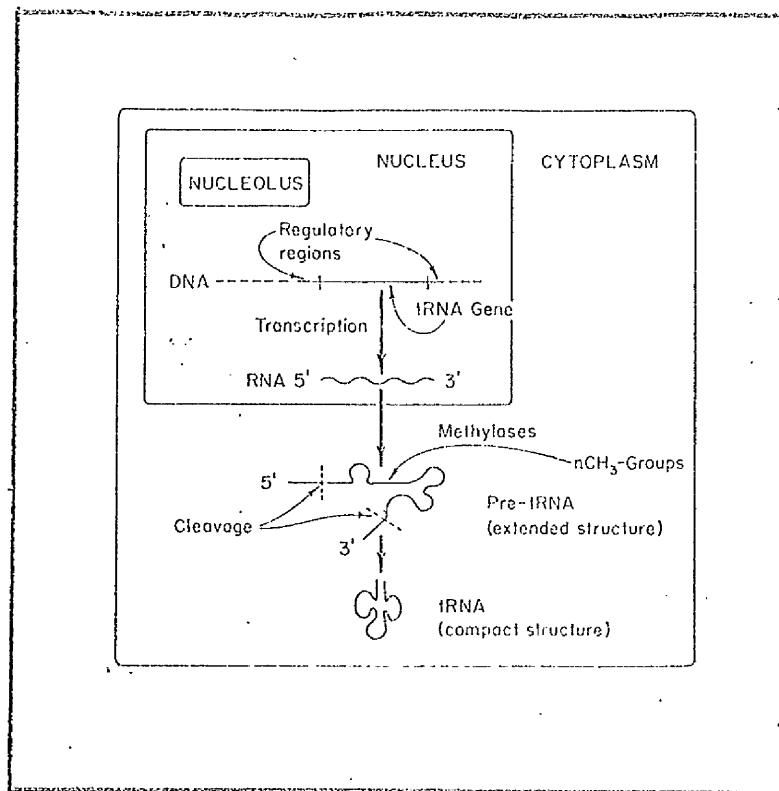


Figure 7. A scheme showing the possible steps involved in the production of mammalian cell tRNA and their intracellular location (from Burdon, 1971).

20

Location of pre-tRNA: Precursors to tRNA have been located in the soluble fraction of the cell sap only and not associated with protein (Burdon & Clason, 1969); in this respect it is of interest that tRNA transmethylase activity also appears predominantly in the cell sap (Burdon, Martin & Lal, 1967). This is in contrast to ribosomal RNA, which does not appear in the cell sap until fully methylated (Weinberg et al., 1967). Thus pre-tRNA does not seem to accumulate in the nucleus after it is transcribed but immediately migrates to the cytoplasm (Weinberg & Penman, 1968). The maturation process of tRNA is diagrammatically illustrated in Fig. 7.

III. 3. (ii) b) Maturation of tRNA in bacterial cells.

The evidence for precursors of tRNA in mammalian cells rests chiefly on the kinetic evidence described in the previous section (III.3.(ii) a)). In bacterial cells, it has not been possible under normal conditions to show the presence of a short-lived tRNA precursor (because of the extremely brief pulse-labelling period that would be necessary). However, indirect kinetic evidence for tRNA precursors in bacteria has been obtained using rifampicin to measure the time taken to transcribe the primary gene product (Vickers & Midgley, 1971). Much work has been done using the bacteriophage $\phi 80$ carrying the gene for tyrosine SU_{III}^{+} tRNA and various mutants of this gene; tyrosine pre-tRNA has been isolated from E.Coli and sequenced (Altman, 1971; Altman & Smith, 1971). The characteristics of this precursor are compatible with what is known about pre-tRNA in mammalian cells, regarding kinetics of maturation and the fact that it is likely to be the unmodified polynucleotide chain that is the substrate for cleavage.

Tyr SU_{III}^{+} pre-tRNA has a long extra sequence at the 5' -end of 38 nucleotides, terminating in pppG; at the 3' -end there seem to be only three/...

three nucleotides beyond the CCA terminus viz UCU_{OH} . It is of interest that the CCA terminus seems to be part of the primary transcription product, contrary to what has been generally believed (section III.3.(ii)e)).

Recently, in vitro synthesising systems have been used successfully. Ikeda (1971) has synthesised in vitro the precursor tRNA which Altman isolated from the in vivo system. Also, the DNA-directed cell-free synthesis of biologically active tRNA has been described (Zubay, Cheong & Gefter, 1971) and this may well be a new fruitful approach for probing the mechanisms of tRNA synthesis.

III.3.(ii)c). Methylation of tRNA

Transfer RNA has a very high proportion of unusual nucleotides; more than thirty different types are known, most of which are derived from the four main nucleotides through methylation of the C, O, or N on the base; methylation of the ribose in the 2'-OH position also occurs and mono- and di-derivatives are also found (see Table I). Net methylation of nucleotides in unfractionated tRNA is 2.5 - 7%, 80 - 90% of this being base modification, unlike ribosomal RNA (Lane & Tamaoki, 1969). The major species of methylated nucleosides in both HeLa and mouse L cells are 1-methyl adenosine, 1-methyl guanosine, N^2 -methyl uridine (thymidine) and, in smaller amounts, 1-methyl hypoxanthine, 3-methyl cytosine and 3-methyl uridine (Iwanami & Brown, 1968).

Methylation occurs at polynucleotide level (see Fig. 7), the donor being the methyl group of methionine - even for the formation of ribothymine (thymidine), and various studies were performed on the methylation process of tRNA even before it was realised that there is a larger precursor.

Since it is known that methylation occurs at polynucleotide level, the question arises whether pre-tRNA is methylated or not. Labelling studies have shown that pre-tRNA is undermethylated and that if methylation is/ ...

is suppressed methyl-deficient 4S RNA accumulates which can subsequently be methylated on restoring methionine (Lal & Burdon, 1967; Bernhardt & Darnell, 1969). This differs from the situation observed in the maturation of ribosomal RNA where unmethylated ribosomal precursor RNA is completely degraded (Vaughan et al., 1967).

Thus it seems that methylation, although it occurs during the processing of pre-tRNA, is not a prerequisite for the production of "4S RNA", since undermethylated 4S RNA can subsequently be methylated. However, for true biological activity as transfer RNA, methylation is almost certainly necessary (Capra & Peterkofsky, 1968; Stern et al., 1970).

III. 3. (ii) d). Pseudouridine formation in tRNA

All tRNAs contain pseudouridine (= 5 ribouracil) which is the most frequently occurring minor nucleoside. In animal cells there are about three pseudouridine residues per tRNA molecule, which is more than occurs in yeast tRNA, which in turn is more modified than bacterial tRNA, but even T4 phage-induced tRNA contains some pseudouridine (Daniel, Sarid & Littauer, 1968). Pseudouridine formation is believed to be a post-transcriptional enzymically-catalysed event (Johnson & Soll, 1970). Pseudouridine formation in pre-tRNA and tRNA has been studied and, like methylation, conversion to pseudouridine occurs during maturation of pre-tRNA but is not a prerequisite of maturation (Smillie & Burdon, 1970; Mowshowitz, 1970).

III. 3. (ii) e). The CCA terminus

It is believed that the 3'-terminal trinucleotide sequence -CCA_{OH} which is common to all tRNA molecules is missing in the newly-formed tRNA, since in both animal cells and microorganisms there is an enzyme which / ...

which attaches the three nucleotides on to tRNA in a DNA-independent reaction (eg. Deutscher, 1972).

The -CCA sequence also seems to be absent from some tRNA cistrons (Daniel, Sassi & Littauer, 1970). However, tyrosine pre-tRNA from E.Coli which has been sequenced by Altman & Smith, 1971 contains the trinucleotide sequence CCA close to the 3'-terminus as part of the primary transcription product.

III.3.(ii) f). Other modifications of tRNA

There are several other modifications of tRNA which are believed to take place at the polynucleotide level, eg. thiolation, formation of isopentenyl adenosine and formation of dihydrouridine; the current knowledge of the biosynthesis of these modified nucleosides has been summarised by Söll, 1971.

III. 4. Heterogeneous Nuclear RNA.

III.4. (i). Structure and synthesis

As discussed in section III.2(ii), when mammalian cells are labelled for a short time, two types of nuclear high molecular weight RNA become labelled. One is ribosomal precursor RNA - a homogeneous 45S peak; the other consists of heterogeneous nuclear RNA (hnRNA) of a very high molecular weight. This hnRNA has the following characteristics which distinguish it from ribosomal precursor RNA. It has a heterogeneous sedimentation profile of 20 - 80S (Warner et al., 1966; Lindberg & Darnell, 1970); it has a nucleotide composition of 44% G+C (Soiero, Birnboim & Darnell, 1966); it has a rapid rate of labelling and it turns over rapidly (Warner et al., 1966); it is unstable in actinomycin-treated cells, unlike ribosomal precursor (Girard et al., 1964; Warner et al., 1966); it is largely found in the nucleoplasm while all of 45S RNA is nucleolar (Soiero et al., 1966).

III. 4. (ii) Function of hnRNA-possible relationship to mRNA

HnRNA must either be a precursor of another RNA fraction (because of its transitory appearance) or else become degraded without leaving the nucleus. Soiero et al., 1968a calculated that only about 10% of hnRNA is exported to the cytoplasm, the rest being degraded. The only cytoplasmic fraction which could logically derive from hnRNA (because of its G+C content) is cytoplasmic messenger RNA. Recent results using a variety of techniques have substantiated this conclusion eg. the presence of covalently-bound poly A sequences in both types of RNA (Edmonds, Vaughan & Nakazato, 1971; Darnell, Wall & Tushinski, 1971; section III.5) and also molecular hybridisation data (Lindberg & Darnell, 1970; Darnell et al., 1970; Georgiev et al., 1972; Melli & Pemberton, 1972).

III. 5. Messenger RNA

The concept of messenger RNA was developed by Jacob & Monod, 1961a before the experimental demonstration; they conceived of it as an unstable direct carrier of genetic information between DNA and the protein-synthesising apparatus. The mRNA is a transcript of the information of the DNA which is translated into protein on the ribosome.

III. 5. (i) The messenger in bacteria.

Since the messenger is the carrier which brings the information to code for protein, there must be a large number of messenger RNAs. This means that mRNA is heterogeneous and this, together with the instability of the bacterial messenger, its small proportion of the cell RNA (2 - 3%) and the short labelling time necessary to distinguish mRNA from rRNA has made study of the messenger difficult. Initial problems were overcome by the use of E.Coli infected with the T-even bacteriophages; in such cells host RNA synthesis stops and no RNA except phage-specific RNA is made; thus longer labelling times could be used (Brenner, Jacob & Meselson, 1961; Gros/...

Gros et al., 1961b). The messenger was originally identified as such since its base composition is similar to that of the infecting virus DNA (Gros et al., 1961a), and it hybridises to virus DNA (Hall & Spiegelman, 1961). Results showing the existence of mRNA were also obtained from uninfected E. Coli (Gros et al., 1961a). Attachment of the messenger to 70S ribosomes and stimulation of the rate of labelling of mRNA during enzymic induction were also shown (Gros et al., 1961a). The methylation of E. Coli mRNA from uninfected or T4 bacteriophage-infected cells is negligible; this lack of methylation seems to be a general property of mRNA in both the infected and uninfected cell (Moore, 1966).

Transport of mRNA is believed to be as a messenger RNA - ribosome complex, probably with the mRNA attached to the 30S ribosomal subunit (Byrne et al., 1964; Bladen et al., 1965; Mangiarotti & Schlessinger, 1967).

III. 5.(ii) The messenger in eukaryotes.

The same difficulties of heterogeneity and turnover that complicate the study of bacterial mRNA have also beset the study of mRNA in eukaryotic cells - except that at least some eukaryotic mRNA is more stable (Revel & Hiatt, 1964; Pitot et al., 1965; Suzuki & Brown, 1972). These difficulties have been minimised by the use of a "model" system - the avian and mammalian erythrocyte where 90% of the cell protein is globin. Several groups of workers have isolated an 8 - 10S RNA with mRNA characteristics, but so far only four groups have shown that their "messenger" can direct the synthesis of a specific protein in a cell-free system (Laycock & Hunt, 1969; Lockard & Lingrel, 1969; Pemberton et al., 1972; Suzuki & Brown, 1972). The presence of covalently-bound poly A sequences seems to be a general phenomenon of eukaryotic mRNA (Lim, Canellakis & Canellakis, 1969; Lim & Canellakis, 1970; Edmonds et al., 1971; /...

Darnell et al., 1971; Lee, Mendecki & Brawerman, 1971; Burr & Lingrel, 1971).

The mammalian mRNA is believed to be transported as ribonucleoprotein (Spirin, 1969; Scherner et al., 1970; Faiferman et al., 1971; Raskas, 1971; Schochetman & Perry, 1972); it migrates from nucleus to cytoplasm where it is believed to become attached to the 40S ribosomal subunit (Holder & Lingrel, 1970).

III. 6. Low molecular weight nuclear RNA.

Apart from ribosomal precursor RNA and hnRNA, the nucleus contains another class of RNA molecules which were not resolved until the use of acrylamide gel electrophoresis (Knight & Darnell, 1967). Nine small molecular weight monodisperse RNA species have been reported in the nucleus of HeLa cells by Weinberg & Penman, 1968, 1969. They are homogeneous species and their size range is 100 - 260 nucleotides; their G+C content is 47 - 54%; they are all methylated; they are all stable or have long half-lives; some are nucleolar, some are also found in the nucleoplasm, but none are cytoplasmic or cytoplasmic RNA precursors; their synthesis is unrelated to DNA synthesis; use of actinomycin, actidione and cordycepin have shown that they are not ribosomal precursor RNA or hnRNA cleavage products (Weinberg & Penman, 1968, 1969). Similar species of RNA have been found in mouse fibroblast cultures (3T3), in the developing chick embryo brain and in L cells (Weinberg & Penman, 1968) and in the Novikoff hepatoma (Prestayko et al., 1970), where one species has been partially characterised and shown to consist of at least three components (Ro-Choi et al., 1970). Thus there are at least nine of these nuclear species and they probably represent a general phenomenon of eukaryotic cells. Their function is unknown and may be that of control, but the fact that they are present in very different amounts suggests that they may have different functions (Weinberg & Penman, 1969).

III. 7. Mitochondrial RNA

The mitochondrion has its own complement of DNA which is believed to be completely transcribed to give mitochondrial RNA (Aloni & Attardi, 1971a). In the HeLa mitochondrion there are mitochondrial ribosomes of 55 - 60S (Attardi & Ojala, 1971; Brega & Vesco, 1971). The heavy and light subunits contain one molecule each of 16S and 12S RNA respectively (Brega & Vesco, 1971) and these RNA species are methylated (Attardi & Ojala, 1971). The mitochondrial ribosomes do not contain 5S RNA (Zylber & Penman, 1971; Lizardi & Luck, 1971) and this fact, together with their smaller size, are main differences between mitochondrial and bacterial ribosomes. However, these two types of ribosomes are similar in their antibiotic sensitivity (Attardi & Attardi, 1971) and in their use of formyl methionyl tRNA for initiation (Smith & Marker, 1968).

One molecule of mitochondrial DNA has genes for 1 molecule of 16S, one of 12S and about 11 molecules of 4S RNA (Aloni & Attardi, 1971b for HeLa cells; similar results have been reported for the mitochondria of Xenopus laevis by Dawid, 1969).

Two classes of mitochondrial-specific 4S RNA (A and B) can be separated by gel electrophoresis; 4S RNA A is methylated and heterogeneous and may be a class of tRNA, but 4S RNA B is probably not tRNA since it contains no methyl groups and is also more homogeneous than tRNA (Knight & Sugiyama, 1969; Knight, 1969).

III. 8. Chromosomal RNA

Chromosomal RNA is a class of RNA 40-60 nucleotides long, which associates with chromatin, described in a wide variety of eukaryotic tissue by Bonner's group (Huang & Bonner, 1965; Huang, 1967; Bonner & Widholm, 1967; Shih & Bonner, 1969); it has been postulated to be involved in gene regulation (Bekhor, Kung & Bonner, 1969; Huang & Huang, 1969; /...

Figure 8

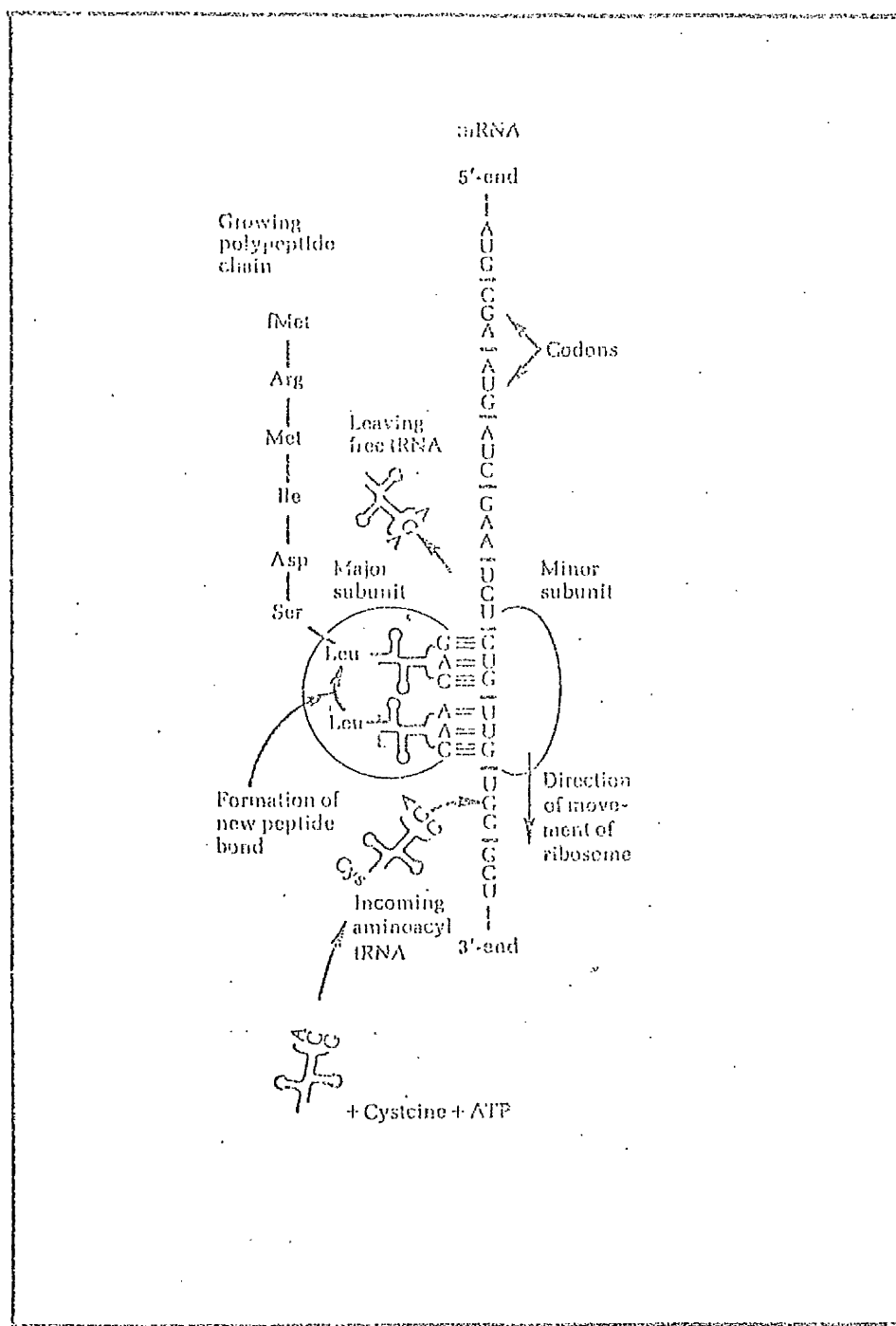


Figure 8. Schematic representation of protein synthesis

(from Lehninger, 1970)

Mayfield & Bonner, 1972). Recent doubt has been cast on this work, and chromosomal RNA may only be tRNA tightly bound to protein (Heyden & Zachau, 1971), with no specific function.

III. 9. RNA and Protein Synthesis

Since most of the RNA species discussed above participate in protein synthesis, any resumé would be incomplete without mentioning this function.

Messenger RNA, transcribed from the DNA, becomes attached to the minor subunit of the ribosome, where it codes for incoming amino acyl tRNAs, which attach themselves to the major subunit; the peptidyl transferase enzyme which catalyses the formation of a peptide linkage between adjacent amino acids is also believed to be located on the larger subunit. The mRNA is transcribed from the 5'- terminus to the 3'- terminus and translated in the same direction. This is summarised in Fig. 8. from Lehninger, 1970.

IV. VIRUSES AND THEIR EFFECT ON CELLULAR METABOLISM

IV.1. General Nature of Viruses.

IV.1.(i) Basic Virus characteristics

Viruses are basically infective nucleoprotein entities, which can replicate only within cells. Thus a primary classification of viruses is by the nature of the hosts they infect - bacterial, plant, animal, etc. The animal viruses can be subdivided into DNA or RNA-containing viruses depending on which nucleic acid forms the genome.

IV.1.(ii) The DNA-containing animal viruses.

The DNA-containing animal viruses are of especial interest in that their DNA has to be transcribed into messenger RNA which then has to be translated into protein in a manner analagous to the uninfected cell.

The/...

Table 2

The groups of DNA-containing animal viruses ^{adapted} (from Fenner, 1968)

Group	Configuration of DNA	Molecular weight of DNA $\times 10^{-6}$	Shape of virion	Size of virion (A°)	Envelope	Symmetry of nucleocapsid	Number of capsomeres
Herpesvirus	Double-stranded	54-92	Roughly spherical	1000-1500	+	Icosahedral	162
Adenovirus	Double-stranded	20-25	Spherical	800-900	-	Icosahedral	252
Adenovirus-associated virus	Single-stranded	1.8	Spherical	200	-	Icosahedral	?
Papovavirus	Double-stranded	3-5	Spherical	300-500	-	Icosahedral	72
Poxvirus	Double-stranded	160-200	Brick-shaped	5000x2000 x 1000	-	Complex	~
Parvovirus	Single-stranded	1.8	Spherical	200	-	Icosahedral	32

The DNA-containing viruses, together with their main characteristics, are summarised in Table 2.

IV.1.(iii) The Herpesviruses

The herpesviruses are a group of viruses with the following characteristics. They are large, enveloped, DNA-containing animal viruses, with double-stranded DNA enclosed within an icosahedral capsid of 162 capsomeres, and they multiply in the nucleus. The herpesvirus group includes the following viruses: pseudorabies virus (PRV); herpes simplex virus (HSV) types I and II, equine abortion virus (EAV), B virus, varicella-zoster, Lucké virus.

Throughout the experimental work of this thesis, the virus used was pseudorabies virus. Much less is known of the biochemical effects of this virus than is known about herpes simplex virus (type I), which is the most widely studied herpesvirus. Thus the basic biochemical characteristics of these two viruses are now outlined since comparison of these viruses occurs in the "Discussion."

a) DNA-density

The buoyant density of the DNA of HSV-I and PRV has been reported by several workers (Russell & Crawford, 1964; Plummer et al., 1969; Subak-Sharpe & Hay, 1965; Subak-Sharpe et al., 1966a; Graham et al., 1972). The reported buoyant density of HSV-I DNA is in the range 1.725 - 1.727 and that of PRV is 1.727 - 1.733; wherever the same group of workers have studied both DNAs together, that of HSV-I is always lower than that of PRV.

b) DNA - base content

The G+C content of the DNAs from HSV-I and PRV have been calculated from the buoyant density to be 68% and 74% respectively (Russell & Crawford, /...

Crawford, 1964; Subak-Sharpe et al., 1966a) and from base composition analysis to be 70 and 77% respectively (Graham et al., 1972); despite this difference in G + C content between the two DNAs, the doublet frequency of the bases is very similar (Subak-Sharpe et al., 1966a). Very recent results obtained by Bronson et al., 1972 using molecular hybridisation techniques have shown that the homology between HSV-I and PRV DNA can be, at most, only 9%.

c) DNA-molecular weight

The molecular weight of the DNA of both HSV-I and PRV has been reported to be 68×10^6 daltons by Russell and Crawford, 1964, although Kaplan & Ben-Porat, 1964, report a value of 70×10^6 for PRV, while Becker, Dym & Sarov, 1968, using electron microscopy, have established a value of 100×10^6 for the molecular weight of the DNA of HSV-I.

d) Biology

The infectious cycles of HSV-I and PRV vary according to the cell type infected, but the cycle of PRV tends to be somewhat shorter (e.g. at low multiplicity of infection in RK cells, the eclipse phase of HSV-I and PRV is 10 hours and 6 hours respectively (Plummer et al., 1969). The difference in eclipse time between these two viruses is, however, slight compared to the long eclipse of many other herpesviruses (Plummer et al., 1969). Both viruses multiply in the same wide spectrum of cells (Plummer et al., 1969).

e) Antigenicity

Antiserum to HSV-I-infected RK cells does not neutralise PRV (Kaplan & Vatter, 1959; Watson et al., 1967) but there may be one common antigen (Watson et al., 1967).

f) / ...

f) Proteins produced P.I.

Gel electrophoresis of proteins produced after infection of cells with PRV or HSV-I produced very different patterns (Kaplan, Shimono & Ben-Porat, 1970), and this tends to support the antigenic evidence.

IV.2. Replication of PRV

IV.2.(i) Infective cycle of PRV

The infective cycle of PRV has been summarised by Fenner, 1968. PRV enters the cell, becomes uncoated and viral DNA replicates in the nucleus between 2 - 8 hours P.I.; viral structural proteins are formed between 2.5 - 10 hours P.I., non-structural proteins (including viral-induced enzymes) between 1 - 6 hours P.I. and mature virions at 5-10 hours P.I. (Kaplan & Ben-Porat, 1963; Hamada & Kaplan, 1965; Hamada *et al.*, 1966). The virus DNA complexes with protein to form the nucleoid of the virus; on this structure the capsomeres form the capsid; the virus membrane is formed as the virus proceeds from nucleus to cytoplasm and the complete virion is then released from the cell (Sydiskis, 1969, 1970).

IV.2.(ii) Effect of PRV on RNA metabolism

Kaplan & Ben-Porat, 1960, showed that infection of PRV caused a sharp decrease in the uptake of ^{14}C -uridine into total RNA; this result has been confirmed by a very recent report from the same laboratory (Rakusanova *et al.*, 1971), and is a similar finding to that reported by other workers using HSV-I (Roizman *et al.*, 1965; Hay *et al.*, 1966; Flanagan, 1967). The decrease of overall RNA synthesis after infection is the resultant of two main effects: decrease in host function (especially in rRNA synthesis) and gradual increase in the synthesis of viral-specific RNA (presumably mostly viral messengers). The synthesis and/...

and metabolism of various species of RNA after infection by HSV-I will now be discussed.

IV.2.(ii).a) Ribosomal RNA

Ribosomal RNA synthesis declines sharply after infection (Hay et al., 1966; Wagner & Roizman, 1969a); it reaches about 10% of the uninfected cell level by 5 hours P.I., and the decline in 28S and 18S RNA synthesis is identical (Wagner & Roizman, 1969a). However 45S RNA synthesis is not affected as greatly as mature rRNA (Hay et al., 1966; Wagner & Roizman, 1969a) and this is accounted for by a decrease in the rate of maturation of 45S RNA and also by abortive processing of 45S RNA to oligonucleotides (Wagner & Roizman, 1969a). Similar results have recently been demonstrated after infection with PRV (Rakusanova et al., 1971). Aberrant processing of ribosomal RNA precursors in poliovirus-infected cells has been reported (Weinberg et al., 1967; Weinberg & Penman, 1970; section III.2.(ii).a)).

IV.2.(ii).b) Messenger RNA

The herpes virus-infected cell is an excellent system in which to study mRNA production since host RNA is greatly depressed and probably most of the viral-specified RNA is informational, and yet the transport problems of the RNA from nucleus to cytoplasm remain.

Viral-specific RNA (i.e. RNA hybridising specifically to virus DNA) in HSV-infected cells was first identified by Hay et al., 1966, who found that most radioactive RNA precursor was incorporated into a heterogeneous RNA species with a sedimentation constant of about 20S; Flanagan, 1967 also reported viral-specific RNA, suggesting a rather higher S-value; Wagner & Roizman, 1969a, b isolated viral-specific RNA of 10-40S (with a peak at about 20S) from the polysome fraction of HSV-infected cells. The/...

The fact that this species of RNA appearing in HSV-infected cells is large, viral-specific and polysome-associated strongly implies that it is viral messenger; thus a similar species (or a larger precursor) should be located in the nucleus, and viral-specific nuclear RNA has indeed been found (Wagner & Roizman, 1969 a,b). This nuclear RNA has a greater S-value than the viral-specific messenger (10-80S), with a significant proportion greater than 50S; this large size is not due to aggregation (Roizman et al., 1970) and must represent the transcription product of at least 10% of the viral genome (Wagner & Roizman, 1969b). The transport of viral mRNA differs somewhat from host mRNA since cycloheximide abolishes the transport of viral-specific but not host-specific mRNA to the cytoplasm (Roizman et al., 1970), and it is possible that viral-specific mRNA "in transit" lingers in the nucleus rather than in the cytoplasm (Roizman et al., 1970).

Further evidence for the transport of viral-specific sequences from nucleus to cytoplasm has recently been obtained in cells infected with another nuclear-replicating virus, adenovirus. Isolated nuclei from adenovirus-infected cells release viral-specific RNA into the medium (in the presence of ATP and an ATP-generating system); this RNA is released as two ribonucleoprotein complexes, the larger of which contains RNA resembling viral mRNA (Raskas, 1971); the nuclear and polysomal adenovirus-specific RNAs both contain poly A sequences (Philipson et al., 1971). Thus it seems that the adenovirus-specific RNA has most of the characteristics of hnRNA and mRNA discussed in sections III.4 and III.5, and it is most probable that viral-specific RNA produced after infection with HSV-I is of a similar nature.

IV.2.(ii).c). 4S RNA

The decrease in 4S RNA synthesis after infection by HSV-I is less than/...

than the decrease in rRNA synthesis (Hay et al., 1966; Wagner & Roizman, 1969a). Similar results have recently been reported for 4S RNA synthesis in PRV-infected cells (Rakusanova et al., 1971). On the basis of the large difference in G + C content of the DNA, and especially the large difference in CpG doublet frequency, it was postulated that new viral tRNAs would be needed after infection with HSV (Subak-Sharpe et al., 1966b). However, although it is possible that there are changes in the tRNA population after infection, this is probably not due to viral-coded tRNAs (Morris, Wagner & Roizman, 1970). Changes in the tRNA population to meet a new need have been reported for adenovirus-infected cells (Raška, Frohwirth & Schlesinger, 1970) but again these tRNAs are not virus-hybridizable; a very recent report has shown that there are alterations in at least two amino-acyl tRNAs after vaccinia virus infection of HeLa cells (Clarkson & Runner, 1971).

IV.2.(ii).d) Other species of RNA produced P.I.

In various infected systems the virus produces new species of RNA of unknown function. The best characterised of these produced by a nuclear-replicating DNA virus is the so-called "VA-RNA" studied by Weissman and his co-workers in adenovirus-infected cells (Rose, Reich & Weissman, 1965; Reich, Forget & Weissman, 1966; Forget & Weissman, 1967a; Ohé, Weissman & Cooke, 1969; Ohé & Weissman, 1970). "VA-RNA" is found free in the cell sap of infected cells; isolated nuclei will synthesise and export this RNA in the presence of all four nucleosides triphosphates and an ATP-generating system (Ohé, Weissman & Cooke, 1969).

It is possible that such species of RNA are produced after infection with herpesviruses.

IV.2.(iii) / ...

IV.2.(iii) Effect of PRV on other macromolecules.

More work has been performed on the effect of PRV on DNA and protein synthesis than on RNA synthesis and so the effect of PRV - with some reference to other herpesviruses - is very briefly summarised here.

IV.2.(iii).a) DNA

Infection of exponentially-growing cells with PRV causes a decline in total DNA synthesis, measured by incorporation of radioactive thymidine (Kaplan & Ben-Porat, 1963; Ben-Porat & Kaplan, 1965); this is similar to the effect of HSV on DNA synthesis (Roizman & Roene, 1964). When cells are in the stationary phase of growth, PRV causes an increase in DNA synthesis, due to synthesis of viral DNA (Ben-Porat & Kaplan, 1963).

Recent results using synchronised cells have shown that in EAV-infected cells there is a relationship between viral DNA synthesis and cell S-phase (Lawrence, 1971) while in HSV-infected cells there is no such relationship (Cohen, Vaughan & Lawrence, 1971), but the question remains open for PRV.

IV.2.(iii).b) Protein

Protein-metabolism in the PRV-infected cell has been fairly extensively studied, especially by Kaplan, Ben-Porat and their co-workers. Overall protein synthesis decreases only slightly after infection (Hamada & Kaplan, 1965); however, overall glycoprotein synthesis is strongly decreased (Ben-Porat & Kaplan, 1970), as is histone synthesis (Stevens, Kado-Boll & Haven, 1969), and cell-specific protein synthesis decreases steadily throughout the infectious cycle (Hamada & Kaplan, 1965).

New viral-specific proteins are produced after infection and can be partially separated by polyacrylamide gel electrophoresis, with a main peak at about 120,000 daltons which is not due to aggregation (Shimono, Ben-Porat & Kaplan, 1969). These proteins made after infection have a different amino acid content than host proteins; they have more arginine and/...

and less lysine, phenylalanine, isoleucine, tyrosine and methionine than host proteins, as would be expected from the differences in base composition and doublet frequency between viral and host DNA (Subak-Sharpe et al., 1966a).

There are two types of proteins made after infection - structural and non-structural proteins. The structural proteins begin to be synthesised about 3 hours P.I., and their production gradually increases throughout the cell cycle (Hamada & Kaplan, 1965); they are synthesised in the cytoplasm and transported to the nucleus for assembly into virus particles (Ben-Porat, Shimono & Kaplan, 1969); inhibition of viral DNA synthesis does not affect the synthesis of these proteins and affects their transport to the nucleus only slightly (Ben-Porat et al., 1969); arginine deprivation prevents the transport but still allows these proteins to be synthesised (Mark & Kaplan, 1971).

About 35% of these structural proteins do not become assembled into the virion (Hamada & Kaplan, 1965). Virions extracted from the nucleus have lower infectivity than mature virus particles (Ben-Porat, Shimono & Kaplan, 1970); this is believed to be due to the lack of two glycoproteins which are supposed to become associated with the virion en route from nucleus to cytoplasm (Ben-Porat et al., 1970; Ben-Porat & Kaplan, 1970). Five nuclear proteins synthesised after infection are acid-extractable (although not histones) and four of them are believed to be structural proteins (Stevens et al., 1969).

The non-structural proteins begin to appear immediately after infection and their synthesis reaches a peak between 4 - 7 hours P.I. (Hamada & Kaplan, 1965); presumably these proteins include a variety of viral-induced enzymes essential for the synthesis of viral components, e.g. the arginine-requiring protein required for the transport of structural proteins to the nucleus (Mark & Kaplan, 1971) and possibly the virus own enzymes for DNA transcription/...

transcription, as occurs in HSV-infected cells (Keir et al., 1966). There has been an intensive study of various enzymes produced after infection with HSV-I and there is now very good evidence that the following enzymes are coded by the virus: DNA polymerase (Keir et al., 1966), thymidine kinase (Klemperer et al., 1967) and DNase (Morrison & Keir, 1968).

V. Aims of the present project

As this "Introduction" has shown, the diversity of RNA species in the mammalian cell is very large, and the problems concerning their production and control are great. As this "Introduction" has also indicated, when a large animal virus infects a cell it proceeds to take over the control and production of all cell macromolecules, including RNA. Thus it was hoped that investigation of the effects of pseudorabies virus on cell metabolism would not only prove valuable as a study of viral metabolism, but might also provide some information on the control and production of some species of cellular RNA.

MATERIALS

I. Virus

The pseudorabies virus (PRV) used was derived by 3x plaque purification from a stock preparation of PRV (Kaplan & Vatter, 1959), and a sample was kindly gifted by Dr. W. Shepherd, Institute of Virology, University of Glasgow.

II. Tissue culture cells

BHK-21/C13 cells were a continuous line of hamster fibroblasts described by Macpherson & Stoker, 1962.

III. Radiochemicals

All isotopically-labelled compounds used were obtained from the Radiochemical Centre, Amersham, Bucks., England.

IV. Polyacrylamide gel materials

Acrylamide, bisacrylamide (methylene bisacrylamide) and Temed (N,N,N^1,N^1 -tetra methylene diamine) were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England; the acrylamide was chloroform-purified as described by Loening, 1967. Ammonium persulphate was obtained from BDH, Poole, England.

V. Chemicals for liquid scintillation counting

1,4 dioxan and PPO(2,5 diphenyloxazole) were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., naphthalene and hyamine hydroxide (1M in methanol) from Nuclear Enterprises Ltd., Sighthill, Edinburgh, 2-methoxyethanol and hydrogen peroxide (100 volumes) from BDH Biochemicals Ltd., and cellulose acetate membrane filters (2.5cm diameter, 45 μ pore size) from Sartorius-Membranfilter GMBH, 34 Gottingen, W.Germany.

VI. Miscellaneous

All other chemicals used were, wherever possible, "Analar" grade or its equivalent and were purchased mainly from BDH Biochemicals Ltd., Poole, Dorset or Sigma Chemical Co., London. Nonidet P40 was provided by Shell Chemical Co., London. Sephadex G100 was bought from Pharmacia Ltd., Uppsala, Sweden, nucleotides, nucleosides and commercial 4 S RNA from Calbiochem Inc., Los Angeles, or Sigma Chemical Co., London, Actinomycin D from Merck, Sharpe and Dohme, N.J., U.S.A. or Calbiochem, Visking dialysis tubing from The Scientific Instrument Centre Ltd., London, Chance No. 1 glass coverslips (13mm diameter) from Macfarlane Robson Ltd., Glasgow, AR.10 fine grain autoradiographic stripping plates and Kodirex film from Kodak Ltd., DePeX from George T. Gurr, Ltd., London, Whatman No.1 2.5cm filter paper discs, 3MM chromatography paper and DEAE-cellulose chromatography paper (DE 81) from H.Reeve Angel & Co., Ltd., London, T_1 ribonuclease from Sankyo Co., Ltd., Tokyo, and cellulose acetate paper from Oxoid Ltd., London.

VII. Composition of media

Eagle's medium. A modification of Eagle's medium (Busby, House & Macdonald, 1964) containing 100 units/ml Penicillin and 100 μ g/ml Streptomycin (Glaxo Laboratories) and 0.002% (W/V) phenol red was used. The vitamins and amino acids were obtained from Flow Laboratories, Irvine, Ayrshire.

Tryptose phosphate broth consisted of a 2.95% (W/V) solution of tryptose phosphate broth in distilled water, and was purchased from Difco Bacto Laboratories, East Molesey, Surrey, England.

EC10 growth medium was composed of 90% (V/V) Eagle's medium and 10% (V/V) /...

(V/V) calf serum (Flow Laboratories).

ETC growth medium was composed of 80% (V/V) Eagle's medium, 10% (V/V) tryptose phosphate broth and 10% (V/V) calf serum.

ECL medium was composed of 99% (V/V) Eagle's medium and 1% (V/V) calf serum.

VIII. Composition of solutions.

RSB (reticulocyte standard buffer) was composed as described by Weinberg & Penman, 1968: 10mM NaCl, 10mM tris-HCl, 1.5mM MgCl₂, pH to 7.4 with HCl. Macaloid was routinely added to a final concentration of 2.5mg%.

Hypotonic buffer was a solution of 10mM KCl, 10mM tris-HCl, 1.5mM MgCl₂, pH to 7.4 with HCl. Macaloid was routinely added to a final concentration of 2.5mg%.

SSC (standard saline citrate) was a solution of 0.15M NaCl, 0.015M sodium citrate, pH to 7.0 with HCl.

4xSSC was a solution of 0.6M NaCl, 0.06M sodium citrate, pH to 7.0 with HCl.

FBS(a) (phosphate-buffered saline (a)) was a solution of 0.17M NaCl, 3.4mM KCl, 10mM Na₂HPO₄ and 2mM KH₂PO₄, pH 7.4 (Dulbecco & Vogt, 1954).

Dioxan-based scintillator consisted of 100g naphthalene and 7.0g PPO per litre of 1,4 dioxan.

Toluene-based scintillator consisted of 0.5% (W/V) PPO in "Analar" toluene.

Toluene/methoxyethanol scintillator was composed of 30% methoxyethanol, 70% toluene-based scintillator (V/V).

BSS (buffered salt solution). The stock solution consisted of 68g NaCl, 4g KCl, 2g MgSO₄·7H₂O, 1.4g NaH₂PO₄, 3.93g CaCl₂·6H₂O, 15 ml phenol red 1%, diluted to 1 litre. This was further diluted 10-fold for use.

Macaloid/...

Macaloid was provided by National Lead Co., Houston, Texas. A solution of 2.5g in 100ml of distilled water was made up, sonicated for 3 - 4min., and dialysed overnight against distilled water; this resulted in a pellet (discarded) and a clear supernatant fluid which was sterilised by autoclaving, and was diluted 1000-fold for use. This stock solution was briefly sonicated every time before removing aliquots for dilution.

Formol saline (10% (V/V)). consisted of 4% (V/V) formaldehyde in 85mM NaCl, 0.1M Na_2SO_4 , and was diluted ten-fold for use.

Giemsa stain. A 1.5% (W/V) suspension of Giemsa (George T. Gurr Ltd., London) in glycerol, heated at 56° for 90 - 120 min and diluted with an equal volume of methanol was used as stock (Dacie, 1956).

Water-saturated phenol was prepared by distilling phenol into distilled water. 8-Hydroxyquinoline (to a concentration of 0.1% (W/V)) was always added to the stock phenol as an anti-oxidant.

Toluidine blue was obtained from BDH Biochemicals Ltd., Poole and was used as a 0.05% (W/V) solution in distilled water.

Blue dextran was purchased from Pharmacia Ltd., Uppsala, Sweden and was used as a 0.5% (W/V) solution in SSC.

Bromophenol blue was supplied by Eastman Kodak Co., Rochester, N.Y. as a 0.1% (W/V) aqueous solution and was diluted ten-fold in distilled water for use.

x% TCA was a solution of x% W/V of trichloroacetic acid in distilled water.

Amfix solution contained 20% (V/V) concentrated Amfix (May & Baker, Ltd., Dagenham, England) in distilled water.

D19b developer contained the following; 72g Na_2SO_3 , 48g Na_2CO_3 , 4g KBr, 8.8g hydroquinone, 2.2g Metol (Kodak Ltd., London) to a total volume of 1 litre.

Gelatine-chrome alum/...

Gelotine-chrome alum consisted of 5g gelatin, 0.5g chrome alum ($\text{CrK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$), 5ml of formaldehyde (40% V/V solution) and 1 ml Photo-Flo (Kodak Ltd., London) in a total volume of 1 litre.

SDS (20% W/V) was a solution of ethanol-recrystallised sodium dodecyl (lauryl) sulphate in distilled water.

Heparin was obtained from Sigma Chemical Co., London. The stock solution was a 10mg/ml solution in distilled water, which was sterilised by filtration.

METHODS

I. Biological

I.1. Growth of cells. The BHK-21/Cl3 cells used throughout this study were grown and maintained in ETC at 37° in rotating (1 r.p.m.) 80 oz roller bottles (burlers), gassed to give an atmosphere of 5% CO_2 , 95% air. Cells were not used beyond a passage number of 12 - 15 (Shedden & Wildy, 1966).

I.1.(i) Normal experimental use. Cl3 cells, which had been passed at least twice in EC10, were seeded at $30 - 40 \times 10^6$ cells per burler in 180 ml EC10, gassed, maintained at 37° and were used 2 - 3 days later, when nearly confluent (approximately 80×10^6 cells per burler). From time to time Roux bottles were used in place of burlers. Some experiments were performed using 50 mm plastic Petri dishes which were seeded at $3 - 4 \times 10^6$ cells per dish; in this case incubation was carried out at 37° in a humidified incubator flushed continuously with 5% CO_2 , 95% air.

I.1.(ii) "Resting" cells. Cl3 cells in which metabolic function was depressed was obtained by serum-depletion using the method of Fried & Pitts, 1968 as modified for Cl3 cells by Nicholas & Pitts, 1971. Cl3 cells were seeded in 100 ml EC10 at $10 - 20 \times 10^6$ cells per burler; about 18 hours later the cell sheet was washed with Eagle's medium and then the medium was replaced with 100 ml EC1. These cells were used 5 - 6 days later. In some experiments 50 mm plastic Petri dishes were used, and the cells were then seeded at 1.3×10^6 cells/plate; Roux bottles were also used, seeded at 4×10^6 cells/Roux. In these cases the amounts of medium used were appropriately scaled down.

I.2. Growth of pseudorabies virus.

I.2.(i) Production of PRV stocks. The virus (see Fig. 9) was propagated as follows./...

Figure 9

Electronmicrograph of the supernatant preparation of
Pseudorabies virus used throughout most of the experimental
work of this thesis (50,000-fold magnification).

(Inset = 128,000-fold magnification)

a = "full" enveloped particles (Inset shows individual capsomeres)

b = "full" enveloped particle (Stain has not entered because of
extra proteins between envelope
and capsid)

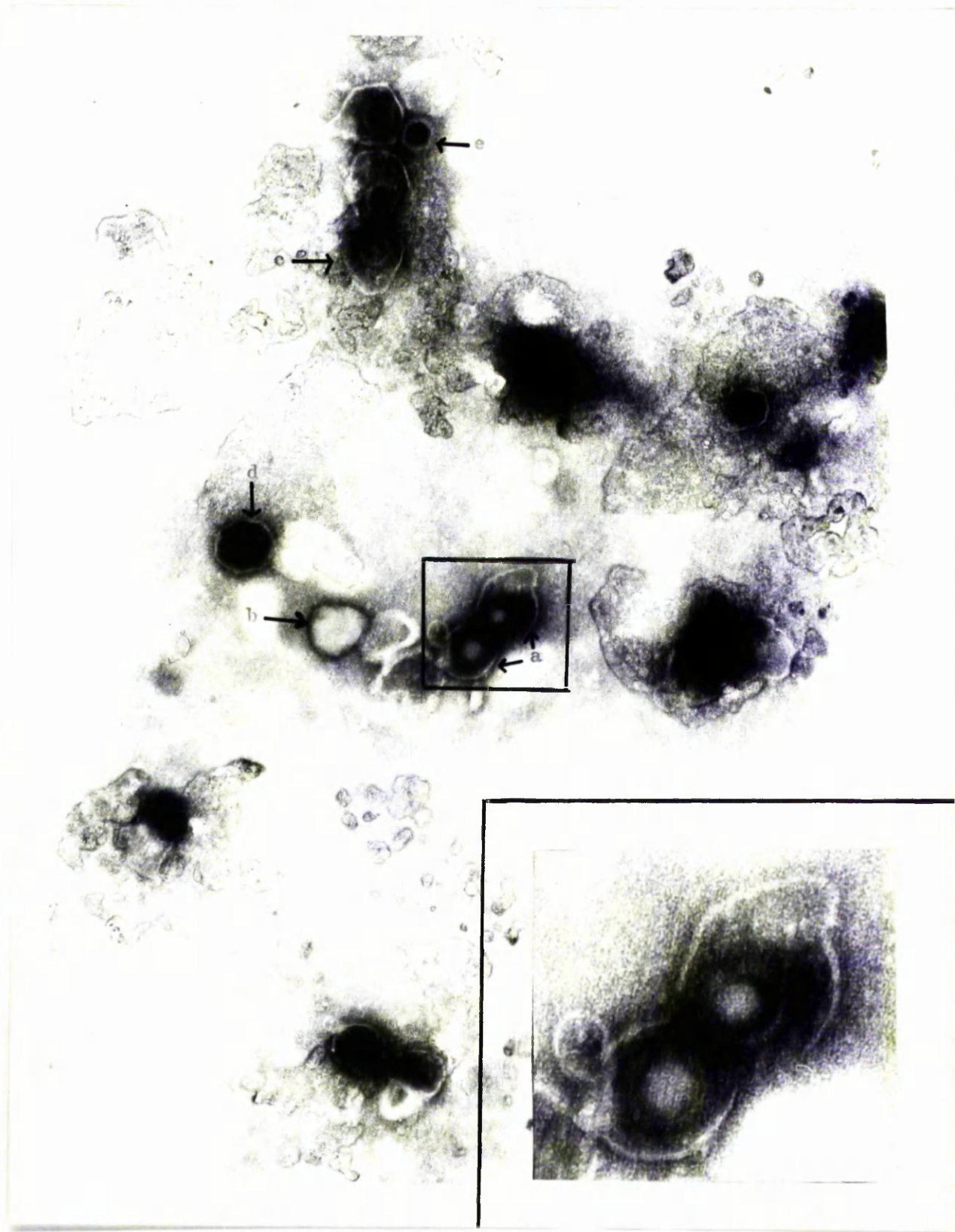
c = "partially full" enveloped particle

d = "empty" enveloped particle

e = "empty" unenveloped particle.

"Full" or "empty" denotes presence or absence of nucleoprotein
within the capsid.

(Electronmicrographs prepared by Mr. H. Elder)



follows. Newly-confluent Cl3 cell cultures, maintained in ETC, were infected in 20 ml ETC at a multiplicity of 1 p.f.u./300 cells. The virus inoculum was allowed to adsorb to the cells for 1 hour at 37° as the burler rotated. After adsorption, the 20 ml ETC was removed, 100 ml of ETC medium was added and the cultures were rolled at 37° for 27-36 hours. The cells were then harvested aseptically by shaking the bottle to dislodge the cells into the medium, the excess medium being removed by centrifugation for 10 min at 600 g. This medium contained the supernatant virus which was concentrated by centrifugation at 15,000 g for 1.5 hours, and resuspended in 1 ml PBS(a) for each original burler used; gentle sonication made the suspension uniform, and it was dispensed into 1 ml aliquots and stored at -70°. The cell pellet contains the cell-associated virus which was released by resuspending it in a small volume of PBS(a) (2-3 ml for every burler used initially) and disrupting the cells by sonication or by 3 cycles of freezing and thawing, and then re-centrifuging; the resulting supernatant contained the cell-associated virus, which was dispensed and stored at -70°. The two fractions were occasionally combined for experimental work, and always for virus production.

I.2.(ii) Plaque assay for PRV. PRV was assayed by infecting cell sheets with dilutions of PRV and counting the resulting plaques produced. Newly-confluent monolayers of Cl3 cells in a series of 50 mm Petri dishes (seeded at 4×10^6 cells/dish 18 hours previously) were infected with various dilutions of PRV in 0.2 ml EC10. After 1 hour of adsorption at 37°, 4 ml EC10 was added and incubation continued; two hours after adsorption, 50 µg/ml heparin was added to the medium to prevent vertical transmission of virus. After 28 hours' incubation at 37°, the cell sheet was washed with/ ...

with PBS(a), fixed with 3ml formol-saline per dish (30 min at room temperature), and stained with 0.5ml Geimsa stain per dish (1 hour at room temperature). Plaques were counted under a microscope at low magnification after excess stain had been removed with water.

I.2.(iii) Infection of cells with PRV for experimental use. Medium was removed from the cell sheet of nearly-confluent C13 cells in EC10, and 20ml fresh EC10 containing 20 p.f.u. PRV/cell was added to each burler. After 1 hour adsorption at 37° , the medium was removed and replaced with 50-100ml fresh EC10. If cells were "resting", infection took place using a small portion of "used EC1" i.e., medium removed from the "resting" burlers, and at the end of the adsorption period, the replacing medium was also "used EC1".

II. Biochemicals.

II.1. Cell fractionation procedures

II.1.(i) Preparation of cell cytoplasm for RNA extraction. The cells were harvested by removing the medium, washing the cell sheet with ice-cold BSS, and then scraping the cells with a rubber blade into ice-cold BSS. The cells were then centrifuged at 600g, resuspended in BSS and centrifuged once more. From the resulting washed cell pellet, the cytoplasm was obtained in one of two ways.

II.(i).a) Homogenisation procedure. The cell pellet was resuspended in 2x packed cell volume of hypotonic buffer or RSB and allowed to swell at 4° for 10 min. The cells were then disrupted with 20 strokes of a Potter homogeniser (operated manually), the disruption being monitored microscopically. The nuclei were removed by centrifugation for 10min at 1000g and the resulting supernatant fluid was used as cell cytoplasm.

II.1.(i).b) Nonidet P40 procedure. The cell pellet was resuspended in 2.0ml RSB + 0.5% (V/V) Nonidet P40 for each 4×10^7 cells (i.e., about 4ml per/...

per burler) and was agitated with a "Vortex" mixer for 5 sec, and centrifuged at 1000 g to precipitate nuclei and yield the supernatant cytoplasm. This was the method used by Wagner & Roizman, 1969a for herpesvirus-infected cells and was first described by O'Brien, 1964.

II.1.(ii). Fractionation of cell cytoplasm. The cytoplasmic fraction, prepared as described in section II.1.(i) was centrifuged at 40,000 g for 10 min to remove mitochondria. The supernatant was then removed and centrifuged at 105,000 g for 1-16 hours to deposit the ribosomes; the top three-quarters of the resulting supernatant was used as the cell sap fraction.

II.2. Preparation of RNA

All glassware used in the preparation of RNA was either flamed, alcohol-washed or autoclaved at 15 p.s.i. for 30 min before use. All solutions and media were sterilised by autoclaving or filtration, and dialysis tubing was boiled for 15 min in dilute EDTA, and rinsed with sterile water followed by dialysis buffer before use. These precautions are essentially those described by Burdon, 1967b to prevent nuclease degradation of RNA.

II.2.(i) Cytoplasmic RNA. Cytoplasmic RNA was prepared by one of the following two procedures.

II.2.(i).a) "Cold phenol" technique. The washed cell pellet was extracted directly with 5 ml water-saturated phenol + 5 ml 0.05M ammonium acetate (pH 5.1) per burler. The solution was mixed well using a "Vortex" mixer, and the RNA was prepared from it as in (b) below. This method, essentially that described by Burdon et al., 1967a for uninfected Cl3 cells, gave an identical cytoplasmic RNA profile to that obtained using the method of (b) below, but was not used for infected cells because of the possible fragility of their nuclei.

II.2.(i).b). The cell cytoplasm was prepared (Section II.1.(i)a,b) and then extracted with an equal volume of water-saturated phenol; this was shaken vigorously or agitated on a "Vortex" mixer and centrifuged at 9,000g for 5 min. The supernatant fluid was removed, macaloid was added to a concentration of 2.5mg% (W/V), and it was then re-extracted with phenol. The resulting supernatant was made 2% (W/V) with respect to sodium acetate, and then two volumes of ethanol were added. The resulting solution was maintained at -20° to allow precipitation of the RNA, which was then centrifuged out at 1200g for 10-30min. The RNA pellet was then washed by resuspension in 2% (W/V) sodium acetate / 67% (V/V) ethanol, and recentrifugation.

II.2.(ii) Cell sap RNA was prepared by extracting the cell sap fraction (section II.1.(ii)), with an equal volume of phenol as described in II.2.(i).b).

II.2.(iii) Ribosomal RNA. The ribosomal pellet (from section II.1.(ii)) was resuspended in 1-2ml RSB for each original burler used. This solution was made 0.5% (W/V) with respect to ethanol-purified SDS and was then extracted twice with an equal volume of water-saturated phenol; the RNA was then pelleted and washed with 2% (W/V) sodium acetate / 67% (V/V) ethanol as described above (II.2.(i)). This method prepared "total RNA" from the ribosomes.

To prepare low molecular weight RNA from the ribosomes, the ribosomal RNA pellet was extracted as follows. To the washed ribosomal RNA pellet was added 1.0ml ice-cold 1M NaCl. This was agitated in a "Vortex" mixer, maintained for 1 hour at $0-4^{\circ}$, mixed once more and centrifuged at 1200g for 10min at 4° . The supernatant was removed and the pellet was extracted/...

extracted once more with 0.5ml 1M NaCl. The combined supernatants contained the low molecular weight ribosome-associated RNA; this RNA could be precipitated by the addition of two volumes ethanol.

II.3. Fractionation techniques for RNA

II.3.(i) Sephadex G100 column chromatography. The RNA preparation, dissolved in a maximum volume of 0.5ml SSC made 10% (W/V) with respect to sucrose, was applied to a 1.5cm x 80cm column of Sephadex G100 (Galibert et al., 1965) which had been swollen as described by the makers and from which the "fines" had been decanted; the void volume of the column had been determined by using 0.5ml of 0.5% blue dextran (in 10% W/V) sucrose). The RNA was eluted from the column at 4° under gravitational pressure and the eluate was collected in 1ml fractions which were measured for absorbancy at 260nm. Radioactivity was determined by adding 70µg heat-denatured DNA and 0.2ml of 50% TCA to each sample, maintaining the samples at 4° for 10min, and then precipitating each sample on to a Sartorius membrane filter under suction; each membrane was then washed three times with 5% TCA, placed in a scintillation vial and dried overnight at 37°. Ten ml of toluene-based scintillator was added to each vial and the samples were counted in a liquid scintillation counter.

II.3.(ii) Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out as described by Loening, 1967 and 1969. The buffer used was 36mM tris-HCl, 30mM NaHPO₄ and 1mM EDTA, pH=7.8. Gels of 10% (W/V) acrylamide and of 2.5% (W/V) acrylamide were used; in the former case the concentration of bisacrylamide was 2.5% and in the latter case 5% that of the acrylamide. The gels contained 0.33% (V/V) Temed /...

Temed and 0.025% (W/V) ammonium persulphate. The gels were run in perspex tubes 10cm long and of 6.0mm internal diameter. 2ml of gel mixture was added to each tube, giving gels 6cm long. The RNA sample was applied to the top of the gel in a total volume of 10-200 μ l as a 10% (W/V) sucrose solution and 20 μ l 0.01% (W/V) bromophenol blue was added as marker. The gels were run for 10min at 2ma/gel and thereafter at 5ma/gel. The 10% gels were run until the bromophenol blue reached the foot of the gel (6cm) while the 2.5% gels were run until the bromophenol blue reached 4cm from the top of the gel.

After electrophoresis, the gels were either stained in 0.05% Toluidine blue (McIndoe & Munro, 1967) and destained in running water, or scanned at 260nm in a Gilford recording spectrophotometer. The gels were then frozen in Drikold, sliced in 1mm slices and prepared for counting as follows. If the isotope used was ^3H , then the method used was a modification of that described by Tishler & Epstein, 1968. The slices were placed in a scintillation vial and dried at 60° for 1 hour. 0.3ml of hydrogen peroxide (100 volumes) was added to each vial and the vials were capped and shaken; the slices usually dissolved after incubation for 6 hours at 60°. Then 10ml toluene/methoxyethanol scintillator was added to each vial and the samples were counted on a liquid scintillation counter.

If the isotope used was ^{14}C or ^{32}P , then each gel slice was placed on a 2.5cm Whatman No.1 filter paper disc and dried before counting in a gas-flow counter.

II.3.(iii) Sucrose gradients. RNA in 0.5ml of 4% (W/V) sucrose was layered on top of 4ml 5-20% sucrose gradients which were centrifuged at 90,000g for 3.5 hours in a swing-out rotor. The fractions were collected dropwise/...

dropwise into scintillation vials (0.3 ml per fraction), and diluted with 0.2 ml distilled water for estimation of absorbancy at 260 nm; then 10 ml dioxan-based scintillator was added to each vial and the samples were counted in a liquid scintillation counter.

II.4. Radioactive labelling and counting of RNA and DNA.

Three isotopes were used to label RNA, ^3H -uridine (uridine-5- ^3H), ^{14}C -uridine (uridine-2- ^{14}C) and ^{32}P as inorganic phosphate; in methylation studies, L-methionine (methyl- ^3H) was also used to label the methyl groups of RNA. Of these isotopes, only uridine-5- ^3H is a specific label for RNA (Hayhoe & Quaglino, 1965), hence, when the level of incorporation into total unfractionated RNA was required, uridine-5- ^3H was the isotope used. Only ^3H -thymidine (methyl- ^3H) was used to label DNA.

Samples were normally prepared for counting in a liquid scintillation counter; a Phillips Liquid Scintillation Spectrometer was normally used and, from time to time, a Nuclear Chicago Series 725. The scintillators used were toluene-based scintillator, dioxan-based scintillator or toluene/methoxyethanol scintillator as described above, and efficiencies were determined by the external standard or channels ratio method, depending on whether the sample was homogeneous or not. "Double-labelled" samples ($^3\text{H}/^{14}\text{C}$ or $^3\text{H}/^{32}\text{P}$) were counted by adjustment of the channels in the scintillation counter to obtain an upper energy channel which measured ^{14}C or ^{32}P only. Samples labelled with ^{14}C only or ^{32}P only were counted in a Nuclear Chicago gas-flow counter (98.7% helium/1.3% butane).

Incorporation/ ...

Incorporation of radioisotope into RNA or DNA in total cells. The procedure used for counting isotopically-labelled fractionated RNA has been described in section II.3. The estimation of incorporation of labelled precursor into RNA or DNA in total cells will now be described. Cells were grown to confluence in 50 mm plastic Petri dishes and were labelled with ^3H -uridine (uridine-5- ^3H) or ^3H -thymidine. The cells were prepared for counting in one of two ways :

(i) TCA precipitation method. The cell sheet was washed with ice-cold BSS, and the cells were scraped off into TCA to give a final concentration of 10% TCA with 250 μg bovine serum albumin (BSA) added as coprecipitant; the cells were then washed three times with 5% TCA at 0° and drained well (overnight). 0.5 ml hyamine was added to the samples which were agitated in a "Vortex" mixer and incubated at 37° for 1 hour. 10 ml toluene-based scintillator was added to each sample for counting in a liquid scintillation counter.

(ii) Coverslip method. In this method, the cell sheet was seeded over three x 13 mm glass coverslips per 50 mm Petri dish; the coverslips had been prepared by being boiled in dilute NaOH for 20 min, washed well with water and finally ethanol and sterilised in an oven at 120° . After the labelling period the coverslips were removed and placed in formol-saline overnight, and then washed well in ice-cold 10% TCA (twice) and finally in ice-cold H_2O . The coverslips were then placed in a scintillation vial with 10 ml toluene-based scintillator and counted in a liquid scintillation counter. If desired, the coverslips could be removed after being counted, washed in PPO-free toluene, dried and used for auto-radiography, as described in section II.9.

Aliquots/ ...

Aliquots of RNA. From time to time aliquots of a preparation of isotopically-labelled RNA were counted. This was achieved by precipitation of the sample on to a Sartorius membrane as described in section II.3.(i)

II.5.(i) Protein estimation was carried out using the method of Lowry et al., 1951, using bovine serum albumin (BSA) as standard.

II.5.(ii) RNA estimation. RNA was estimated by its absorbancy at 258nm (40 μ g RNA/ml gives an absorbancy of 1.0).

II.6. In vitro incubations

II.6.(i). Preparation of extract. All the following procedures were carried out at 4°. Buntlers of mock-infected or PRV-infected cells were harvested at 5 hours P.I. by scraping into ice-cold PBS(a); the cells were washed by centrifugation and resuspension in PBS(a) and finally in 5mM 2'-mercaptoethanol/20mM tris-HCl buffer pH 8.0. The cells were then resuspended in 4 volumes of this buffer and homogenised (20 strokes in a Potter homogeniser operated manually). The resulting lysate formed the crude cell extract which was normally used without centrifuging out the large particulate material. The protein concentration of the extract was measured and the extract was used within a few hours of its preparation and without its having been frozen.

II.6.(ii) Assay conditions for in vitro incubation studies. ^3H -RNA was incubated at 37° with 2.0mg (protein) of the above extract in a total volume of 1.0ml. From time to time other cofactors were supplied as described in the "Results" section. After the desired incubation period, the reaction mixture was extracted twice with an equal volume of water-saturated/...

water-saturated phenol and RNA was prepared as described in section II.2.(i).b).

II.7. Elution of RNA from gels. RNA was eluted from 10% polyacrylamide gels by a modification of the method described by Marcaud *et al.*, 1971. The gel slice was incubated overnight at 60° in 1.0ml (sterile) 4xSSC pH 7.0 to which macaloid had been added to give a concentration of 2.5mg% (W/V). The salt was then removed by dialysis against two changes of distilled water or by diluting the solution 4-5 fold with distilled water; commercial 4S RNA was then added as coprecipitant, and the RNA was precipitated in 2% (W/V) sodium acetate/67% (V/V) ethanol.

II.8. Nucleotide analysis of RNA. The nucleotide content of RNA was determined by the method of Sebring & Salzman, 1964. ³²P-labelled RNA was eluted from gels as described above (section II.7) precipitated with 1.0mg commercial 4S RNA, washed twice in a 3:1 ethanol:ether mixture, desiccated for 30 min *in vacuo* and hydrolysed for 18 hours with 0.25ml 0.3N KOH at 37° and the pH adjusted to pH 3.5 (2.5-4.5) with perchloric acid; the pellet of KClO₄ was removed by centrifugation at 0° and the supernatant was recentrifuged. This supernatant was applied to a sheet (46cm x 57cm) of Whatman 3MM paper and electrophoresed in pyridine-acetate-EDTA buffer pH 3.5 (16ml pyridine, 17ml glacial acetic acid, 18.5g disodium EDTA, water to 5 litres); electrophoresis was carried out at 300V for 20min and then at 3kV for 2.75 hours. The dried electropherograms were then autoclaved for 15min at room temperature to remove pyridine and the nucleotides were located under ultraviolet/...

ultraviolet light. The spots were cut out from the paper, placed in 10ml toluene-based scintillator in scintillation vials, and counted in a liquid scintillation counter.

II.9. Autoradiography. Determination of the percentage of cells in a given culture incorporating ^3H -thymidine was performed by the method of Subak-Sharpe, Bürk & Pitts, 1969. Cells in 50mm Petri dishes were seeded over 13mm glass coverslips; ^3H -thymidine was used to label the cells, and after the labelling period the coverslips were fixed and TCA-washed as described in section II.4.(ii). After being dried the glass coverslips were mounted with DePeX (cells uppermost) on 0.8-1mm glass microscope slides which had been degreased in ethanol and coated with a film of gelatine-chrome alun. The slides were covered with AR.10 stripping film and exposed for several weeks before being processed. The autoradiographs were developed in D19b for 5min, rinsed in water, fixed with Amfix (no hardener) for 4min and rinsed again with water. They were immediately stained with Giemsa (freshly-diluted 1:20 (V/V) in water) for 5min, rinsed with water and finally dried in air.

Microscopic examination revealed that cells which had incorporated ^3H -thymidine had silver grains over their nuclei.

II.10. Fingerprinting of RNA. "Fingerprints" of T_1 ribonuclease digests of ^{32}P -labelled RNA were obtained essentially as described by Sanger, Brownlee & Barrell, 1965 with the slight modifications described by Salim, Williamson & Maden, 1970 and Maden, Salim & Summers, 1972. The T_1 ribonuclease digestion was carried out at a 1:20 enzyme to substrate ratio in 5-20 μl total volume for 30min at 37° .

RESULTS

I. Studies on incorporation of ^3H -uridine into total cell RNA.

I.1. Effect of uridine concentration on uptake of ^3H -uridine.

Before investigating the production of RNA in uninfected and infected Cl3 cells, the effect of uridine concentration in the medium on the uptake of ^3H -uridine was examined; this aspect of RNA metabolism was investigated since it was proposed to use isotopically-labelled uridine throughout these studies as a measure of RNA synthesis as described in "Methods", II.4.

Plates of Cl3 cells were set up as described in "Methods", I.1.(i) and then labelled for 30 min with ^3H -uridine (uridine - 5 - ^3H) in the presence of amounts of unlabelled uridine varying from 0.2 μM to 25 μM . The incorporation of isotope into total RNA was measured by TCA precipitation of the cells ("Methods", II.4.(i)). The results of such an experiment are shown in Fig. 10, which indicates that, between about 1.0 μM and 6.0 μM uridine, incorporation of radioisotope is independent of the molarity of uridine in the medium. A similar independence of incorporation from molarity of uridine at this level of uridine was also observed for ^{14}C -uridine.

The effect of intracellular pool size at this "plateau" level of uridine (1.0 - 6.0 μM) was next examined. Different amounts of ^3H -uridine were added to the medium while maintaining a constant molarity of uridine in the medium. If pool size is negligible relative to the amount of uridine added, the incorporation of label should increase by the same factor that isotope in the medium is increased. The results of such an experiment when

Figure 10

Incorporation of ^3H -uridine into total cell RNA at
various uridine concentrations

50 mm plastic Petri dishes of C13 cells were set up ("Methods", I.1.(i)) and when almost confluent were each labelled for 30 min with 1 $\mu\text{Ci}/\text{ml}$ of ^3H -uridine (uridine-5-T) in the presence of unlabelled uridine varying from 0.2 μM - 25 μM . At the end of the labelling period the cells were scraped off, TCA-precipitated and hyamine digested; the radioactivity was determined by liquid scintillation spectrometry ("Methods", II. 4.(i)).

Incorporation of ^3H -uridine (d.p.m. $\times 10$)

Figure 10

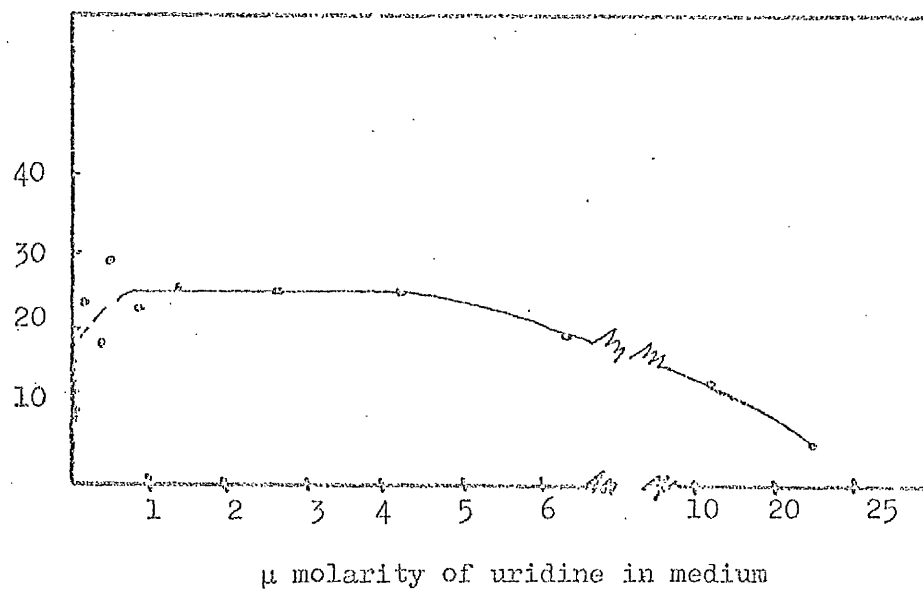


Table 3

Effect on incorporation into total RNA of varying amounts of ^3H -uridine at constant molarity of uridine ($2.7 \mu\text{M}$)

$\mu\text{Ci/ml}$ of ^3H -uridine added	Incorporation (d.p.m. $\times 10^{-3}$)
1	24.8
4	99.7
8	220.8

Incorporation is expressed as d.p.m. incorporated into RNA, obtained as described in the legend to Fig. 10.

the medium concentration of uridine is equivalent to the "plateau" level of Fig. 10 (in this case $2.7 \mu\text{M}$) is shown in Table 3. This indicates that, as the amount of added label increases, so does the amount of label incorporated, by a similar factor. Hence at this concentration of uridine in the medium, the effect of the intracellular pool of uridine on incorporation of labelled uridine is negligible.

Thus this "plateau" level of around $3 \mu\text{M}$ uridine was used in subsequent experiments and was adjusted from time to time for experiments in which longer labelling times were used; however, the concentration of $6 \mu\text{M}$ (the maximum concentration on the "plateau" in Fig. 10) was never exceeded.

I.2. Uptake of ^3H -uridine into PRV-infected cells.

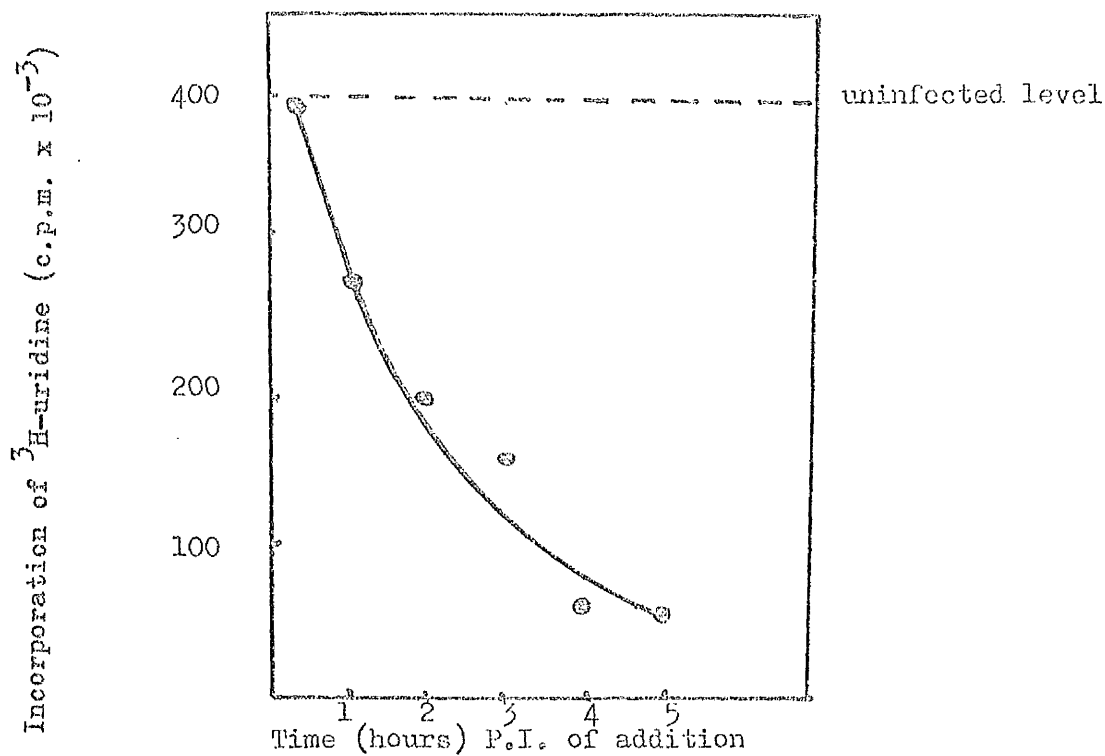
To investigate incorporation of tritiated uridine into total RNA in the PRV-infected cell, plates of Cl3 cells were set up and infected or mock-infected with PRV, as described in "Methods", I.1. At various times after infection, the cells were labelled for 1 hour with uridine - $5 - ^3\text{H}$ and harvested immediately after labelling ("Methods", II.4.(ii)). The results are given in Fig. 11, which shows that the incorporation of ^3H -uridine into RNA decreases steadily after infection, and reaches 15% of the mock-infected (or uninfected) level after 5 - 6 hours.

II. Cytoplasmic RNA synthesis in exponentially-growing uninfected and PRV-infected cells.

II.1. In vivo labelling studies of exponentially-growing cells.

II.1.(i). Sephadex G100 column chromatography.

A commonly-used method of separating the low molecular weight species of RNA from the larger species is by gel filtration on a Sephadex G100



of isotope.

Figure 11. Incorporation of ^3H -uridine into total cell RNA
at various times after PRV-infection

50 mm plastic Petri dishes of Cl3 cells were set up, seeded over 3 x 13 mm glass coverslips per dish. Plates were either mock-infected or infected with PRV at 20 p.f.u./cell, and were labelled for 1 hour periods from 1 - 5 hours P.I. with 1 $\mu\text{Ci}/\text{ml}$ of ^3H -uridine (uridine - 5 - ^3H). At the end of the labelling period the coverslips were removed, fixed and counted ("Methods", II.4. (ii)). The plates were set up in duplicate, and hence 6 coverslip results were obtained for each point; the figure graphed is the average of the 4 median counts.

column (see "Methods", II.3.(i); Galibert et al., 1965). A typical separation of cytoplasmic RNA on such a column is shown in Fig. 12; there are three peaks of absorbancy at 260 nm. The first, eluting between 25 - 45 ml elutes with the void volume (obtained by the use of Blue Dextran - "Methods", II.3.(i)); the second peak elutes between 50 - 60 ml and the third between 70 - 90 ml. The three peaks are respectively the two largest ribosomal RNAs (also messenger RNA), 5S RNA and 4S RNA (Galibert et al., 1965). It can be seen from Fig. 12 that, in our hands at least, the separation of 5S RNA from ribosomal RNA is poor. However, this method does allow large amounts of RNA to be separated, and this is especially convenient if incorporation of radioisotope is low.

Earlier work using Sephadex G100 chromatography (Shepherd, 1969) had shown that the elution profile of cytoplasmic RNA labelled with ^3H -uridine for 30 min periods was considerably changed by infection with PRV at any time from 1 - 7 hours P.I.. It was decided to repeat this experiment as a preliminary to characterising the nature of these changes in RNA synthesis induced by PRV-infection. Cells were labelled at 5 hours P.I. since by then host DNA synthesis has virtually ceased (Kaplan and Ben-Porat, 1963), virus DNA synthesis is well under way (Kaplan and Ben-Porat, 1963; also Fig. 41) and progeny virus have begun to appear inside the cells (Sydskis, 1969); similar results have been obtained in this department.

Thus PRV-infected cells were labelled for 30 min with ^3H -uridine at 5 hours P.I., while mock-infected cells were labelled for 30 min with ^{14}C -uridine; these cells were then harvested together and cytoplasmic RNA was prepared ("Methods", II.1.(i)a and II.2.(i)b) and fractionated on a Sephadex G100 column. The results are shown in Fig. 13 and reveal that,

65

Figure 12.

Sephadex G100 column chromatography

Burlers of Cl3 cells were harvested, the cytoplasmic fraction was prepared ("Methods", II.1.(i)a) and RNA was extracted from it by phenol-extraction ("Methods", II.2.(i)b), and was chromatographed on a 1.5 x 80 cm column of Sephadex G100 ("Methods", II.3.(i)). SSC was used as eluant and 1.0 ml fractions were collected. The absorbancy at 260 nm of each fraction was measured on a SP 500 spectrophotometer.

Fig. 12

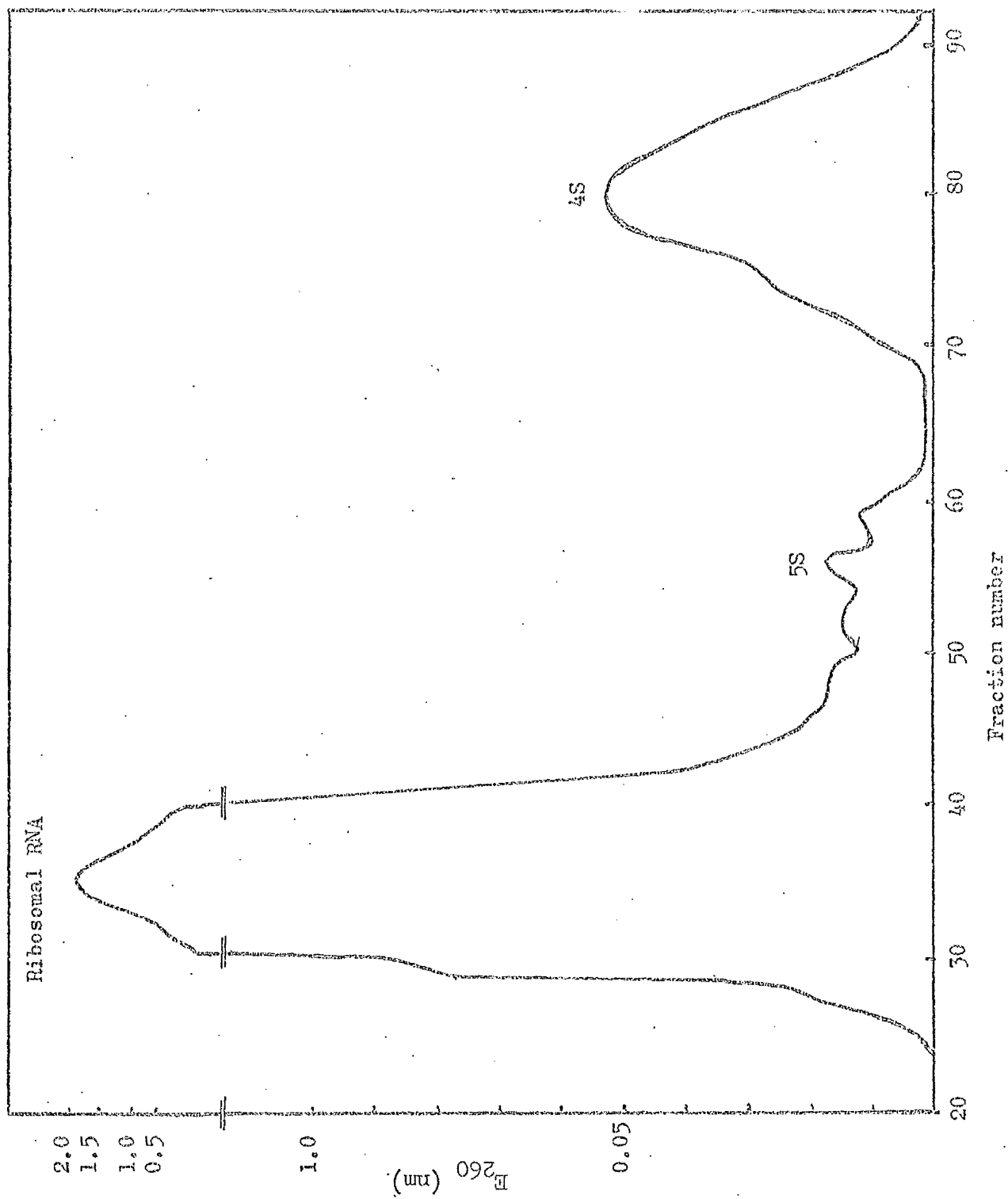


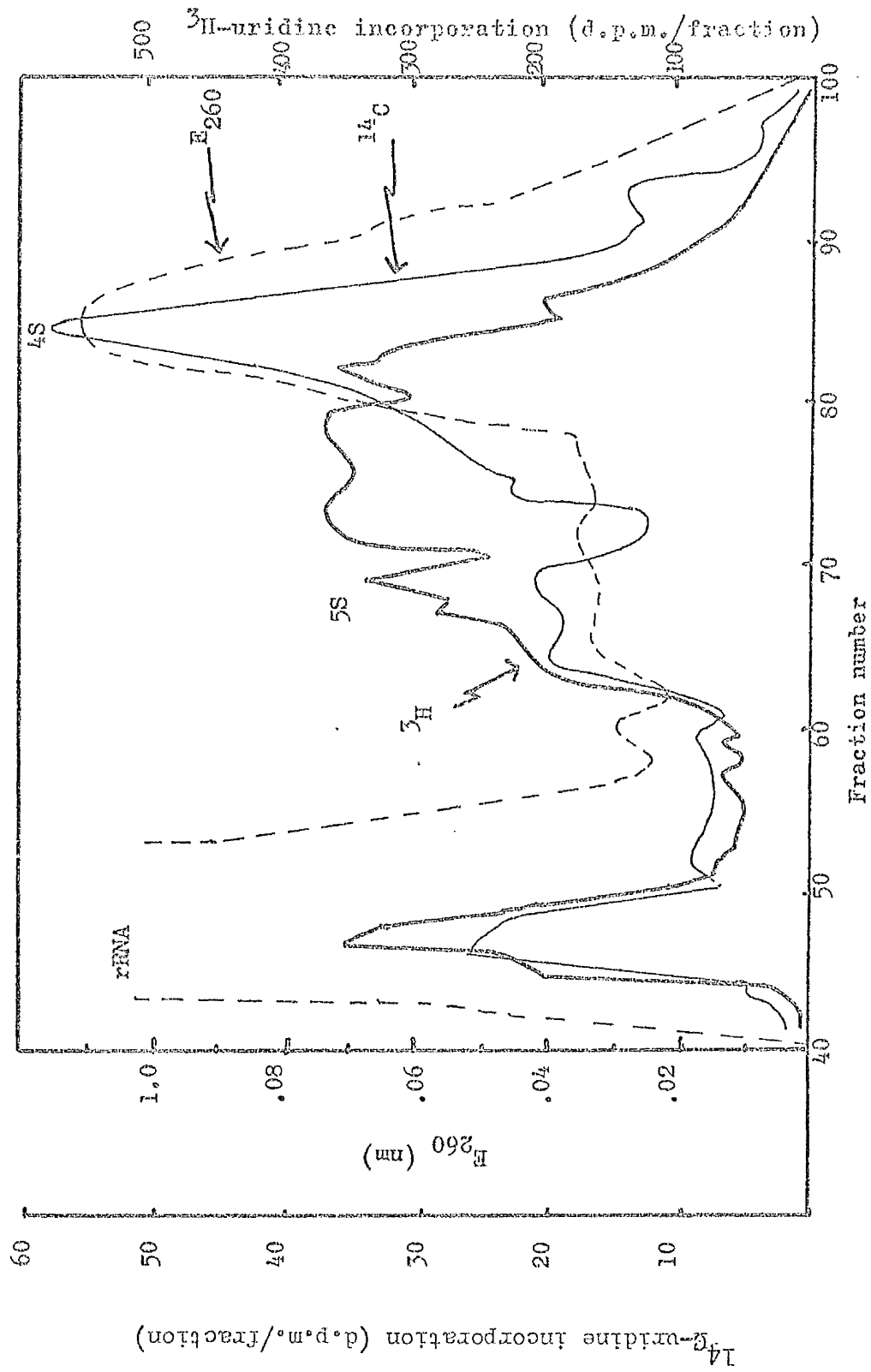
Figure 13.

30 min pulse-label of PRV-infected and uninfected cell cytoplasmic
RNA chromatographed on a Sephadex G100 column

Nearly-confluent burlers of C13 cells were infected or mock-infected and 5 hours P.I. were labelled for 30 min with ^3H -uridine or ^{14}C -uridine respectively. The burlers were harvested together with unlabelled cells. Cytoplasmic RNA was prepared by pelleting the nuclei and phenol-extraction ("Methods", II.2.(i)b) and was fractionated on a 1.5 x 80 cm Sephadex G100 column ("Methods", II.3.(i)). SSC was the eluant and 1.0 ml fractions were collected, their absorbancy at 260nm estimated on a SP500 spectrophotometer, and the d.p.m. in each fraction obtained by TCA precipitation of the fractions on to cellulose acetate membranes and subsequent liquid scintillation spectrometry.

— — — — —, absorbancy at 260nm
 —————, ^3H d.p.m. from ^3H -uridine
 (PRV-infected cells)
 —————, ^{14}C d.p.m. from ^{14}C -uridine
 (mock-infected cells)

Figure 13.



after a 30 min pulse-label, the position of the three peaks of radioactivity in the uninfected cell RNA is very similar to the absorbancy profile, i.e. there are peaks of rRNA, 5S RNA and 4S RNA; the only radioactive peak which does not correspond exactly in position to that of the absorbancy is that of 4S RNA, which is very slightly displaced towards the 5S position. This slight displacement is probably owing to the very small amount of pre-tRNA labelled after 30 min in uninfected Cl3 cells (see "Introduction", III.3.(ii)a; also Smillie, 1970). There are, however, large differences in the relative amounts of isotope in the three peaks, notably the rRNA is present in a small amount relative to 5S RNA and 4S RNA. This is to be expected since the processing of ribosomal RNA from nucleus to cytoplasm is slow - about 30 min for 18S and longer for 28S (Penman et al., 1966; Penman, 1966; Fig. 48).

The radioactive profile of the infected cell RNA is very different from that of the uninfected cell RNA; it contains rRNA but the material in the low molecular weight region is very poorly fractionated. Most of the material elutes between the 5S and 4S RNA absorbancy peaks and only a very small fraction of material coelutes with 4S RNA; there is a sharper 5S RNA peak in the infected cell RNA than in the uninfected cell RNA. One further difference between the infected and the uninfected pulse-labelled profiles in Fig. 13 is the relatively even smaller amount of rRNA present in the infected cell; this is due to the strong inhibitory effect of pseudorabies virus on rRNA synthesis and will be discussed later.

Thus Fig. 13 shows that PRV-infection causes marked changes in the synthesis of cytoplasmic RNA species, especially in the region of 5S to 4S RNA. These differences in elution profile between the infected and uninfected

Figure 14

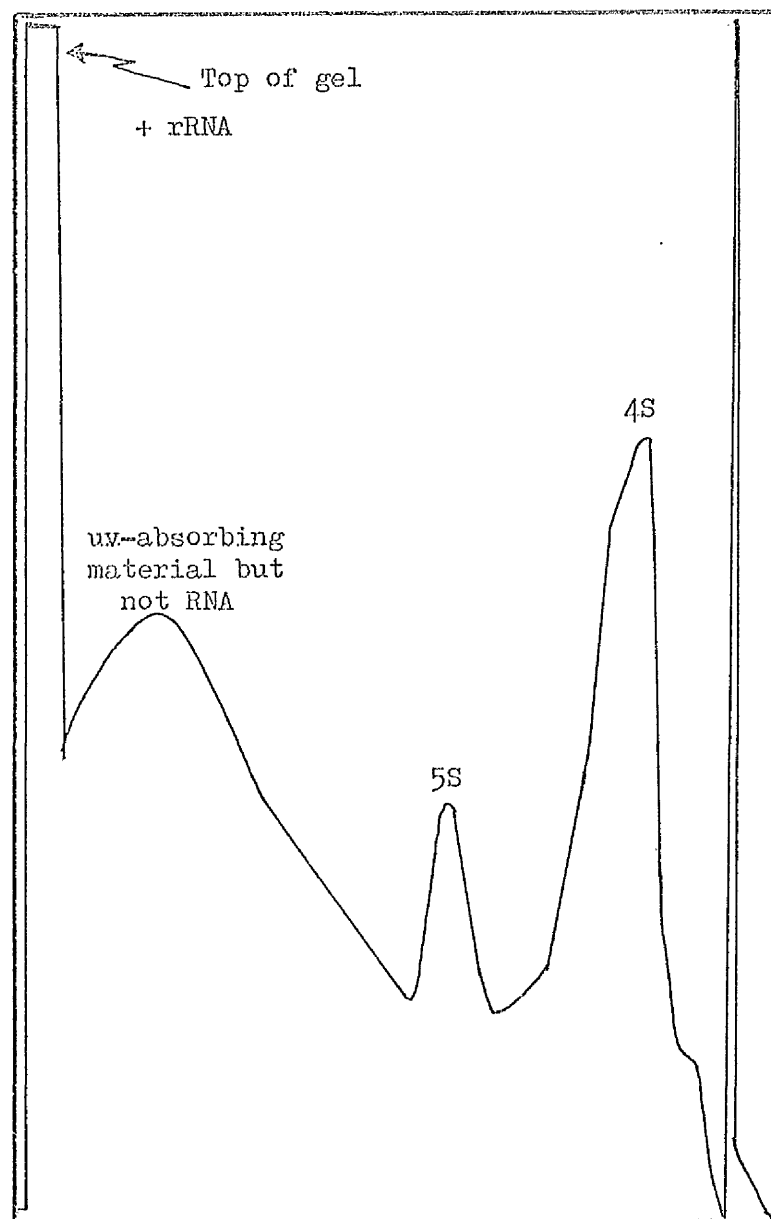


Figure 14. Absorbancy trace at 260 nm of a 10% polyacrylamide gel electrophoretic separation of cell cytoplasmic RNA

Cytoplasmic RNA was prepared from Cl3 cells ("Methods", II.2.(i)b) and was electrophoresed on a 10% polyacrylamide gel ("Methods", II.3.(ii)). The absorbancy trace at 260 nm was obtained using a Gilford model 240 spectrophotometer with a linear transport attachment (model 2410).

cytoplasmic RNA must be due to the effect of PRV-infection since the "double-label" technique ensured that the method of preparation was identical; (by "double-label" is meant the use of two different isotopes of the same radioactive precursor - here ^3H -uridine and ^{14}C -uridine for the infected and uninfected cell respectively). Differences due to isotope effects were excluded by reversing the labels in a subsequent experiment in which ^{14}C -uridine was used to label infected cells and ^3H -uridine was used to label uninfected cells. Artefacts due to isotope differences were further ruled out by subsequent experiments in which ^3H -uridine was used to label both infected and uninfected cell cytoplasmic RNA (section II.1.(iii)etc.).

Investigation of the differences in RNA synthesis between the infected and the uninfected cell - especially of the striking differences in the low molecular weight region - is complicated by the poor resolution by Sephadex G100 columns of 5S RNA from rRNA, and of the region between 5S and 4S RNA. Thus, to achieve better resolution, 10% polyacrylamide gels (Loening, 1967) were used to fractionate RNA in all subsequent work on low molecular weight RNAs.

II.1.(ii). Calibration of the 10% polyacrylamide gel system.

The separation of a cytoplasmic preparation of RNA (prepared as described in "Methods", II.2.(i)) is shown in Fig. 14, in which absorbancy at 260 nm is measured in the intact, unstained gel ("Methods", II.3.(ii)). There are three peaks. The 28S and 18S rRNAs do not enter a 10% gel; they remain on top of the gel and are hence liable to be lost in the processing of the gel, making their quantitation difficult. The smaller species of RNA do enter the gel, and it can be seen that there is good separation

between 5S and 4S RNA, which were identified by reference to published data (e.g. Loening, 1966); the identity of 4S RNA with the 4S RNA peak of Sephadex G100 column chromatography was independently confirmed in a subsequent experiment (Fig. 16b).

Choice of isotope for future experiments. While there are clearly advantages in the interpretation of results from experiments in which a double-label technique (as in Fig. 13) has been used, we had experienced low levels of radioactivity from ^{14}C -uridine incorporation. This was the result of: (a) the low specific activity of ^{14}C -uridine preparations commercially available, and (b) the decrease in RNA synthesis after PRV-infection. Thus it was decided that in the following series of experiments ^3H -uridine should be used to pulse-label both infected and uninfected cell cytoplasmic RNA; this necessitates that the material is worked-up and electrophoresed separately. To facilitate comparison of gels, cytoplasmic RNA labelled to equilibrium with ^{14}C -uridine was prepared (Fig. 15), and was co-electrophoresed routinely with the sample of ^3H -labelled RNA. This "double-label" technique allowed the radioactive elution profile of the ^3H -RNA to be compared directly with this internal ^{14}C -marker RNA, and circumvented the difficulty of relating the absorbancy trace to the d.p.m. obtained from each gel slice. In addition, in later work it became important to be able to pinpoint the exact relative position of the radioactivity and absorbancy peaks.

The gel profile of the ^{14}C -marker RNA fractionated on a 10% polyacrylamide gel is shown in Fig. 15. The material was prepared by labelling cells with ^{14}C -uridine for 20 hours (i.e. for about one Cl3 cell generation). This long incubation gives rise to radioactively-labelled RNA equivalent to the absorbancy profile shown in Fig. 14, as comparison of the

Figure 15.

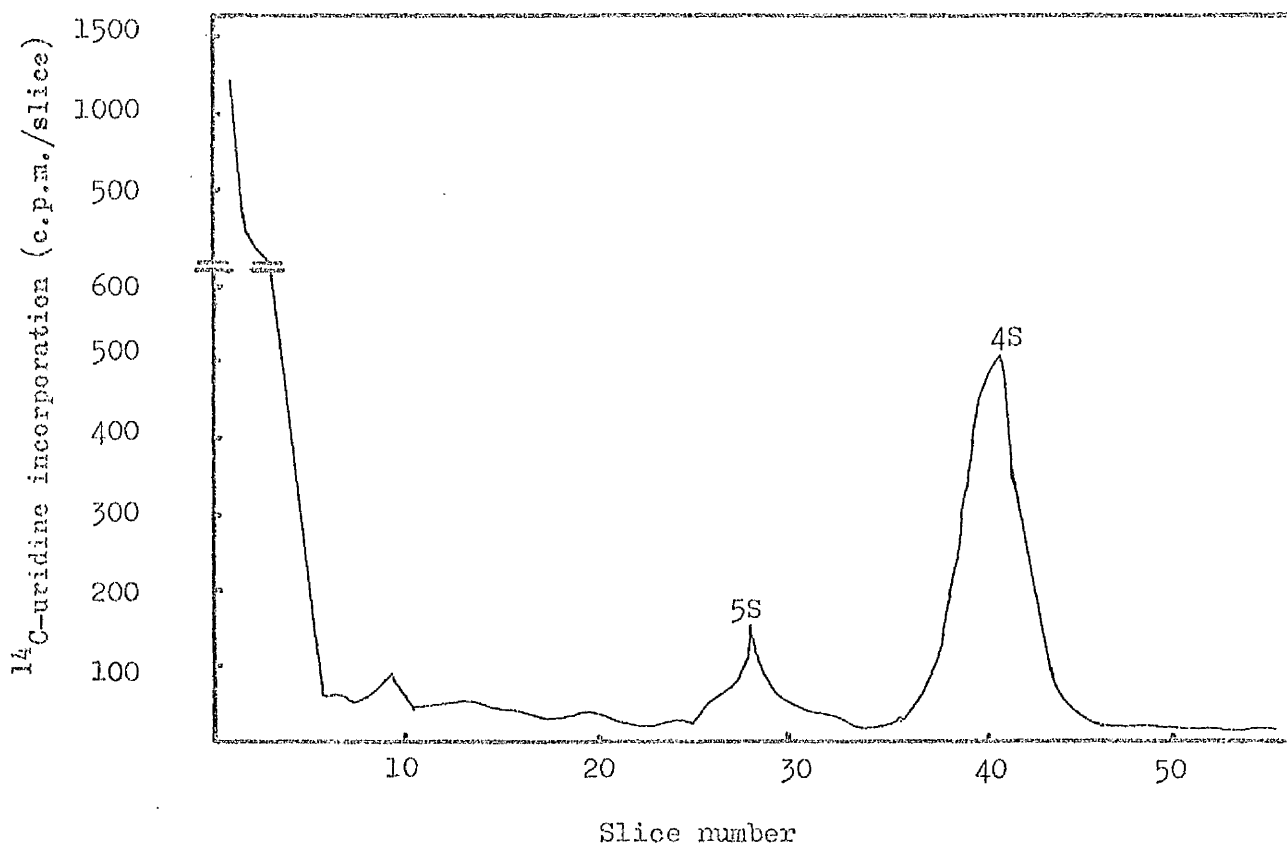


Figure 15. ^{14}C -internal marker RNA electrophoresed on a 10% polyacrylamide gel

One newly-confluent burler of Cl3 cells was labelled for 20 hours with ^{14}C -uridine ($0.25 \mu\text{Ci/ml}$) and RNA was extracted by the "cold phenol" technique ("Methods", II.2.(i)a) and dissolved in 2.0 ml distilled water. 100 μl was electrophoresed on a 10% polyacrylamide gel, which was sliced, and the slices were counted on a gas-flow counter ("Methods", II.3.(ii)).

two figures indicates. It should be pointed out that the ^{14}C -marker RNA is so highly labelled that the amount added to each gel has d.p.m. but no absorbancy. Thus absorbancy traces of the sample RNA alone can be obtained even when the internal ^{14}C -marker is co-electrophoresed.

To confirm that the larger (in amount) of the two peaks which enters the gel is indeed 4S RNA, the pooled 4S RNA fractions from a Sephadex G100 column of C13 cell cytoplasmic RNA labelled with ^3H -uridine for 2 hours were prepared (Fig. 16a). This pooled 4S RNA material was co-electrophoresed with the ^{14}C -marker RNA and the result of this is shown in Fig. 16b. It is clear that 4S RNA from Sephadex G100 is identical to the peak designated as 4S RNA on 10% polyacrylamide gels, since the two peaks exactly coincide.

Once more, comparison of Figs. 16a and 16b shows that the separation of the low molecular weight RNA species afforded by 10% polyacrylamide gel electrophoresis is superior to that obtained by Sephadex G100 column chromatography.

II.1.(iii). 10% polyacrylamide gel electrophoresis of pulse-labelled RNA.

Now that the labelling and fractionating systems had been satisfactorily worked out, RNA metabolism in the PRV-infected cell could be investigated.

Infected and mock-infected cells were pulse-labelled at 5 hours P.I. with ^3H -uridine and the cytoplasmic RNA was prepared in an experiment similar to that described earlier (section II.1.(i)); the two samples of ^3H -RNA were co-electrophoresed separately with the ^{14}C -internal marker. In this experiment a 45 min pulse-label was used instead of a 30 min label in order to decrease the already very small amount of pre-tRNA in the uninfected

Figure 16a.

Sephadex G100 column chromatography of uninfected cell cytoplasmic
RNA labelled with ^3H -uridine for 2 hours

Nearly-confluent burlers of Cl3 cells were labelled with ^3H -uridine for 2 hours; RNA was then prepared and chromatographed on a Sephadex G100 column as before (Fig. 13). The fractions indicated by hatching were combined and the RNA was precipitated from them by 2% sodium acetate/67% ethanol and resuspended in a small volume of distilled water.

————— , absorbancy at 260nm
—— — , ^3H d.p.m. from ^3H -uridine.

Figure 16b.

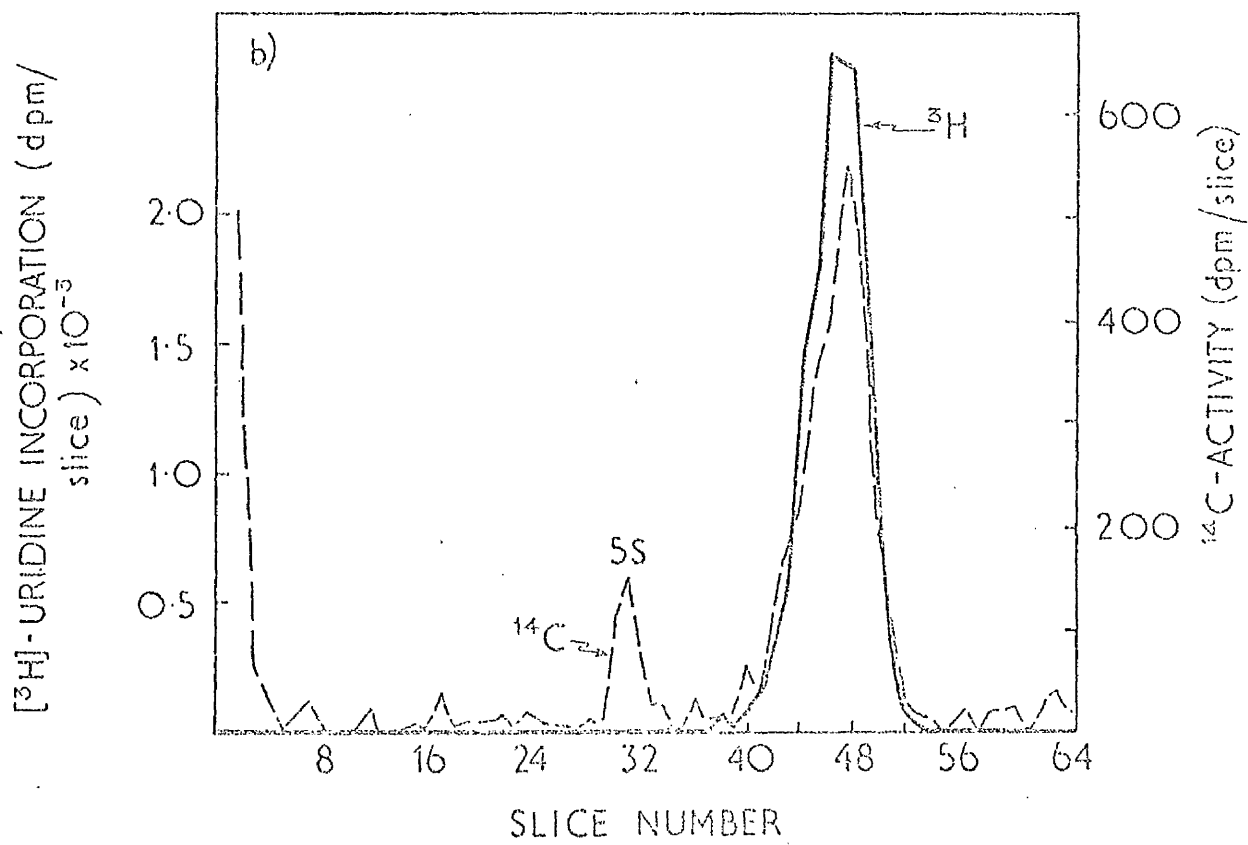
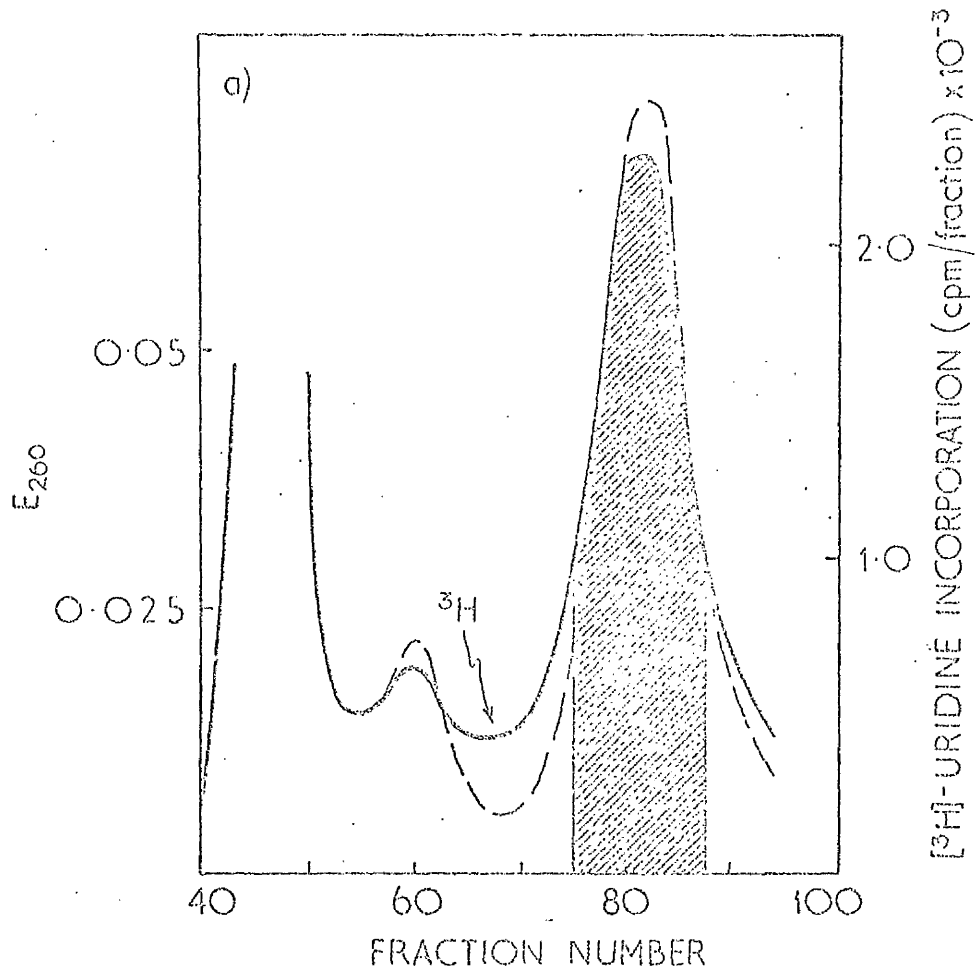
10% polyacrylamide gel electrophoresis of column-purified 4S RNA

The sample of ^3H -RNA prepared as described above was co-electrophoresed on a 10% polyacrylamide gel with 20 μl of ^{14}C -internal marker. The gels were run, sliced and counted on a liquid scintillation counter as described in "Methods", II.3.(ii).

————— , ^3H d.p.m. from ^3H -uridine
—— — , ^{14}C dp.m. from ^{14}C -internal marker

Figure 16

16



cell cytoplasm. The results from this experiment are shown in Fig. 17a and b. It can be seen that after a 45 min label the RNA from uninfected cell cytoplasm electrophoreses in a very similar manner to the internal ^{14}C -marker, with only a very small amount of material between 5S and 4S, i.e. after 45 min, the low molecular weight cytoplasmic RNA of uninfected cells has been labelled almost to equilibrium. In the infected cell, however, the 45 min-labelled cytoplasmic RNA gave a polyacrylamide gel electrophoresis profile similar to that obtained on a Sephadex G100 column (Fig. 13); the characteristics of the profile are a relatively large 5S peak, a great deal of material between 5S and 4S, and very little material in the 4S position. The material between 5S and 4S (Fig. 17b) consistently eluted as two main peaks, which have been designated throughout these studies as peak I (closer to 5S) and peak II; peak II often contains a second minor peak.

The total d.p.m. incorporated into rRNA, 5S RNA, 4S RNA and peaks I + II are tabulated in Table 4. The small amount of material between 5S and 4S in the RNA from uninfected cell cytoplasm is tabulated as I + II for purposes of comparison only. The table shows that rRNA production is very strongly inhibited by PRV infection, 4S RNA synthesis is less inhibited and 5S RNA synthesis is even less inhibited.

In the uninfected Cl3 cell, ^3H -labelled RNA appears between 5S and 4S after very short incubations (about 5 -- 10 min) with ^3H -uridine precursor; this material has been shown to be the precursor to tRNA ("Introduction", III.3.(ii); Smillie and Burdon, 1970). In the uninfected Cl3 cell, the maturation of this precursor is rapid and so a short pulse-label is necessary to label this precursor rather than 4S RNA; hence a 45 min label (Fig. 17a) shows only a very small amount of material that could be pre-tRNA. Now,

Figure 17

Comparison of uninfected and infected cell cytoplasmic RNA
electrophoresed on 10% polyacrylamide gels

Nearly-confluent burlers of Cl3 cells were either (a) mock-infected or (b) infected with PRV, and at 5 hours P.I. were labelled for 45 min with ^3H -uridine ($3\mu\text{Ci/ml}$). The cytoplasmic RNA was prepared by phenol-extraction of the cytoplasmic fraction ("Methods", II.2.(i)b) and an aliquot of each sample was co-electrophoresed with 20 μl of ^{14}C -internal marker on 10% polyacrylamide gels, which were run, sliced and counted as described in "Methods", II.3.(ii).

—————, ^3H d.p.m. from ^3H -uridine
- - - - -, ^{14}C d.p.m. from ^{14}C -internal marker.

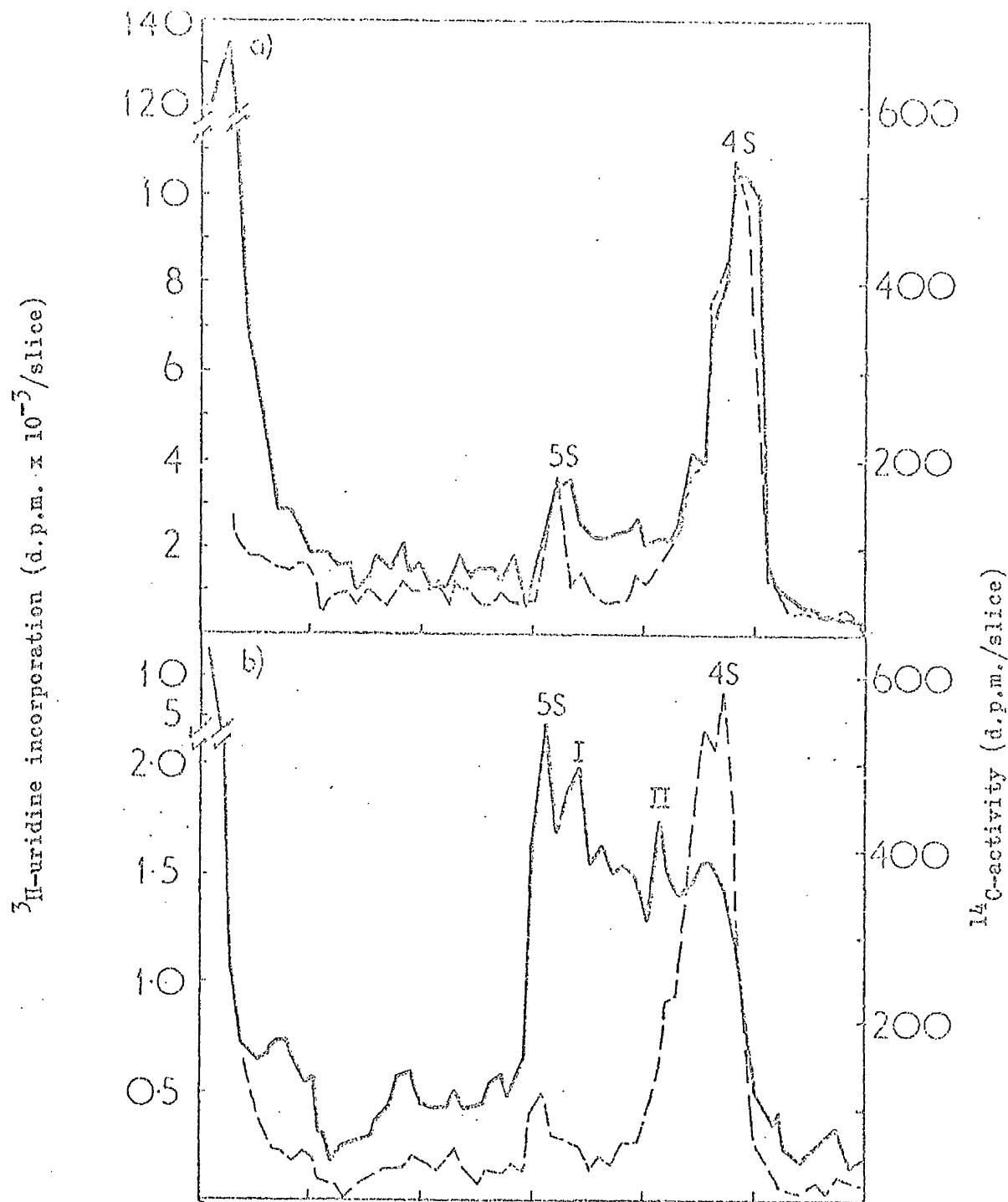


Table 4

Incorporation of ^3H -uridine into cytoplasmic RNA species
in uninfected and PRV-infected cells during a 45 min
pulse-label

	rRNA	5S	I + II	4S	I + II + 4S
Uninfected (d.p.m. $\times 10^{-3}$)	257	10.5	17.8	48.4	66.2
Infected (d.p.m. $\times 10^{-3}$)	21	5.7	14.0	7.7	21.7
$\frac{\text{Infected}}{\text{Uninfected}} \times 100\%$	8	54	79	16	33

Data taken from Fig. 17.

it is known that PRV decreases overall RNA synthesis after infection (Fig. 11), and that 4S RNA synthesis is decreased to 16% by 5 hours P.I. (Table 4); a reason for this decrease might be a retardation of the rate of maturation of pre-tRNA. If this were the case, then pre-tRNA would remain labelled in the infected cell after longer labelling times than it does in the uninfected cell. Thus the material between 5S and 4S RNA (designated as I and II) in the infected cell cytoplasm could be pre-tRNA; the final column in Table 4 indicates what would be the true level of "4S RNA" in the infected cell if I and II were precursors to tRNA. This then suggests that we can distinguish two effects of PRV on 4S RNA synthesis: (a) inhibition of maturation of pre-tRNA, and (b) a decrease in the synthesis of pre-tRNA.

Gel electrophoresis of briefly-labelled RNA from uninfected cells. The following series of experiments was designed to examine the material designated I + II in the PRV-infected cell, and to try to determine if this material behaves as pre-tRNA.

It seemed important at this stage to establish the electrophoretic properties on 10% polyacrylamide gels of material known to contain a high proportion of pre-tRNA. Thus uninfected Cl3 cells were pulse-labelled for 5 min and for 10 min with ^3H -uridine; this length of label in Cl3 cells is known to produce predominantly pre-tRNA rather than tRNA (Smillie and Burdon, 1970; Smillie, 1970). The cytoplasmic RNA from these experiments was prepared as described in "Methods", II.2.(i).b and electrophoresed separately on 10% polyacrylamide gels with the ^{14}C -marker RNA. The results are shown in Figs. 18a and b. The 5 min pulse-labelled RNA (Fig. 18a) shows material predominantly between 5S and 4S; it appears as two main peaks, possibly analogous to peaks I and II of uninfected cell cytoplasmic RNA. The 10 min

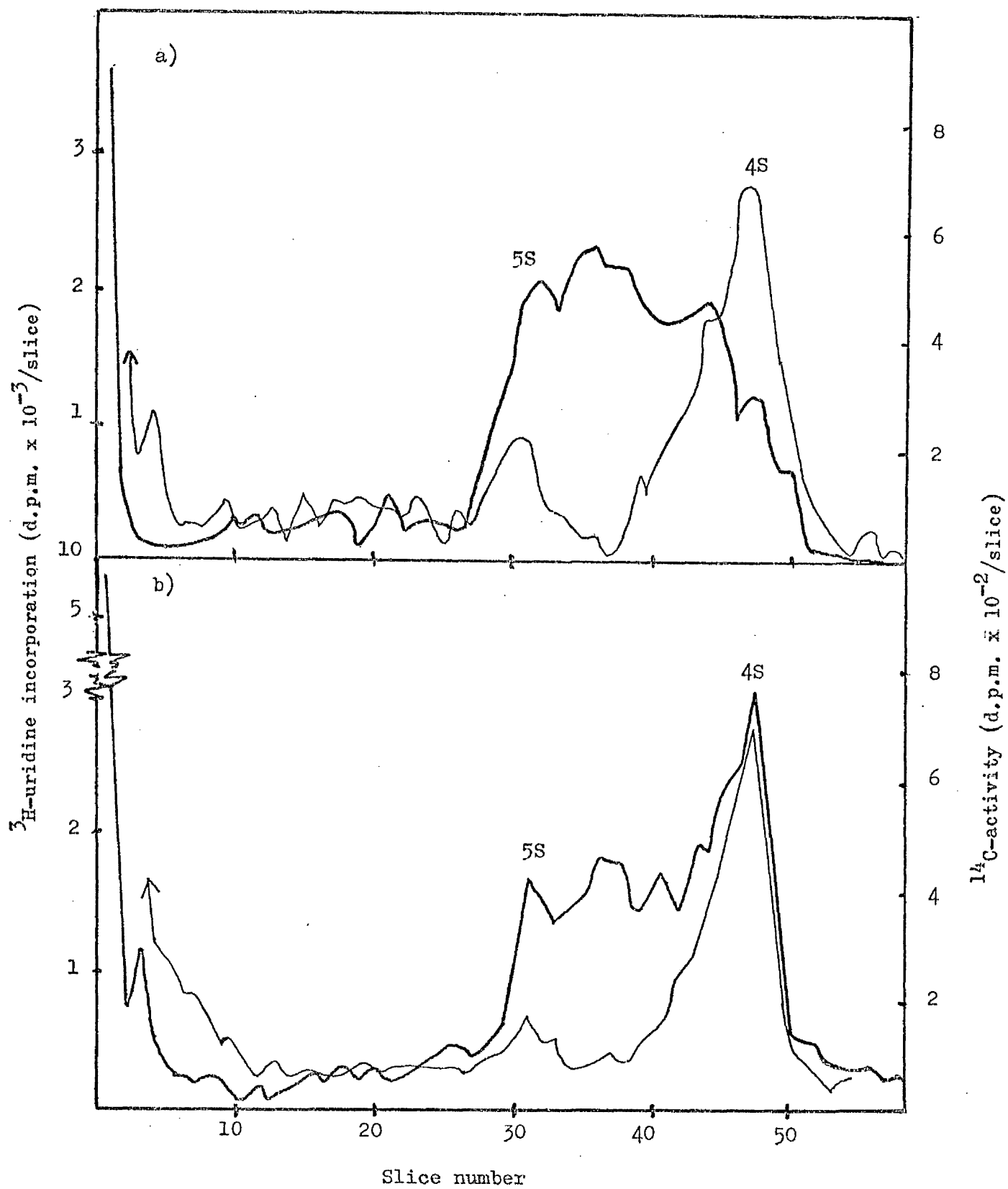
Figure 18.

Uninfected cell cytoplasmic RNA labelled for 5 min and for
10 min electrophoresed on 10% polyacrylamide gels

Nearly-confluent Cl3 cells were labelled for (a) 5 min and (b) 10 min with ^3H -uridine ($3\mu\text{Ci/ml}$) and the cytoplasmic RNA was prepared by phenol-extraction of the cytoplasmic fraction ("Methods", II.2.(i)b). An aliquot of each sample was electrophoresed on 10% polyacrylamide gels with $20\mu\text{l}$ ^{14}C -internal marker; the gels were run, sliced and counted in a liquid scintillation counter as described in "Methods", II.3.(ii).

—————, ^3H d.p.m. from ^3H -uridine
 —————, ^{14}C d.p.m. from ^{14}C -internal marker.

Figure 18



labelled RNA (Fig. 18b), in contrast, contains a significant amount of 4S RNA, but over half the material smaller than 5S RNA electrophoreses between 5S and 4S - also as two peaks. A 5 min or a 10 min pulse-label of uninfected Cl3 cells has been reported to produce predominantly pre-tRNA in the low molecular weight cytoplasmic RNA fraction, eluting as one peak from a Sephadex G100 column (Smillie and Burdon, 1970; Smillie, 1970). The superior resolving power of the polyacrylamide gel system has probably enabled this peak to be fractionated into two species.

Gel electrophoresis of pre-tRNA from uninfected cells. Since the presence of two peaks in the pre-tRNA region is novel, it was decided to electrophorese on the 10% polyacrylamide gel system material established to be pre-tRNA by the criteria used by Smillie and Burdon, 1970, but which appeared as a single peak on G100 Sephadex.

Pre-tRNA from uninfected Cl3 cells was kindly prepared by Dr. J. Smillie by labelling Cl3 cells for 10 min with ^3H -uridine, preparing cytoplasmic RNA ("Methods", II.2.(i).a), and fractionation of the RNA on a Sephadex G100 column. Fractions were taken from the column as indicated in Fig. 19a to correspond to partially-purified pre-tRNA. This material can be converted to 4S RNA in vitro (Smillie and Burdon, 1970; similar results have been reported by Mowshowitz, 1970). This partially-purified pre-tRNA was co-electrophoresed on a 10% polyacrylamide gel with ^{14}C -internal marker. The result is shown in Fig. 19b, and it is clear that the single diffuse peak of pre-tRNA on a G100 column has been resolved on the 10% polyacrylamide gel into two main peaks, electrophoresing between 5S and 4S RNA. These two peaks of pre-tRNA appear on the gel in a similar position to the material designated peaks I and II observed in the infected cell cytoplasm after a 30 or 45 min label. If then this infected cell RNA is pre-tRNA, its appearance after a 30 - 45 min pulse-

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Figure 19a

Sephadex G100 column chromatography of uninfected cell cytoplasmic
RNA labelled with ^3H -uridine for 10 mins

Nearly-confluent burlers of Cl3 cells were labelled with ^3H -uridine for 10 mins; the cells were harvested and RNA prepared by the "cold phenol" method ("Methods", II.2.(i)a) and chromatographed on a Sephadex G100 column, and the hatched fractions combined as described in Fig. 16a.

———— , absorbancy at 260 nm
— — — , ^3H d.p.m. from ^3H -uridine.

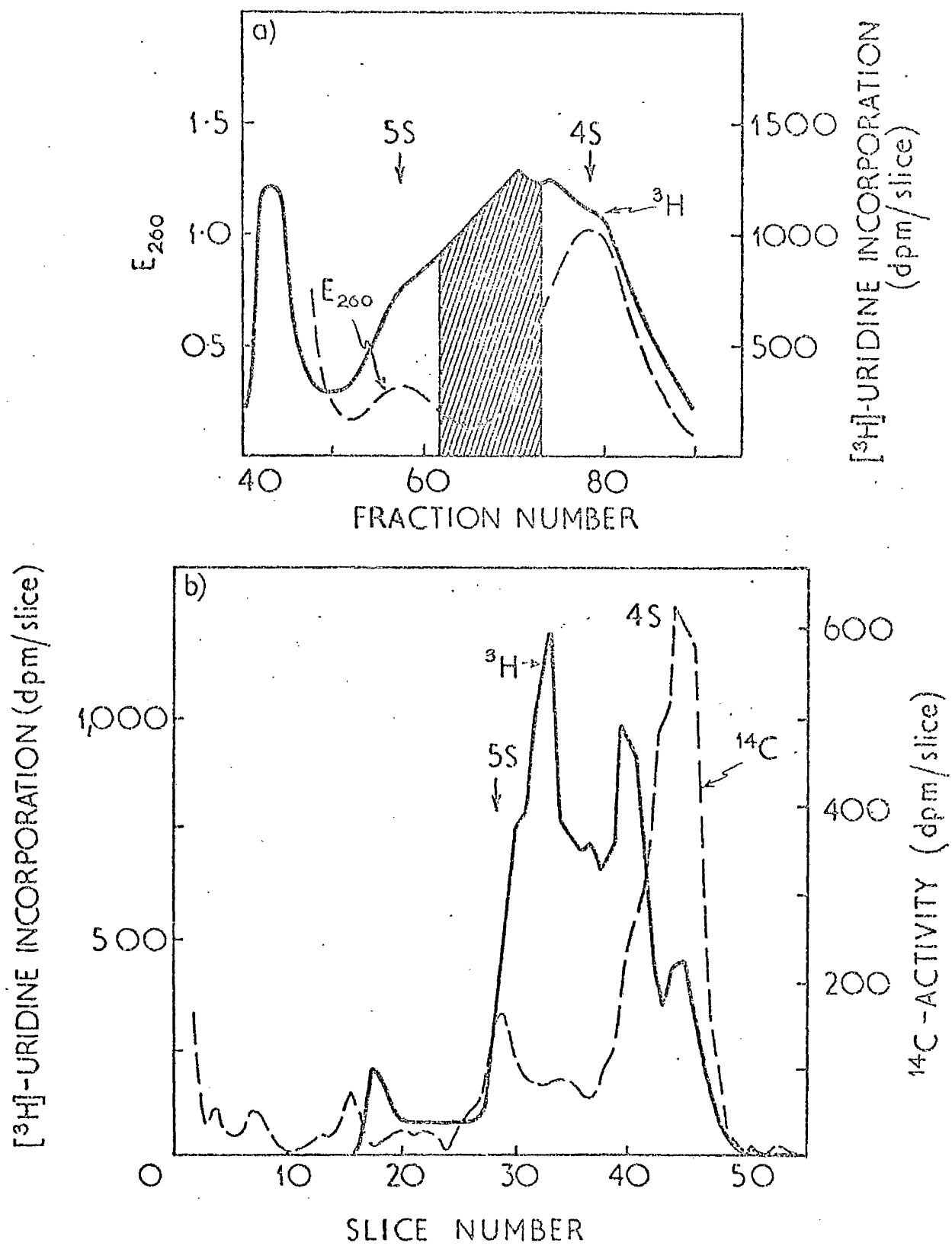
Figure 19b

10% polyacrylamide gel electrophoretic separation of partially-
purified pre-tRNA

The sample of ^3H -RNA prepared as described above was co-electrophoresed on a 10% polyacrylamide gel with 20 μl of ^{14}C -internal marker. The gels were run, sliced and counted in a liquid scintillation counter as described in "Methods", II.3.(ii).

———— , ^3H d.p.m. from ^3H -uridine
— — — , ^{14}C d.p.m. from ^{14}C -internal marker.

Figure 19



label could be explained by a retardation of the maturing process. This hypothesis was tested by the following series of experiments designed to examine the kinetics of production of low molecular weight cytoplasmic RNA.

Time-course of cytoplasmic RNA from the infected cell. The kinetics of appearance of this 4 to 5S material in the infected cell was investigated. Infected cells were labelled for various lengths of time at 5 hours P.I. with ^3H -uridine. The cytoplasmic RNA was extracted ("Methods", II.2.(i)b) and co-electrophoresed with the ^{14}C -marker RNA. The results obtained from pulse-labelled periods of 10 min, 30 min, 45 min and 2 hours are shown in Fig. 20. In all cases there are 5S and 4S RNA peaks and also, between 5S and 4S, the two peaks designated as peaks I and II. After a 10 min pulse-label (Fig. 20a) there is a distinct 5S RNA peak, peak I, peak II and virtually no 4S RNA. As the length of labelling time is increased the amount of label in peaks I and II increases and the 4S peak also increases, eventually becoming the most highly labelled low molecular weight RNA species in the cytoplasm; in addition, at these longer labelling times there is more peak II relative to peak I than in the samples pulse-labelled for shorter times.

These results suggest that there may be a flow of labelled RNA from peaks I and II to the 4S position, or possibly from peak I to peak II to 4S. This does suggest a 4S RNA precursor function for peaks I and II, although these results could be explained by breakdown of peaks I and II and reutilisation, coupled with separate synthesis of 4S RNA. These possibilities are examined in the next series of experiments using actinomycin D and also in the in vitro chase experiments described in sections II.2 and III.2.

The results of Fig. 20 also show that, if there is a maturation of

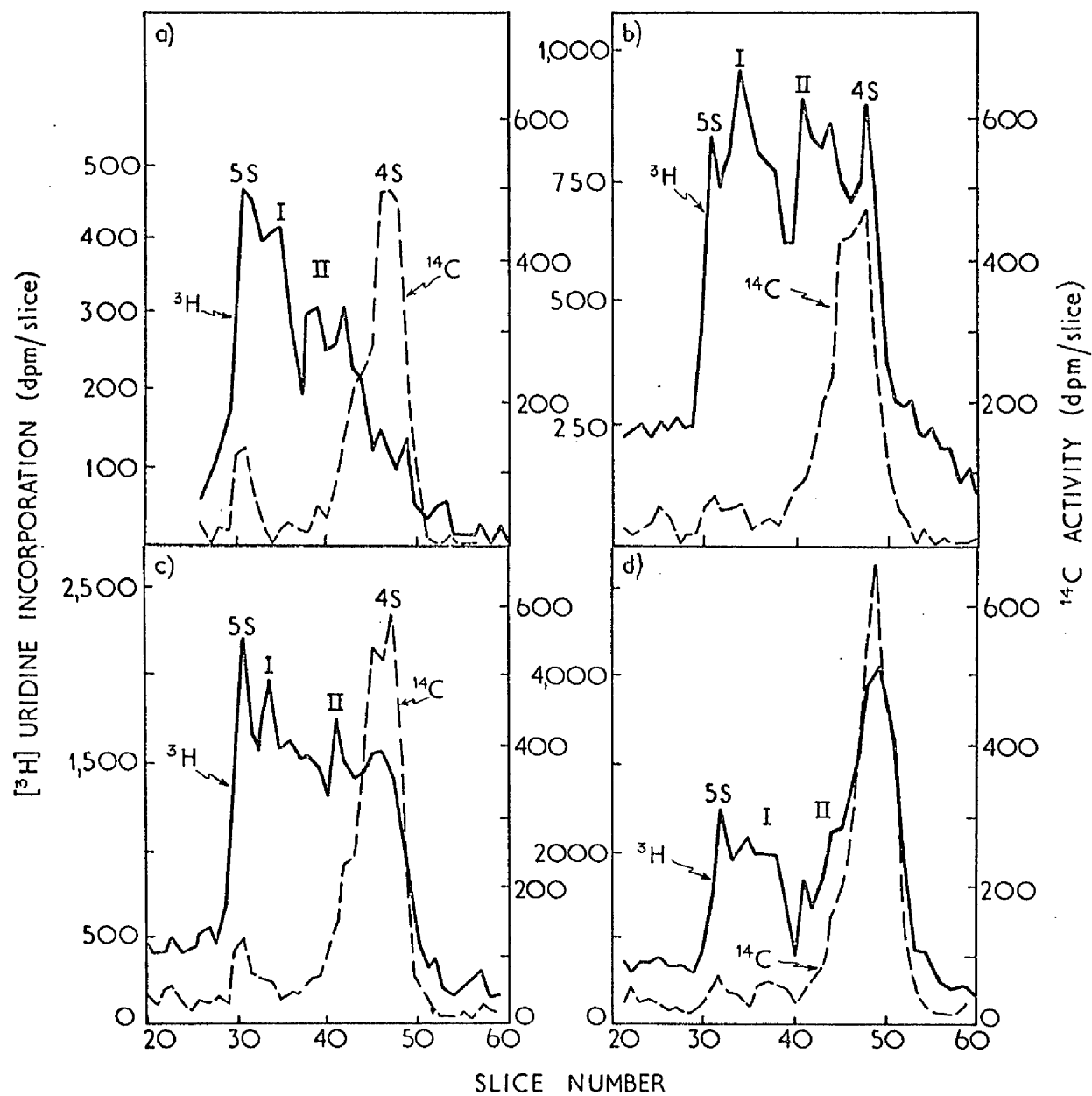
Figure 20

Time-course of infected cell cytoplasmic RNA

Newly confluent burlers of Cl3 cells were infected with PRV, and at 5 hours P.I. were labelled for (a) 10 min, (b) 30 min, (c) 45 min, and (d) 2 hours with ^3H -uridine ($3\mu\text{Ci/ml}$). Cytoplasmic RNA was prepared by phenol extraction of the cytoplasmic fraction and electrophoresed on 10% polyacrylamide gels as described in Fig. 17.

———— , ^3H d.p.m. from ^3H -uridine
— — — , ^{14}C d.p.m. from ^{14}C -internal marker.

Figure 20



peaks I and II to 4S in the PRV-infected cell, it is much slower than in the uninfected cell. In Fig. 20 there is virtually no 4S RNA after a 10 min label, while there is a substantial amount in the uninfected cell (Fig. 18b); in fact, this infected cell 10 min-labelled material is very similar to the 5 min-labelled material of the uninfected cell (Fig. 18b). In these present results (Fig. 20) the amount of peaks I and II reaches a maximum between 30 - 45 min, and distinct amounts of peaks I and II remain after a 2 hour label; however, in the uninfected cell a pulse-label of 2 hours gives a radioactivity profile virtually identical to that of the absorbancy trace (see Fig. 16a) and even at 45 min the labelling pattern is very similar to the ^{14}C -marker (see Fig. 17a).

One additional feature of the labelled infected cell RNA (Fig. 20) is the high level of 5S RNA relative to the amount of 4S RNA. This will be discussed and investigated later.

Further studies on peaks I and II by use of actinomycin D. The investigation of the nature of the RNA in peaks I and II was continued by studying their behaviour in the absence of new RNA synthesis. If peaks I and II are labelled for a period with ^3H -uridine in vivo and then RNA synthesis is prevented, the fate of the labelled RNA can be followed; if they are precursors to tRNA then, after a suitable length of time, the radioactive label incorporated into peaks I and II should appear in the position of 4S RNA. New RNA synthesis can be prevented by the use of 5 $\mu\text{g/ml}$ actinomycin D in the medium (Perry and Kelley, 1968, 1970).

Thus two burlers of Cl3 cells were infected with PRV and at 5 hours P.I. both were pulse-labelled for 30 min with ^3H -uridine. One burler was harvested immediately, while the other burler was "chased" for 30 min.

The "chase" conditions consisted of replacing the medium with fresh medium containing 5 $\mu\text{g/ml}$ of actinomycin D to prevent new RNA synthesis; the new medium also contained 20 times the previous level of unlabelled uridine to dilute out the radioisotope in the cellular uridine pools. Cytoplasmic RNA was prepared from both burlers ("Methods", II.2.(i)b), and co-electrophoresed on 10% polyacrylamide gels with ^{14}C -marker RNA; the results of the 30 min pulse-label and the 30 min pulse-label followed by a 30 min "chase" are shown in Figs. 21a and b respectively. Fig. 21a - which gives the result of a 30 min pulse-label of infected cell cytoplasmic RNA - resembles previous Figs. 17b, 20b and 20c showing similar 30-45 min labels, although in this case peak I has separated more clearly than before from 5S RNA. It is of interest to note that there appears to be a slightly smaller relative amount of 4S RNA in this experiment than appears, for example in Fig. 20b; the most probable explanation for this would be an effective incubation time slightly shorter than 30 min.

The 30 min-labelled infected cell cytoplasmic RNA "chased" for 30 min is shown in Fig. 21b. This indicates that the amount of material in the 4S RNA peak has increased during the "chase" while the amount of label in peaks I and II has decreased. This result strongly indicates that peaks I and II are precursors to 4S RNA. Note also that there has been a small decrease in the amount of 5S RNA.

Tabulation of the counts in each peak is shown in Table 5; peaks I and II are taken together because of the difficulty in resolving these two species accurately. (It should be noted that species I is imperfectly resolved from 5S RNA, as is species II from 4S RNA; hence a degree of error may thus arise when calculating the counts per peak). This table shows

Figure 21.

In vivo 30 min-"chase" of infected cell cytoplasmic RNA

Two nearly-confluent burlers of Cl3 cells were infected with PRV. At 5 hours P.I. both burlers were labelled with $3\mu\text{Ci/ml}$ of ^3H -uridine (final concentration of uridine = $1\mu\text{M}$) for 30 min. One of the burlers was then harvested (a) while the other was "chased" for 30 min before harvesting (b). "Chase" conditions consisted of replacing the medium with fresh medium containing $5\mu\text{g/ml}$ actinomycin D, $20\mu\text{M}$ with respect to uridine.

Cytoplasmic RNA was prepared from both these samples and electrophoresed on 10% polyacrylamide gels as described in Fig. 17.

——, ^3H d.p.m. from ^3H -uridine
——, ^{14}C d.p.m. from ^{14}C -internal marker.

Figure 21

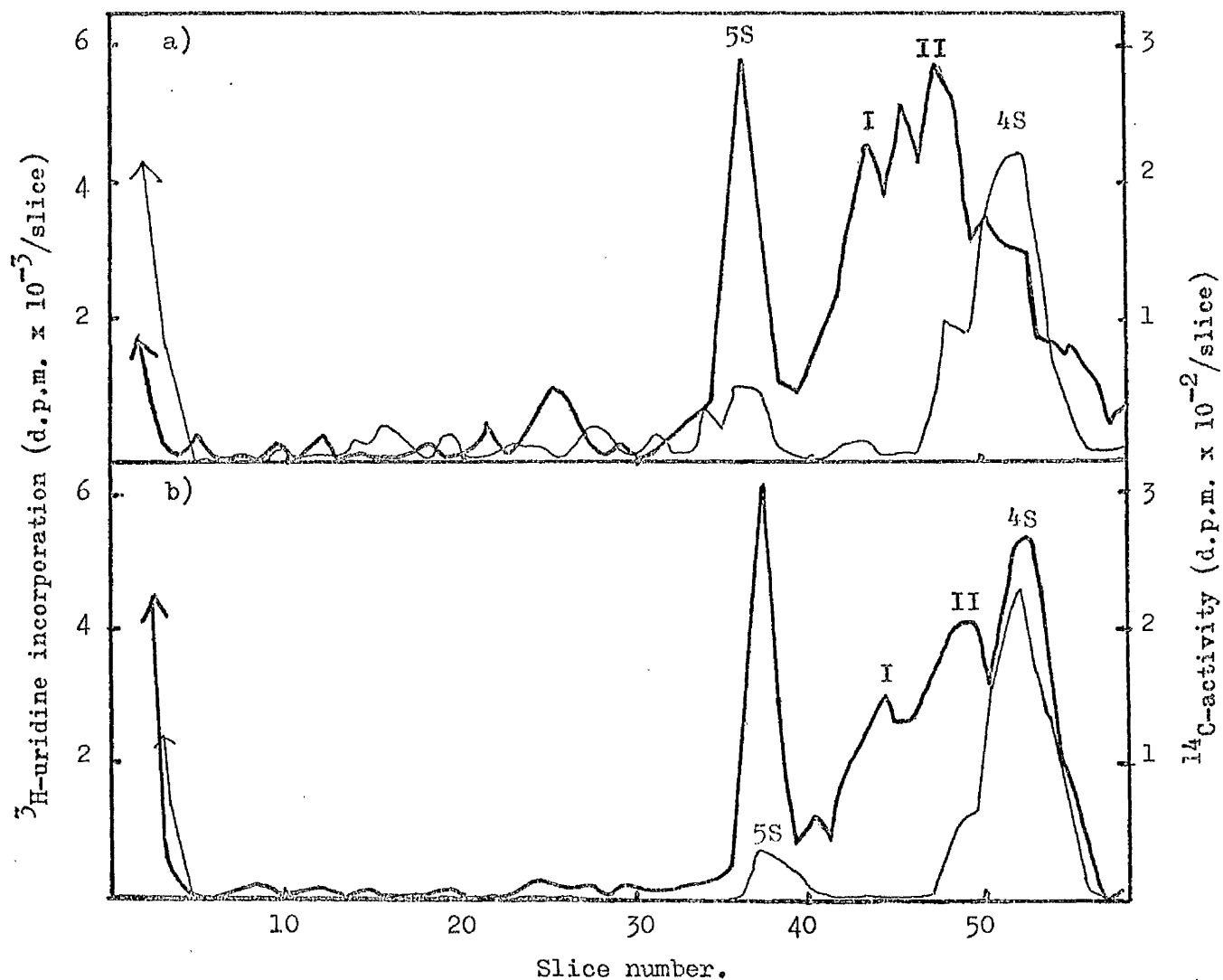


Table 5

Incorporation of ^3H -uridine into low molecular weight cytoplasmic RNA species in PRV-infected cells during a 30 min pulse-label and a subsequent 30 min "chase"

	5S	I + II	4S	I + II + 4S	Total d.p.m.
30 min pulse-label	1410	3855	1550	5405	6815
30 min pulse-label + 30 min "chase"	1280	2640	2190	4830	6110

Data taken from Fig. 21.

that the labelled material in the 4S region increases by 40%, and this is paralleled by the fall in the amount of peaks I and II. The overall recovery of counts is 90%. This suggests that some labelled material may have been lost in the processing of I and II to 4S, as occurs in the maturation of pre-tRNA in the uninfected cell (Smillie and Burdon, 1970). Clearly, the appearance of labelled material in the 4S RNA region can not be explained simply by a breakdown of labelled material and its subsequent reutilisation.

This result indicates that at least a portion of the RNA in peaks I and II moves into the 4S position, but that about 70% of the material in peaks I and II still remains after a 30 min "chase". Thus, a similar experiment to that described above was performed using a longer "chase" period. If peaks I and II are precursors to 4S RNA, then a longer "chase" period should result in a greater loss of material from peaks I and II and a corresponding increase in the amount of 4S RNA.

The pulse-labelling period used was 45 min and the cells were "chased" for 1 hour. The results of this experiment are shown in Fig. 22, and the counts in each peak are tabulated in Table 6. The 45 min pulse-label of infected cell RNA gives the now familiar pattern of a sharp 5S peak, peaks I and II, and a 4S peak. After the one-hour "chase", about 87% of the counts in these low molecular weight species are retained but have been markedly redistributed such that once more there is a large increase in 4S RNA with a concomitant decrease in species I and II.

There is also apparently a decrease in this amount of 5S RNA. This will be discussed later.

Correlation of data from "pulse-chase" experiments. Fig. 23 is an attempt

Figure 22.

In vivo 1 hour "chase" of infected cell cytoplasmic RNA

Two nearly-confluent burlers of C13 cells were infected with PRV. At 5 hours P.I. both burlers were labelled with 2 μ Ci/ml of ^3H -uridine (final concentration = 1 μM) for 45 min. One of the burlers was then harvested (a), while the other was "chased" for 1 hour before harvesting (b). "Chase" conditions were as described in Fig. 21.

Cytoplasmic RNA was prepared from both these samples and electrophoresed on 10% polyacrylamide gels as described in Fig. 17.

———, ^3H d.p.m. from ^3H -uridine
———, ^{14}C d.p.m. from ^{14}C -internal marker.

Figure 22

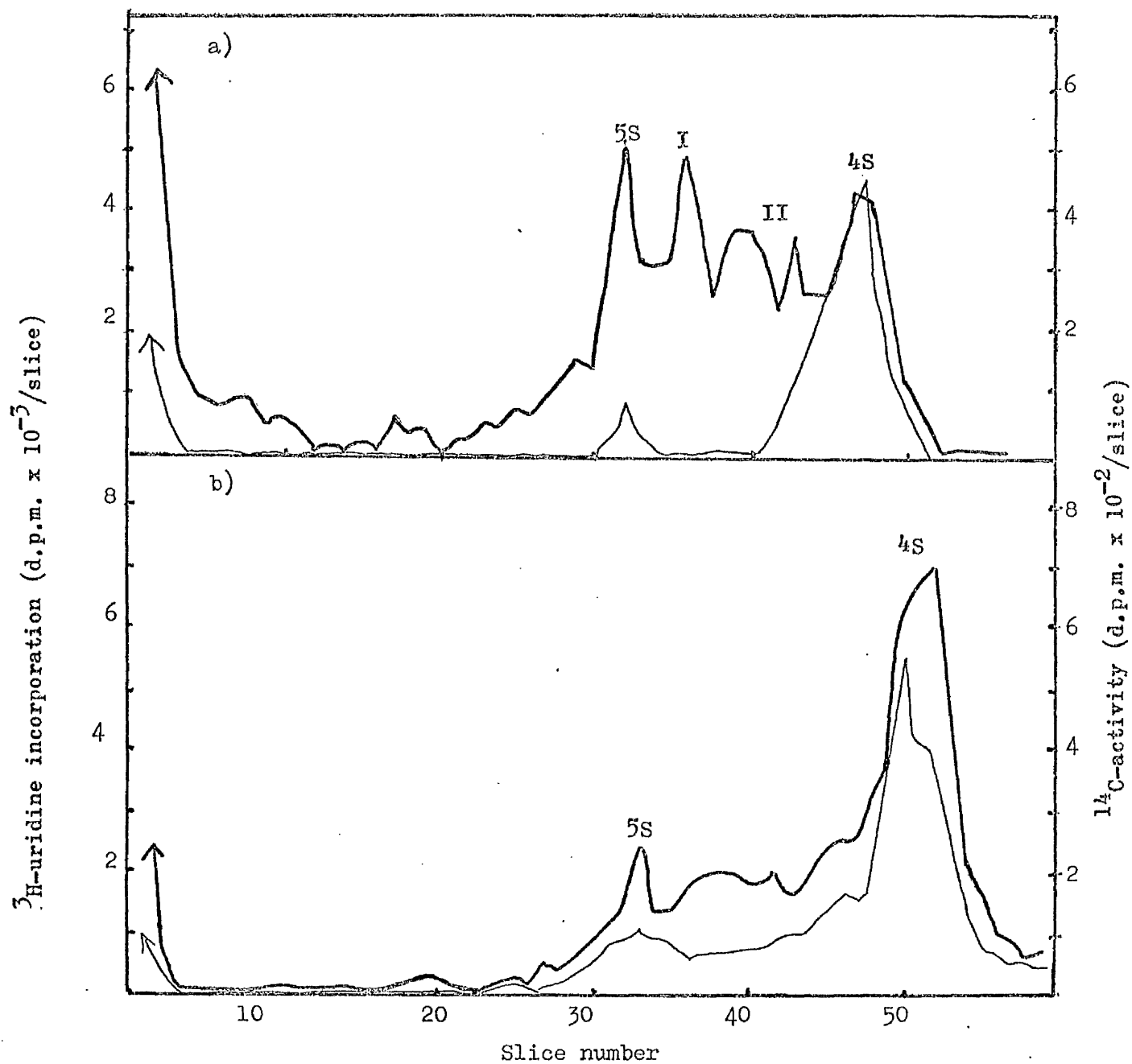


Table 6

Incorporation of ^3H -uridine into low molecular weight cytoplasmic RNA species in PRV-infected cells during a 45 min pulse-label and a subsequent 1 hour "chase"

Data taken from Fig. 22.

	5S	I + II	4S	I + II + 4S	Total d.p.m.
45 min pulse-label	14750	40200	20350	60550	75300
45 min pulse- label + 1 hour "chase"	6400	26100	33850	59990	66390

Figure 23

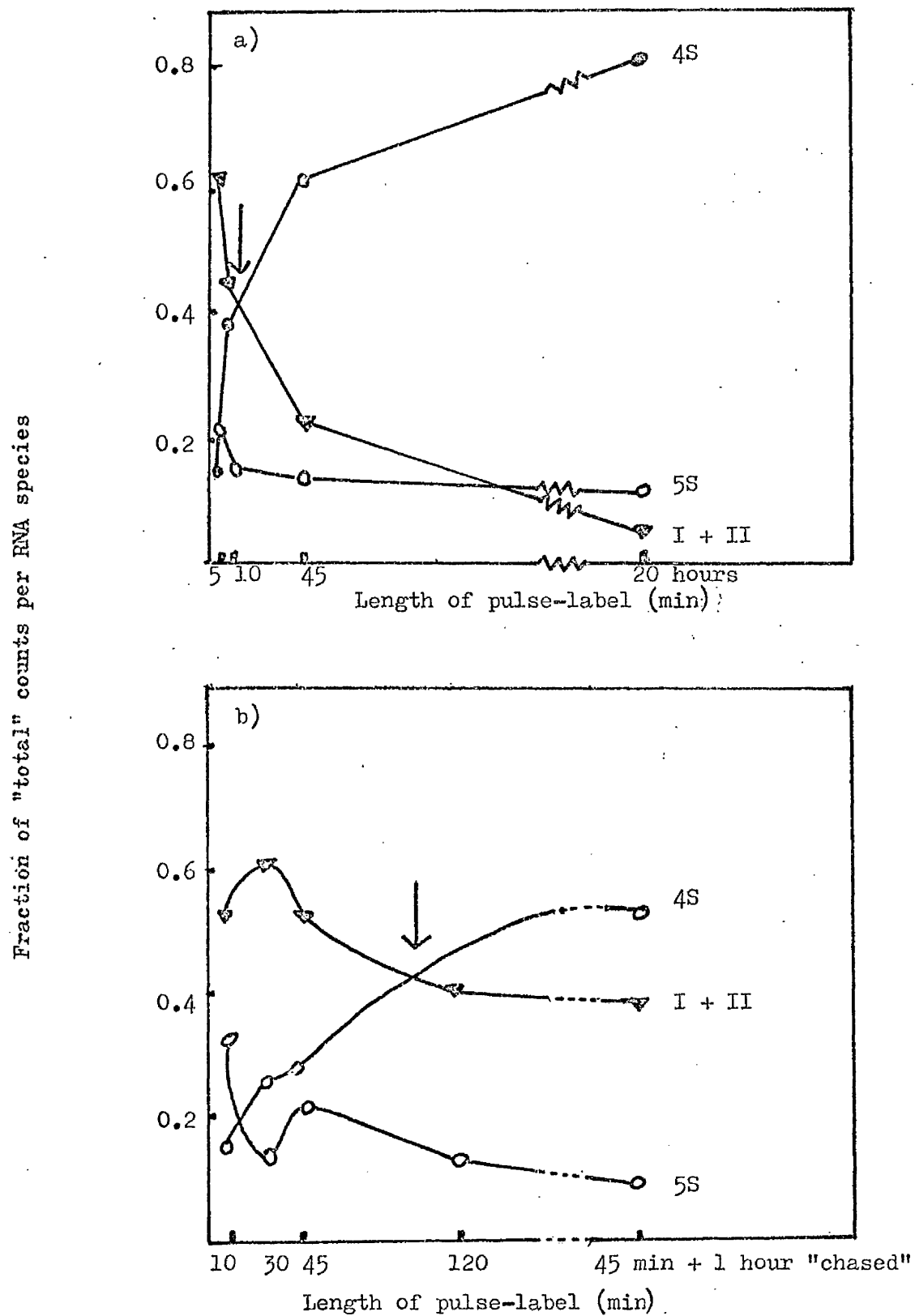


Figure 23. Composite figure of cytoplasmic RNA synthesis in the uninfected and PRV-infected cell

- a) Cytoplasmic RNA synthesis in the uninfected cell
- b) Cytoplasmic RNA synthesis in the PRV-infected cell.

Data taken from Figs. 15, 17, 18, 20 and 22.

to correlate some of the above data into a composite picture of the behaviour of cytoplasmic low molecular weight RNA before and after infection with PRV. The "total" number of counts is taken as the sum of 5S + I + II + 4S, and the fraction of counts in any one species is related to this "total", plotted against length of time of incubation of the cells with radioactive uridine. Peaks I and II are summated throughout for convenience, and to avoid the difficulty of separation of these closely electrophoresing species. The difficulties in calculating the counts per peak discussed in relation to Table 5 must also be borne in mind here.

Fig. 23a refers to uninfected cell RNA. The labelling times of 5 min, 10 min and 45 min are taken from Figs. 18a, 18b and 17a respectively. The last time point is 20 hours, taken from the ^{14}C -marker (Fig. 15) where the equilibrium state almost certainly has been reached. The fraction of counts in 4S increases with time and the fraction of counts in I + II decreases - as is expected in a precursor-product relationship. The level of counts in 5S is virtually constant except at 5 min when it is slightly elevated.

Fig. 23b is the composite graph of infected cell cytoplasmic RNA. In the infected cell it is practically impossible to label to equilibrium for two reasons: (a) PRV has a short lytic cycle, and (b) the amounts and types of the various RNA species in the infected cell are not constant but change throughout the infectious cycle. Hence, in Fig. 23b the final time point is not a long label but the 45 min label + 1 hour "chase", taken from Fig. 22b. The infected cell pulse-label times of 10, 30, 45 and 120 min are taken from Fig. 20. In the composite graph the amount of label in peaks I and II rises initially and then gradually falls, while 4S RNA climbs

gradually. 5S RNA has an initial fall and rise and then levels off. This suggests that the flow of material could be $5S \rightarrow (I + II) \rightarrow 4S$, raising the possibility that part at least of the infected cell 5S RNA is a tRNA precursor; another possibility is that peak I overlaps into the 5S RNA position. These possibilities, and further discussion of 5S RNA in the infected cell, will be more fully considered later.

Comparison of the two sets of graphs (Figs. 23a and b) shows that the relative amounts of the various RNA species is almost the same after about 15 min pulse-label in the uninfected cell as at about 100 min in the infected cell - see positions indicated by arrows. This lends weight to the suggestion that the production of mature 4S RNA from species I and II in the infected cell is slowed down, relative to the uninfected cell.

II.2. In vitro incubation studies.

As a result of the work discussed in the previous section (II.1. (iii)), it appears that one effect of PRV infection of Cl3 cells is to slow down the production of mature 4S RNA from precursor species. The extract of Cl3 cells which matures tRNA contains nuclease activity (Smillie and Burdon, 1970); an endonuclease performs this maturing function in bacterial cells (Altman and Smith, 1971). Retardation of maturation of the infected cell 4S RNA could occur in one or both of the following ways: PRV-infection could inhibit production of host cell "maturing" nuclease, or it could produce an inhibitor of the host cell "maturing" nuclease. In either case, it should be possible to show that an extract of uninfected cells can mature pre-tRNA in vitro whereas an extract of infected cells should be unable to do so. Thus it should be possible to show a difference in "maturing ability" of uninfected and infected cell extracts, by incubating ^3H -labelled precursor

RNA with cell extracts and electrophoresing the RNA after incubation.

The following in vitro incubation reaction mixture was used.

1 ml contained :-

- 5 μ moles 2'-mercaptoethanol
- 20 μ moles tris-HCl pH = 8.0
- 20 μ moles $MgCl_2$
- 10 μ g heparin
- 200 μ g 3H -RNA
- Crude cell extract containing 2.0 mg protein.

Mercaptoethanol was used to prevent oxidation of enzymic thiol groups; magnesium chloride was added to provide the correct ionic strength for tRNA secondary structure; heparin was added (hopefully) to prevent non-specific RNase degradation; the preparation of the cell extract was as described in "Methods", II.1.(iii).

In this first in vitro experiment 10 min-labelled uninfected cell cytoplasmic RNA was used since this is known to contain a large amount of pre-tRNA which matures to tRNA in conditions similar to those described above (Mowshowitz, 1970; Smillie and Burdon, 1970). The incubation was carried out for 1 hour with infected or uninfected cell extract, and the RNA was re-extracted and co-electrophoresed on 10% gels with ^{14}C -marker. The results are shown in Fig. 24. Figs. 24a and b show that material between 5S and 4S has moved almost exactly into the 4S position and that there is very little difference in the effect of the uninfected and infected cell extracts. This can be seen in Fig. 24d where the graphs have been superimposed by alignment of the 5S peaks of the ^{14}C -internal marker. Fig. 24c shows the non-incubated 10 min-labelled infected cell RNA. As

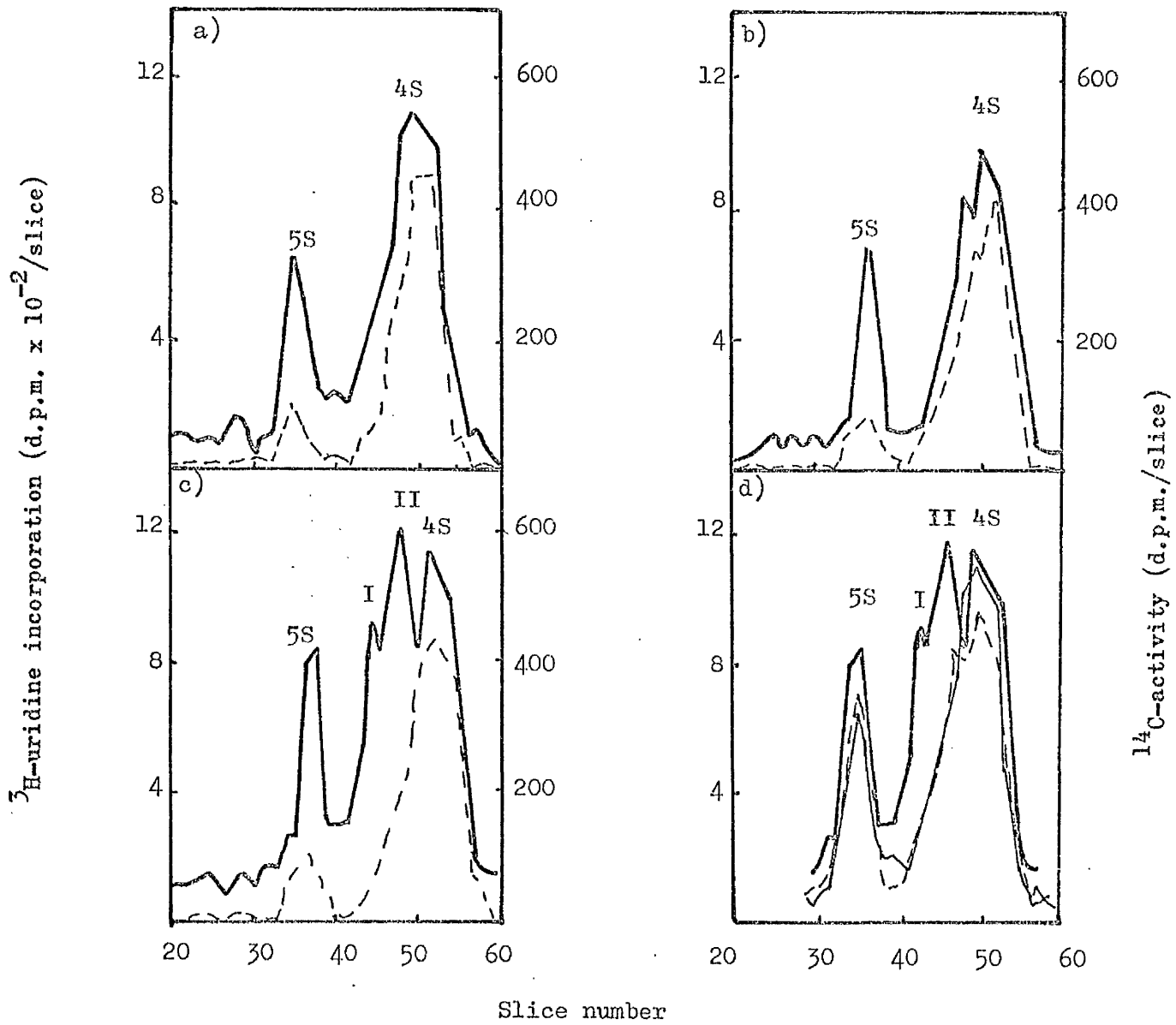
Figure 24.

Effect of *in vitro* incubation with PRV-infected or uninfected cell
extracts on cytoplasmic RNA from uninfected cells labelled for 10 min
with ^3H -uridine

Cytoplasmic RNA from uninfected Cl3 cells labelled for 10 min was prepared as described in Fig. 18b. 200 μg aliquots of the ^3H -RNA were then incubated for 1 hour with cell extracts obtained from uninfected or PRV-infected cells ("Methods", II.6.(i)), in the 1.0 ml reaction mixture described in the text; RNA was then extracted from this mixture ("Methods", II.6.(ii)). 10% polyacrylamide gels were run of (a) extracted RNA from incubation with infected cell extract, (b) extracted RNA from incubation with uninfected cell extract and (c) of 200 μg of the ^3H -RNA which had not been incubated. 20 μl of the ^{14}C -internal marker RNA was co-electrophoresed with each sample and the gels were run, stained with toluidine blue, sliced and counted by liquid scintillation spectrometry ("Methods", II.3.(ii)). (d) is a composite graph of (a), (b) and (c) drawn by alignment of the 5S peak of the ^{14}C -internal marker RNA.

—————, ^3H d.p.m. from ^3H -RNA
 —————, ^{14}C d.p.m. from ^{14}C -marker RNA.

Figure 24



sometimes occurs, peak I is barely present although there is a distinct shoulder on peak II. This could be because peak I is unstable or because the labelling period was longer than usual and most of peak I had become peak II or 4S RNA.

Nonetheless, it is quite clear that the material between 5S and 4S does move towards the 4S position producing a single peak with a discrete leading edge, i.e. there seems to be little evidence for non-specific breakdown or production of material smaller than 4S RNA.

Fig. 24 shows that maturation of the material between 5S and 4S in the uninfected cell (pre-tRNA) does indeed occur in vitro but shows no difference between the uninfected and infected cell extract. This may be because uninfected cell RNA was used; it is possible that the postulated inhibitor of maturation affects RNA made after infection only. Hence the above experiment was repeated using 45 min-labelled infected cell RNA. The same buffer conditions as described above were used since they have been shown to allow maturation of the 10 min labelled RNA from uninfected cells (Fig. 24). Thus the results shown in Fig. 25 were obtained.

These results show that after a 1 hour incubation of 45 min-labelled infected cell RNA with either cell extract, the bulk of the material between 5S and 4S now elutes as one diffuse peak much closer to 4S although not exactly in the 4S position. In this experiment there does seem to be a slight difference in the effect of uninfected and infected cell extracts. The new "4S" peak after incubation with infected cell extract has its peak four slices to the left of the 4S marker and a shoulder in the true 4S RNA position. The new "4S" peak after incubation with uninfected cell extract has its peak only two slices to the left of the 4S marker and the leading

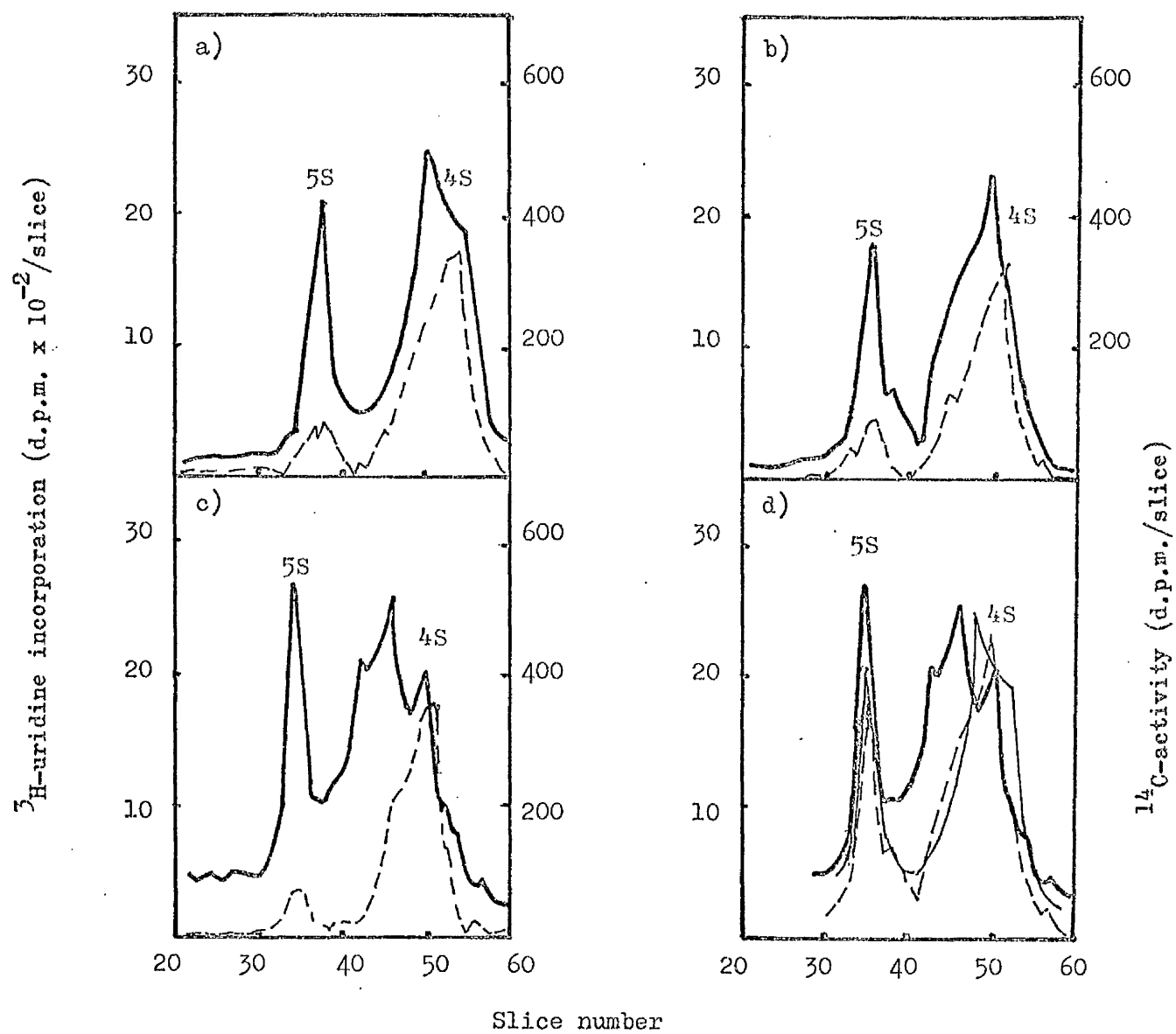
Figure 25.

Effect of *in vitro* incubation with PRV-infected or uninfected
cell extracts on cytoplasmic RNA from PRV-infected cells labelled
for 45 min with ^3H -uridine.

Cytoplasmic RNA from PRV-infected cells labelled for 45 min at 5 hours P.I. with ^3H -uridine was prepared as described in Fig. 17b. 200 μg aliquots of this ^3H -RNA were then incubated for 1 hour with cell extracts from uninfected and PRV-infected cells, and the RNA was extracted and electrophoresed on 10% polyacrylamide gels, as described in Fig. 24. Thus gels were obtained of (a) RNA incubated with infected cell extract, (b) RNA incubated with uninfected extract, (c) RNA which had not been incubated. (d) is a composite graph of (a), (b) and (c) drawn by alignment of the 5S peak of the ^{14}C -internal marker RNA.

—————, ^3H d.p.m. from ^3H -RNA
 —————, ^{14}C d.p.m. from ^{14}C -marker RNA.

Figure 25



edge is almost exactly coincident with the 4S marker.

The results shown in Fig. 25 indicate that the uninfected cell extract has moved the material between 5S and 4S slightly further towards 4S than the infected cell extract. This could be explained if the infected cell extract had incomplete inhibition of maturing ability which would be largely overcome by the long (1 hour) incubation; this hypothesis would seem reasonable since production of 4S RNA from precursor species is not completely inhibited in vivo but simply retarded.

Time-course of "chase" in vitro. Thus, for the above reasons, ^3H -RNA was incubated for short periods of time with infected or uninfected cell extract, and a time-course of "chase" in vitro was performed. In this series of experiments the incubation conditions were slightly altered. 1 ml of reaction mixture contained :-

5 μ moles 2'-mercaptoethanol
 20 μ moles tris-HCl pH = 8.0
 20 μ moles MgCl_2
 3 μ moles ATP
 20 μ moles methionine
 200 μg ^3H -RNA
 Crude cell extract containing 2.0 mg protein.

The heparin was omitted because of its unknown effect on enzyme activity and on the postulated inhibitor. ATP and methionine were added because methylation may be involved in maturation - even although maturation is not dependent on methylation (Bernhardt and Darnell, 1969); also the postulated inhibitor may need ATP as a cofactor.

The results of incubating the 45 min-infected cell RNA for

0, 10 min and 20 min and re-extracting the RNA and electrophoresing it with ^{14}C -internal marker are shown in Fig. 26. For clarity the graphs have been superimposed by alignment of the 5S RNA peak of the ^{14}C -internal marker.

The results of incubation show a gradual progression of material from between 5S and 4S RNA towards the 4S position after incubation with either infected cell extract (Fig. 26a) or uninfected cell extract (Fig. 26b). Once more there is no significant difference between the ^3H -RNA incubated with infected and uninfected cell extracts; even after only 10 min incubation both samples have moved towards 4S at an identical rate (Fig. 26c).

However, this in vitro time-course does show a gradual progression of the material between 5S and 4S towards the 4S, and there is negligible degradation beyond 4S. This tends to confirm that the material in peaks I and II in the infected cell is indeed precursor to 4S RNA, and supports the conclusions reached from in vivo "chase" experiments.

In vitro incubation of column-purified pre-tRNA. A further attempt to show a difference in maturing ability between infected and uninfected cell extracts was made by using as substrate the Sephadex G100 column-purified pre-tRNA from uninfected cells. This is the material described in Fig. 19; we felt that the effect of the extracts on material known to be partially purified pre-tRNA should give unequivocal results. This material was incubated with infected and uninfected cell extracts for 1 hour. 1 ml of reaction mixture contained :

20 μ moles tris-HCl pH = 7.5

10 μ moles MgCl_2

Crude cell extract containing 2.0 mg protein

^3H -pre-tRNA.

Figure 26.

Time-course of chase in vitro of infected cell cytoplasmic RNA

labelled for 45 min with ^3H -uridine.

Cytoplasmic RNA from PRV-infected cells labelled for 45 min at 5 hours P.I. with ^3H -uridine was prepared as described in Fig. 17b. 200 μg aliquots of this ^3H -RNA was incubated with uninfected and infected cell extracts as described in Fig. 24 except that the incubation times were 0, 10 and 20 mins. RNA was extracted from the reaction mixtures and was electrophoresed on 10% polyacrylamide gels as described in Fig. 24.

Composite graphs were drawn by alignment of the 5S peak of the ^{14}C -marker RNA, the position of which has been indicated on each graph

(a) Composite graph of RNA incubated for 0, 10 and 20 min with infected cell extract.

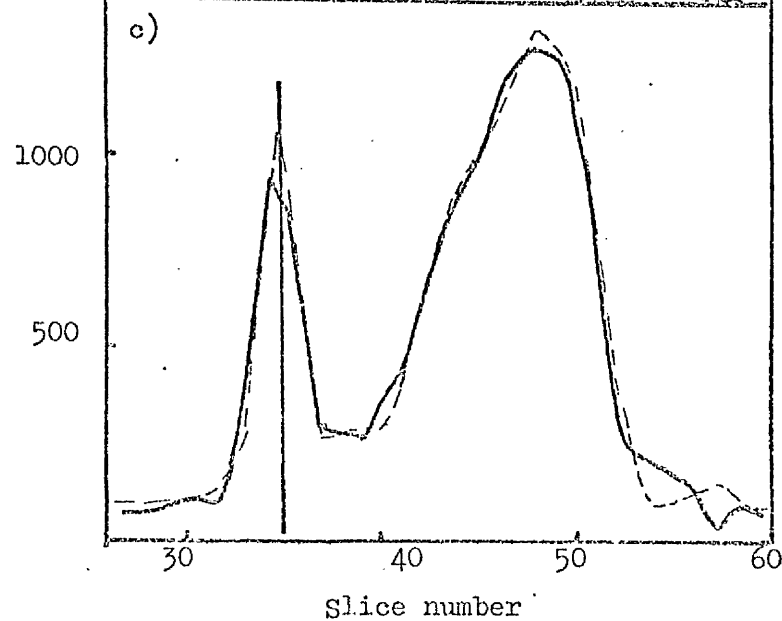
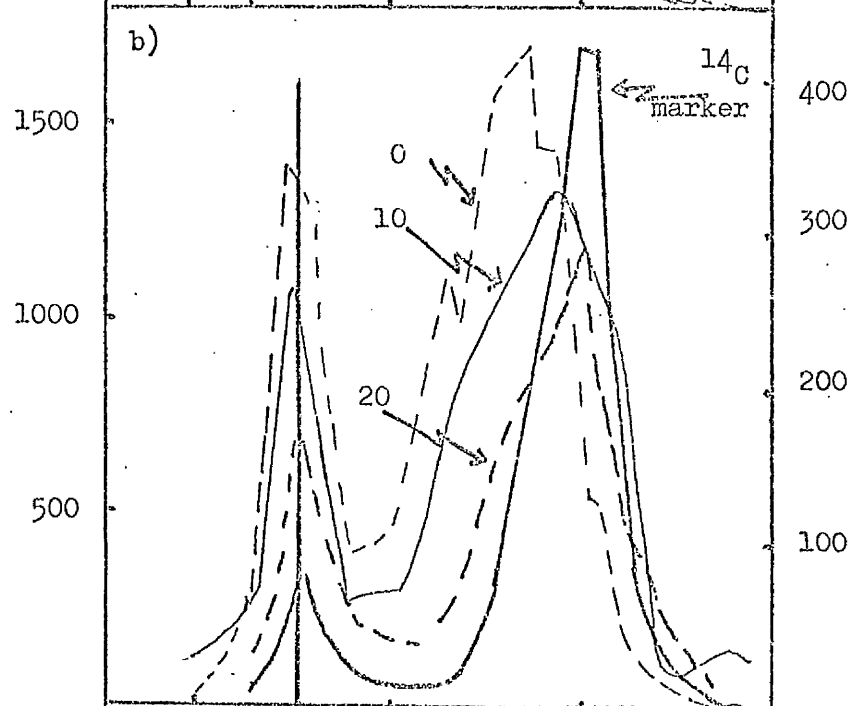
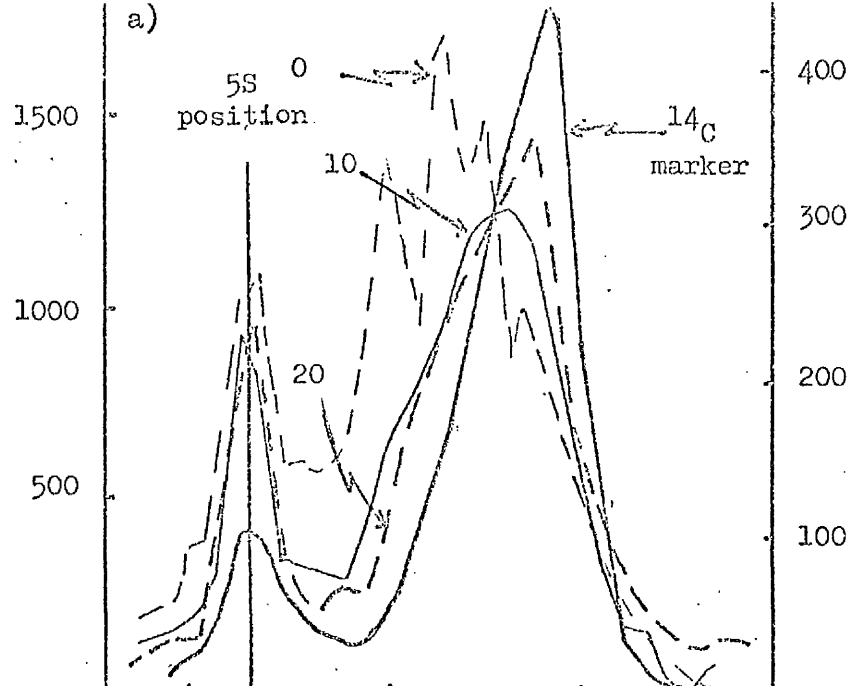
(b) Composite graph of RNA incubated for 0, 10 and 20 min with uninfected cell extract.

----- , ^3H d.p.m., 0 min incubation
————— , ^3H d.p.m., 10 min incubation
----- , ^3H d.p.m., 20 min incubation
————— , ^{14}C d.p.m. from ^{14}C -marker RNA

(c) Composite graph of RNA incubated for 10 min with infected and uninfected cell extracts.

----- , ^3H d.p.m. after incubation with infected
cell extract
————— , ^3H d.p.m. after incubation with uninfected
cell extract.

^3H -uridine incorporation (d.p.m./slice)



^{14}C -activity (d.p.m./slice)

These are similar to the conditions used by Smillie and Burdon, 1970 for maturation of pre-tRNA. The results are shown in Fig. 27. On incubation the two peaks between 5S and 4S disappear, to be replaced by a single peak which almost, but not quite, co-electrophoreses with the ^{14}C -internal marker; comparison of the results of uninfected and infected cell incubations (Fig. 27d) shows no significant difference in the effect of the two extracts. This experiment does demonstrate clearly that here it is indeed the material between 5S and 4S RNA that forms 4S RNA on incubation. This is clear both because there is no RNA in this experiment other than 4-5S RNA and also because the recovery of this material after incubation was greater than 90%. It seems that for some reason it is not possible to reproduce in vitro the effect of inhibition of maturation of the material between 5S and 4S RNA in the PRV-infected cell; possibly this is due to the disruption of the intracellular organization in the preparation of the extract. Another explanation is the possibility that, in the in vitro conditions used, too much precursor RNA is being supplied for the postulated inhibitor to prevent its maturation. It was thus reasoned that decreasing the amount of added ^3H -RNA may give the inhibitor the opportunity to act. For this reason an in vitro incubation was performed at the normal ^3H -RNA:protein concentration and at a lower ^3H -RNA:protein concentration.

Further studies on the in vitro maturation process. The specific effect of the extract was studied by incubating ^3H -RNA under the following conditions:- a) with buffer only for 40 min; b) with extract for zero minutes; c) with a high ^3H -RNA:protein ratio for 40 min; d) with a low ^3H -RNA:protein ratio for 40 min.

The in vitro conditions described in the "chase" in vitro were

Figure 27.

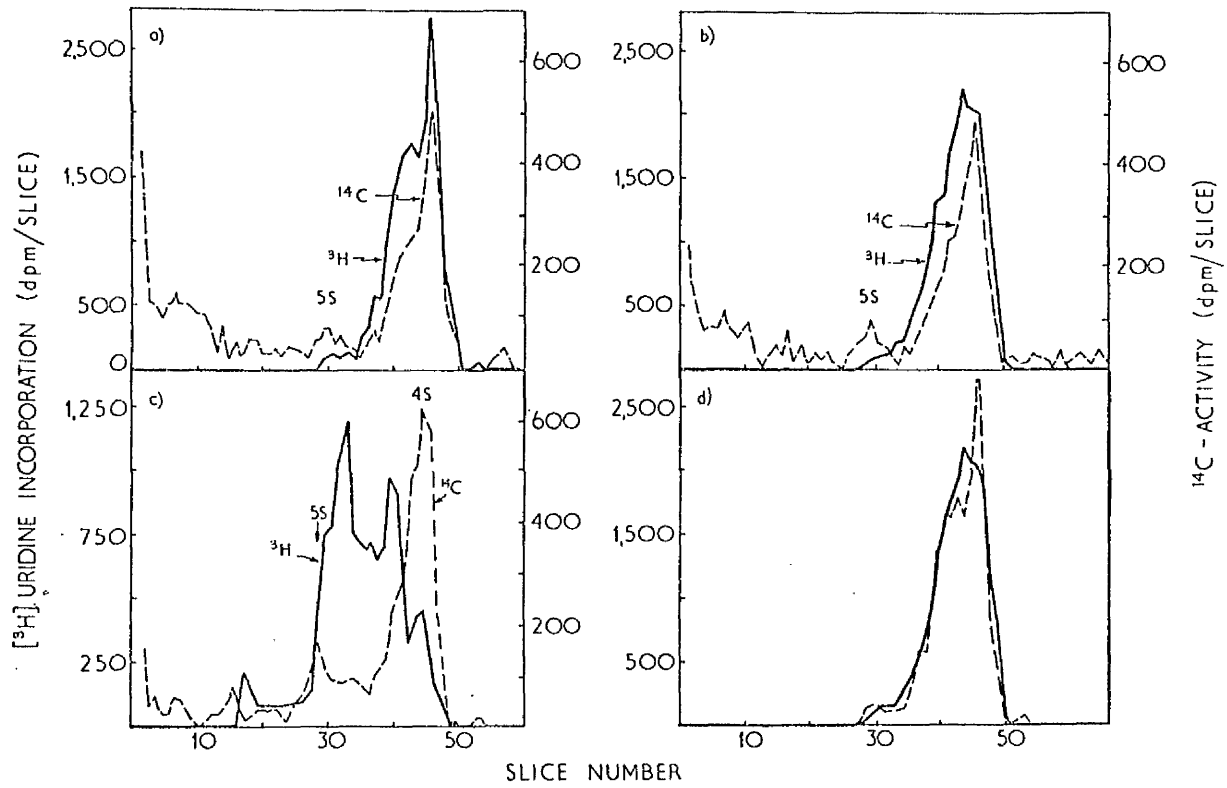
In vitro incubation of partially-purified pre-tRNA with uninfected
and PRV-infected cell extracts

^3H -labelled pre-tRNA from uninfected C13 cells was partially purified as described in Fig. 19. Cell cytoplasmic extracts from uninfected and PRV-infected cells were prepared and incubated for 1 hour at 37° with this partially-purified pre-tRNA; the RNA was then extracted and electrophoresed on 10% polyacrylamide gels, as described in Fig. 24.

Thus gels were obtained of (a) pre-tRNA incubated with uninfected cell extract, (b) pre-tRNA incubated with PRV-infected cell extract, and (c) pre-tRNA which had not been incubated. (d) is a composite graph of (a) and (b), drawn by alignment of the 5S peak of the ^{14}C -marker RNA.

—————, ^3H d.p.m. from ^3H -RNA
- - - - -, ^{14}C d.p.m. from ^{14}C -marker RNA.

Figure 27



used (P. 105) with the following amounts of RNA.

- a) buffer only - 40 μg ^3H -RNA
- b) zero incubation - 40 μg ^3H -RNA
- c) high ^3H -RNA:protein - 200 μg ^3H -RNA
- d) low ^3H -RNA: protein - 40 μg ^3H -RNA.

In this case the ^3H -RNA used as substrate was infected cell cytoplasmic RNA labelled for 30 min from 5 - 5.5 hours P.I. The results are shown in Fig. 28. The results were so similar for the uninfected and infected cell extracts that only the results using infected cell extract are shown. The results obtained after incubation of ^3H -RNA with buffer for 40 min or zero min incubation with extract were also virtually identical, and thus only the 40 min buffer-incubated sample is shown.

The results are corrected for the different amounts of d.p.m. used in varying the amount of ^3H -RNA and hence the different graphs can be directly compared. In this experiment the d.p.m. were rather low and this accounts for the uneven radioactive profile.

This experiment shows that incubation of 30 min labelled infected cytoplasmic RNA for 40 min with buffer did not produce any ^3H -RNA in the 4S position. Incubation with cell extract produced movement of this material towards the 4S RNA position. Incubation with a low ^3H -RNA:protein ratio caused more movement than a higher ^3H -RNA:protein ratio. This result would be compatible with a situation in which a specific, probably enzymic, process causes maturation of a precursor to 4S RNA, and this result also demonstrates that it is a specific effect of the cell extract which is causing maturation. Once more, however, there is no evidence for a difference in maturing ability between the infected and uninfected cell extracts.

Figure 28.

Effect of varying the ratio of ^3H -RNA to protein in the in vitro
incubation studies

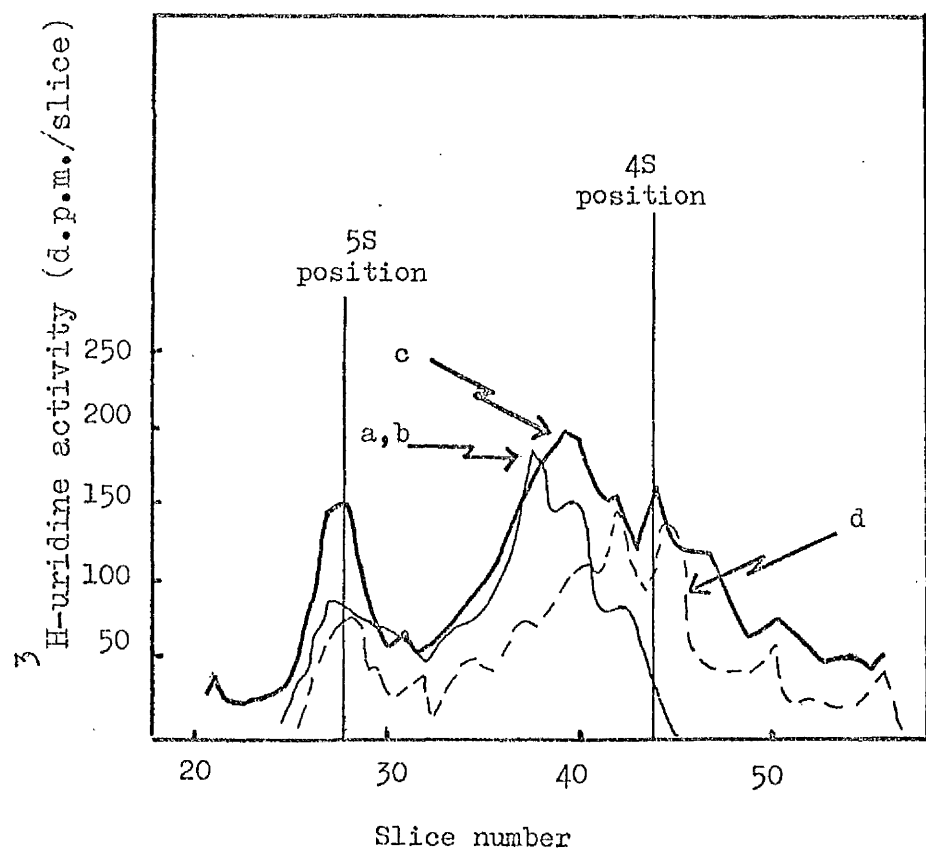
Cytoplasmic RNA from PRV-infected cells labelled for 30 min at 5 hours P.I. with ^3H -uridine was prepared as described in Fig. 17b. Aliquots of this ^3H -RNA were then incubated with cell extracts from uninfected and PRV-infected cells, and then the RNA was extracted and run on 10% polyacrylamide gels as described in Fig. 24.

The amounts of ^3H -RNA incubated and conditions of incubation were as follows.

a	—————	40 μg ^3H -RNA	:	40 min incubation with buffer only
b	—————	40 μg ^3H -RNA	:	0 min incubation with extract
c	—————	200 μg ^3H -RNA	:	40 min incubation with extract
d	-----	40 μg ^3H -RNA	:	40 min incubation with extract.

A composite graph was drawn by alignment of the 5S peak of the ^{14}C -internal marker RNA, the position of which has been indicated. Since the result of incubating the ^3H -RNA with uninfected and PRV-infected cell extracts was so similar, only the results from incubation with infected cell extract have been shown.

Figure 28



Thus it has not been possible to show an in vitro difference in maturing ability between infected and uninfected cell extracts, in spite of the striking in vivo effect. However, this series of experiments has shown that the RNA between 5S and 4S in the infected cell (peaks I and II) behaves in vitro as true pre-tRNA does, producing 4S RNA with negligible non-specific degradation. This specificity strongly suggests that peaks I and II in the infected cell are pre-tRNA species.

III. Cell sap experiments using exponentially growing cells.

III.1. In vivo labelling studies to determine the location of low molecular weight RNA species in the PRV-infected cell.

It is clear that after PRV-infection there is a distinct 5S RNA peak (e.g. Fig. 20); at least some of this material is unstable (Fig. 22). There are various possibilities for the appearance of 5S RNA after infection. The 5S RNA peak might be heavily contaminated with peak I RNA, or there could be an even larger precursor to 4S RNA electrophoresing at 5S; other possibilities are that a new virus-induced RNA species co-electrophoreses with 5S RNA, or that the cell continues to produce its own ribosomal 5S RNA after infection.

In an attempt to clarify the situation, cell sap RNA was examined; by "cell sap" is meant the post-ribosomal supernatant. The cell sap should contain few ribosomes and hence ribosome-associated 5S RNA should not be present. It was of course inevitable that on investigating the appearance and location of 5S RNA, data should be gathered on the appearance of the other low molecular weight RNA species (species I, II and 4S RNA), which has thrown some light on the nature of these species (especially species I and II).

Figure 29

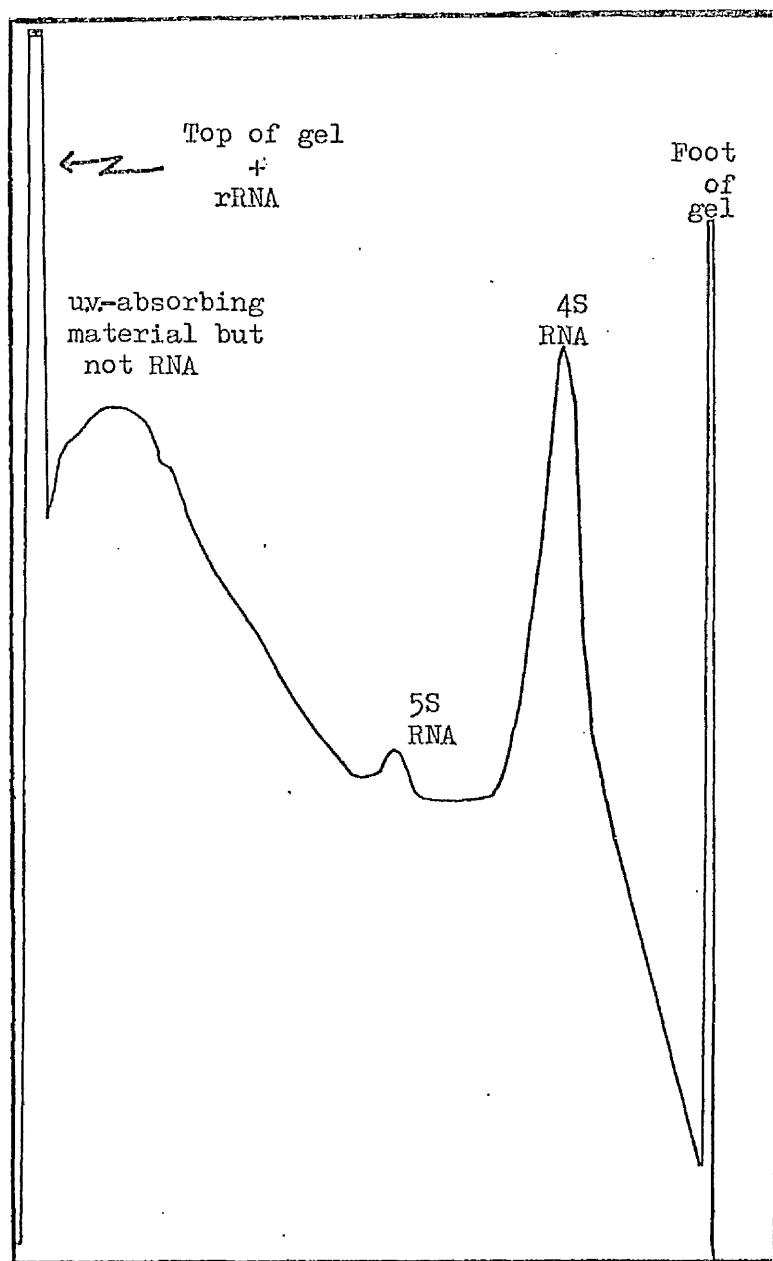


Figure 29. Trace of absorbancy at 260 nm of a 10% polyacrylamide gel separation of cell sap RNA.

The gels described in Fig. 30 were scanned at 260 nm in a Gilford model 240 spectrophotometer with a linear transport attachment (model 2410).

A time-course of the appearance of cell sap RNA was performed using both PRV-infected and uninfected cells, i.e. the cells were labelled with ^3H -uridine for the following periods of time at 5 hours P.I. or after mock-infection: 10 min, 30 min, 1 hour and 3 hours. In addition, the infected cells were pulse-labelled for 1 hour at 7 hours P.I. to check that during the 3 hour label (5 - 8 hours P.I.) the pattern of RNA synthesis was not changing markedly.

The cells were harvested and in this experiment were ruptured by the use of 0.5% Nonidet P40 instead of by mechanical homogenisation ("Methods", II.1.(i)b); this allowed for even greater sterility in the preparation of the RNA samples. The nuclei were precipitated and then the mitochondria and ribosomes were removed by a 105,000 g centrifugation for 90 min. The RNA from the resulting cell sap was extracted and co-electrophoresed on a 10% gel with the ^{14}C -internal marker RNA. A trace of the absorbancy at 260 nm of each gel was obtained (Fig. 29). This showed some ribosomal RNA still on top of the gels and an amount of 5S RNA consistent with its having come from the fraction of unprecipitated ribosomes. A longer centrifugation than 90 min may have obviated this problem but it was decided that any longer could allow nuclease to act and might even allow some maturation to take place.

The results obtained from uninfected cells are shown in Fig. 30; those from PRV-infected cells are shown in Fig. 31. In Fig. 30 there is clearly a substantial amount of "pre-tRNA", labelled as I and II, after a 10 min label, but the amount of this material decreases on longer labels so that after a 1 hour label the greatest amount of radioactive material is in 4S RNA. There is some 5S RNA and this can be accounted for by the presence

Figures 30 and 31

Time-course of mock-infected and PRV-infected cell sap RNA

Newly confluent burlers of Cl3 cells were mock-infected (Fig. 30) or infected with PRV (Fig. 31), and at 5 hours P.I. were labelled for (a) 10 min, (b) 30 min, (c) 1 hour and (d) 3 hours; PRV-infected cells were also labelled at 7 hours P.I. for 1 hour (Fig. 31e).

The cytoplasmic fraction was prepared by the Nonidet P40 method ("Methods", II.1.(i)b) and then the cell sap was prepared by pelleting the mitochondria and ribosomes ("Methods", II.1.(ii)); the final centrifugation to pellet the ribosomes was at 105,000g for 90 min. RNA was phenol-extracted from the cell sap ("Methods", II.2.(ii)) and electrophoresed in the usual way on 10% polyacrylamide gels, as described in Fig. 17.

—————, ^3H d.p.m. from ^3H -uridine
-----, ^{14}C d.p.m. from ^{14}C internal marker.

The absorbancy trace obtained from these gels is shown in Fig. 29.

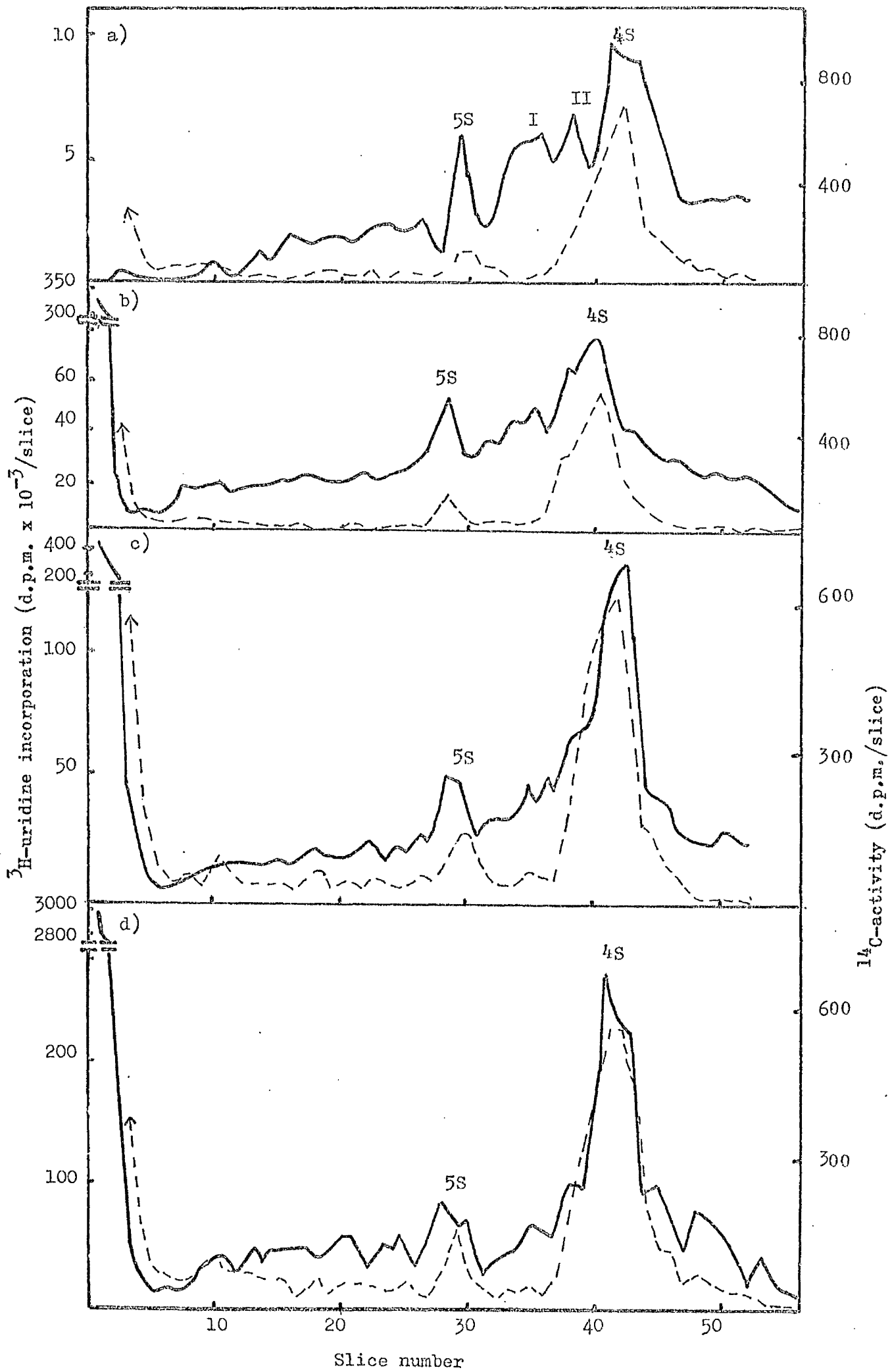


Figure 31

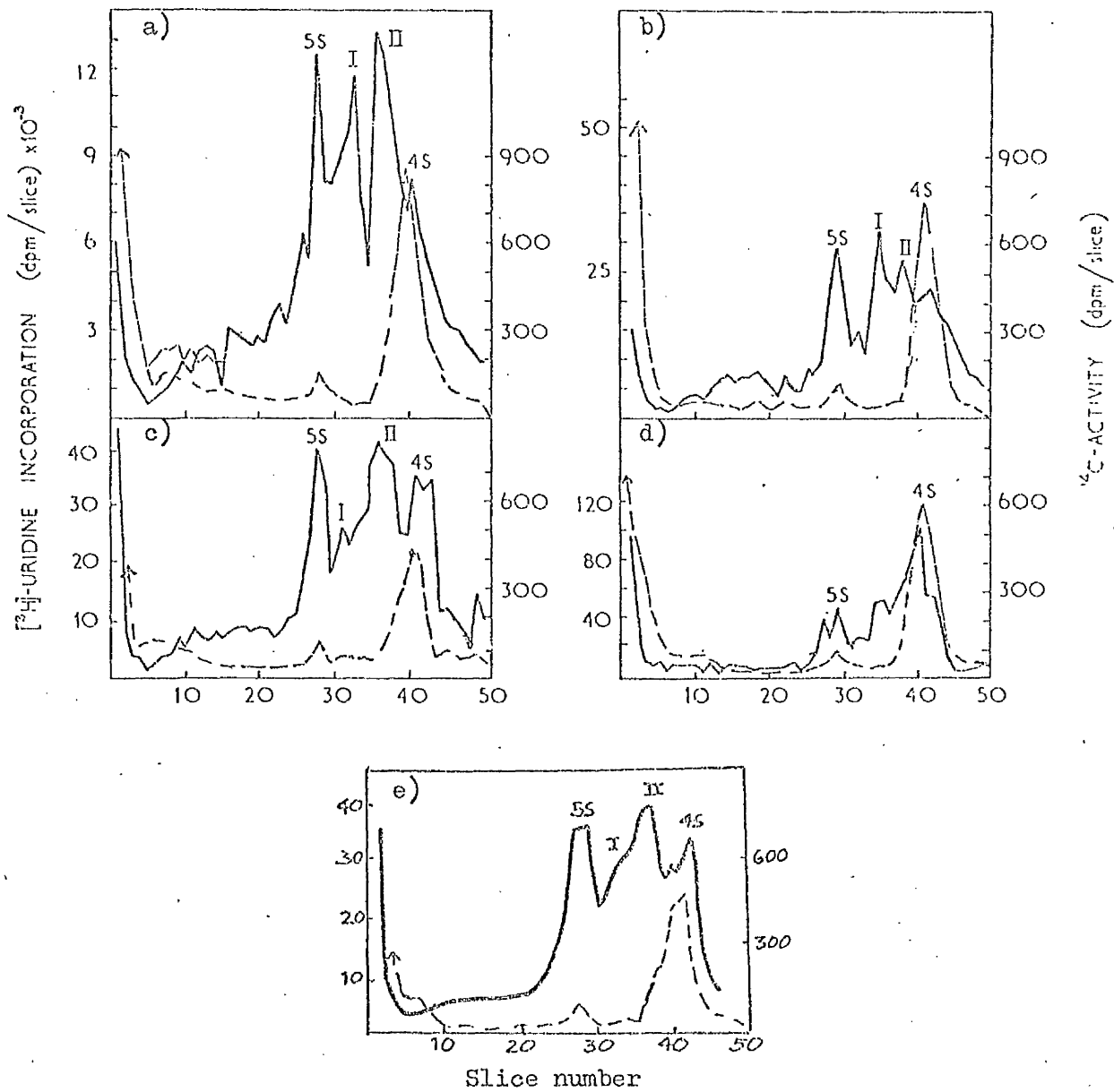


Fig. 31. Legend to this figure is opposite Fig. 30

of some ribosomes in the preparation; however there is a slight drop in the relative amount of 5S RNA in these cell sap preparations compared to the cytoplasmic preparations, e.g. compare the composite figure of uninfected cell sap RNA (Fig. 32a) to that already shown for uninfected cell cytoplasmic RNA (Fig. 23a).

In Fig. 31 the time-course of infected cell sap RNA is shown. The overall pattern is very similar to the uninfected cell in that there is a gradual movement of material from peaks I and II to the 4S position. However, as we have consistently observed, this movement is slower in the case of the infected cell, e.g. at 1 hour there is still more material in peak II than in the 4S RNA peak, and even after a 3 hour pulse-label there is still a clear peak II. This series of results suggests that the flow of material may be from peak I \longrightarrow peak II \longrightarrow 4S RNA and not peak I \longrightarrow 4S RNA \longleftarrow peak II, since the relative amount of peak II gradually increases as the amount of peak I decreases. Note also that the amount of 5S RNA in relation to 4S RNA decreases as length of labelling increases; this is compatible with the suggestion mentioned earlier that some "5S RNA" may be a large precursor to tRNA.

It is of interest that the 1 hour pulse-label at 7 hours P.I. produced a gel electrophoresis profile similar to that obtained on pulse-labelling from 5 - 6 hours P.I.

These results have been compiled in two sets of composite figures in Fig. 32, on the same principles as Fig. 23; note that the same reservations about calculation of the d.p.m. per RNA species that apply to Fig. 23 also apply to this figure.

Figure 32

Fraction of "total" counts per RNA species

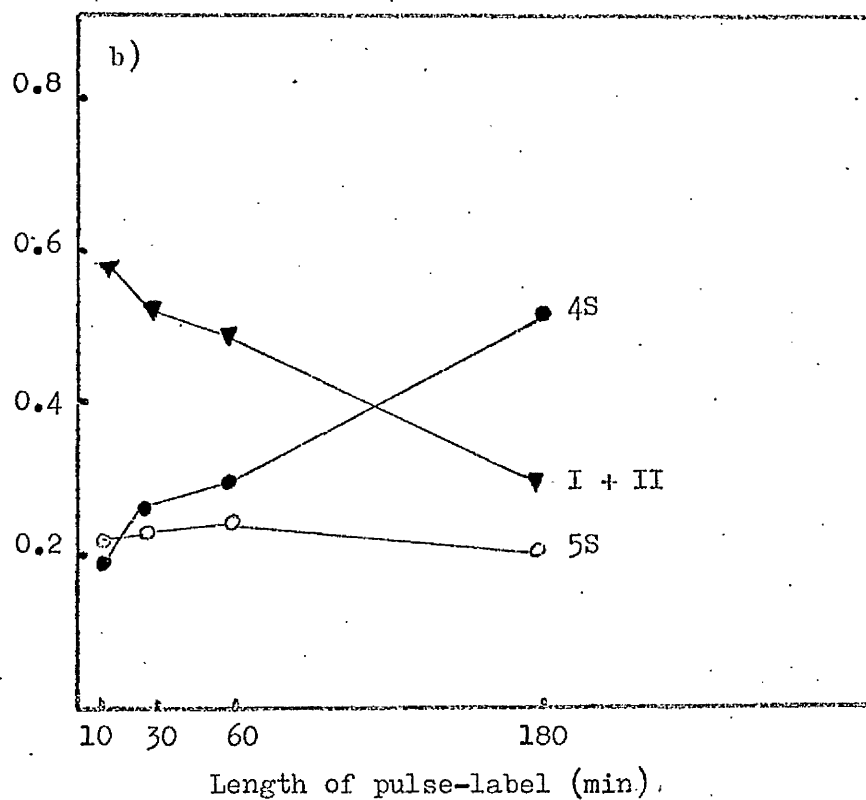
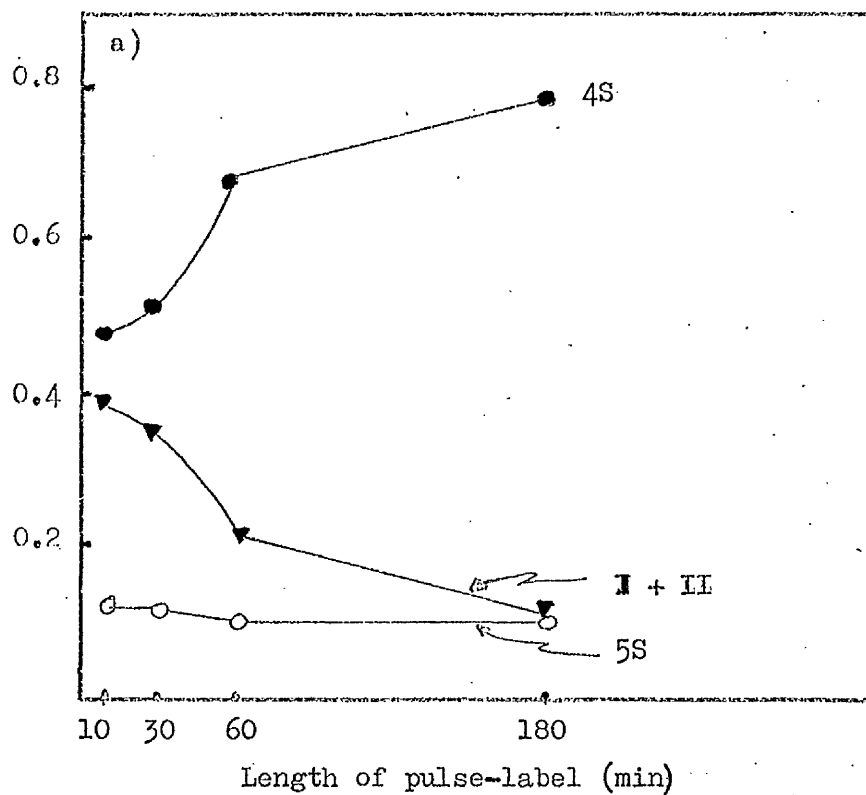


Figure 32. Composite figure of cell sap RNA synthesis in the uninfected and PRV-infected cell

- a) Cell sap RNA synthesis in the uninfected cell
- b) Cell sap RNA synthesis in the PRV-infected cell.

Data taken from Figs. 30 and 31.

Fig. 32a - uninfected cell sap RNA - shows that the fraction of d.p.m. in 5S RNA stays constant while the fraction of counts in peaks I + II decreases. This graph is very similar to the composite graph of cytoplasmic RNA (Fig. 23a) in the relative amounts of the species after various lengths of pulse-label, with slightly less 5S RNA.

Fig. 32b - PRV-infected cell sap RNA - once more shows that the fraction of counts in 4S RNA rises much more slowly (indicating slower maturation kinetics) than in the uninfected cell; concomitant with the rise in 4S RNA there is a decrease in the fraction of counts in species I and II. This time, as opposed to Fig. 23b, the initial rise in amount of species I + II is not observed. However, the striking difference between this figure and Fig. 23 is the increased level of 5S RNA observed in the present experiment. This increase in the fraction of 5S RNA is entirely at the expense of a decrease in the fraction of 4S RNA. This can be explained by the fact that there is some 4S RNA associated with the ribosomes, but that precursor tRNA is believed to be in the cell sap only; hence sedimentation of ribosomes and therefore of some 4S RNA increases the relative amount of other low molecular weight soluble RNA species. Thus it does seem that a considerable proportion of 5S RNA in the infected cell must not be ribosome-associated, as it is in the uninfected cell.

III.2. In vitro incubation studies to investigate the stability of isolated low molecular weight RNA species.

Whether or not 5S RNA is a stable species after infection is obviously an important issue; another important point also unresolved is whether peak I passes through a stage as peak II en route to 4S RNA, as the previous experiment might suggest. To clarify these questions, the four

different low molecular weight RNA species (5S, I, II, 4S) were each eluted separately from gels and the effect of incubation with cell extract (which we have shown to effect maturation of pre-tRNA in vitro) on each species was compared.

The RNA from the previous experiment (Figs. 30 and 31) was used. It was decided to use the samples pulse-labelled for 1 hour since in the infected cell there was an adequate amount of all four RNA species (5S, I, II, 4S) while in the uninfected cell the equilibrium position had almost been reached, which makes a good comparison.

Thus 10% polyacrylamide gels were run of infected cell sap RNA pulse-labelled for 5 - 6 hours P.I. and uninfected cell sap RNA labelled for 1 hour at 5 hours after mock-infection. A sufficient quantity of ^{14}C -internal marker RNA was co-electrophoresed so that the ^{14}C in the slices could be counted in the gas-flow counter ("Methods", II.4) and thus the position of 5S and 4S RNA could be obtained accurately without solubilisation of the gel slices. The ^{14}C counts obtained by this method are shown in Fig. 33a and b. A small fraction of the ^3H -RNA sample and ^{14}C -internal marker was co-electrophoresed at the same time as these gels, and these gel slices were solubilised and counted in the usual way in a liquid scintillation counter ("Methods", II.3.(ii)). Using these results (Fig. 33c and d) - which are essentially identical to the original results of Figs. 30c and 31c - the slices which must contain peaks I and II were pinpointed. The slices from each peak were combined as indicated on Fig. 33. Three slices were used for 5S from both gels, 5 - 6 slices for peak I, 3 - 4 slices for peak II and 4 slices for 4S RNA. The RNA was eluted from the gels as described in "Methods", II.7, and the resulting material was divided into three portions -

Figure 33.

Production of partially-purified 5S RNA, 4S RNA, and RNA species

I and II from uninfected and PRV-infected

cells

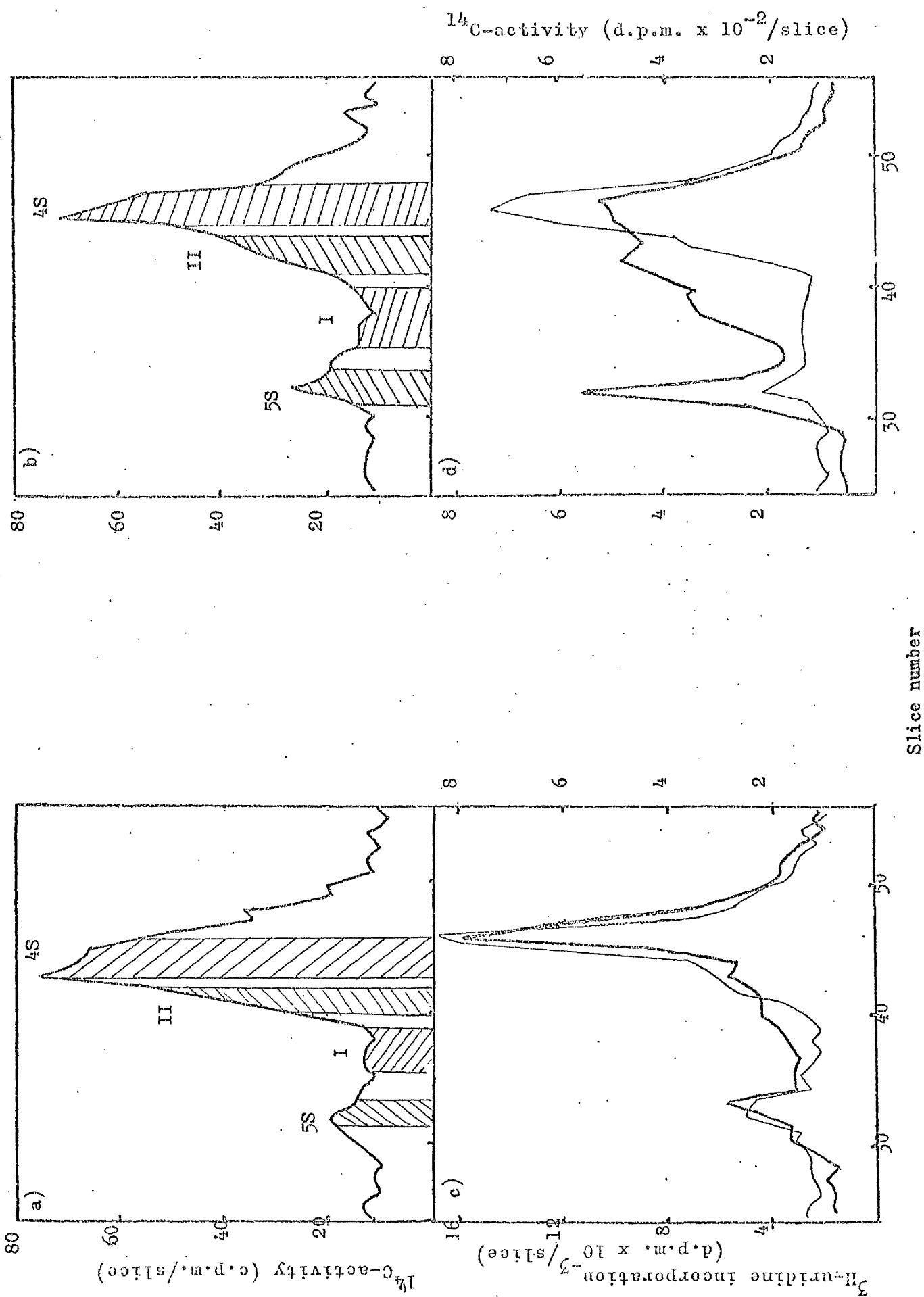
Uninfected and PRV-infected C13 cells were labelled with ^3H -uridine for 1 hour at 5 hours P.I. and the cell sap RNA was prepared as described in Figs. 30 and 31(c). The ^3H -RNA samples were co-electrophoresed on 10% polyacrylamide gels with 50 μl ^{14}C -marker RNA; the gels were sliced and the dried slices were counted in a gas-glow counter which measured the ^{14}C -emissions only. (a = uninfected cell RNA, b = infected cell RNA).

Smaller aliquots of the ^3H -RNA were also electrophoresed on 10% polyacrylamide gels with 20 μl ^{14}C -marker RNA which were sliced and counted by liquid scintillation spectrometry (c = uninfected cell RNA, d = infected cell RNA). Using these results, the positions of 5S RNA, 4S RNA and RNA species I and II in gels (a) and (b) were pinpointed, the slices indicated by hatching were combined, and the RNA was eluted from them as described in "Methods", II.7.

—————, ^3H d.p.m. from ^3H -uridine
—————, ^{14}C d.p.m. from ^{14}C -marker RNA.

(Note: A background level of 70 d.p.m. for ^{14}C across all gels counted by liquid scintillation spectrometry in Figs. 33-39 has not been subtracted).

Figure 33



part was immediately electrophoresed to check the effects of elution, part was incubated with buffer only and part was incubated with cell extract; in all cases the ^{14}C -internal marker was co-electrophoresed with the ^3H -RNA sample.

The effect of elution on the uninfected cell RNA species is shown in Fig. 34. It must be remembered that in the original gel from which this ^3H -RNA was eluted, the predominant material was 4S RNA, with very little material in the pre-tRNA position. What was eluted as "I" or "II" or even as "5S RNA" probably contained a large amount of 4S RNA. Fig. 34d shows that the eluted 4S RNA co-electrophoreses exactly with the 4S RNA of the marker. Eluted species II (Fig. 34c) electrophoresed almost exactly with the 4S marker, but with its peak 3 slices ahead of 4S RNA; this is because it is largely 4S RNA. The eluted species I runs as two peaks, which probably represent peak I and peak II + some 4S RNA; again this is to be expected since the amount of true peak I in the infected cell after a 1 hour label is very small indeed. Eluted 5S RNA electrophoreses in an unusual fashion; most of the eluted d.p.m. do coincide with the ^{14}C -marker 5S RNA, but a substantial portion co-electrophoreses with 4S RNA.

In Fig. 35, the eluted species of ^3H -RNA from the infected cell are co-electrophoresed with the ^{14}C -marker. Once again the eluted 4S RNA co-electrophoreses with the 4S of the ^{14}C -internal marker. In this figure (b and c) it is seen that both species I and II electrophorese in the position from which they came. This shows that these species are not just "cuts" from 4S RNA but distinct species of RNA. Eluted 5S RNA electrophoreses mainly in the 5S position but there is some trailing of material, suggesting that, perhaps, part of the eluted 5S is unstable. However, not nearly so

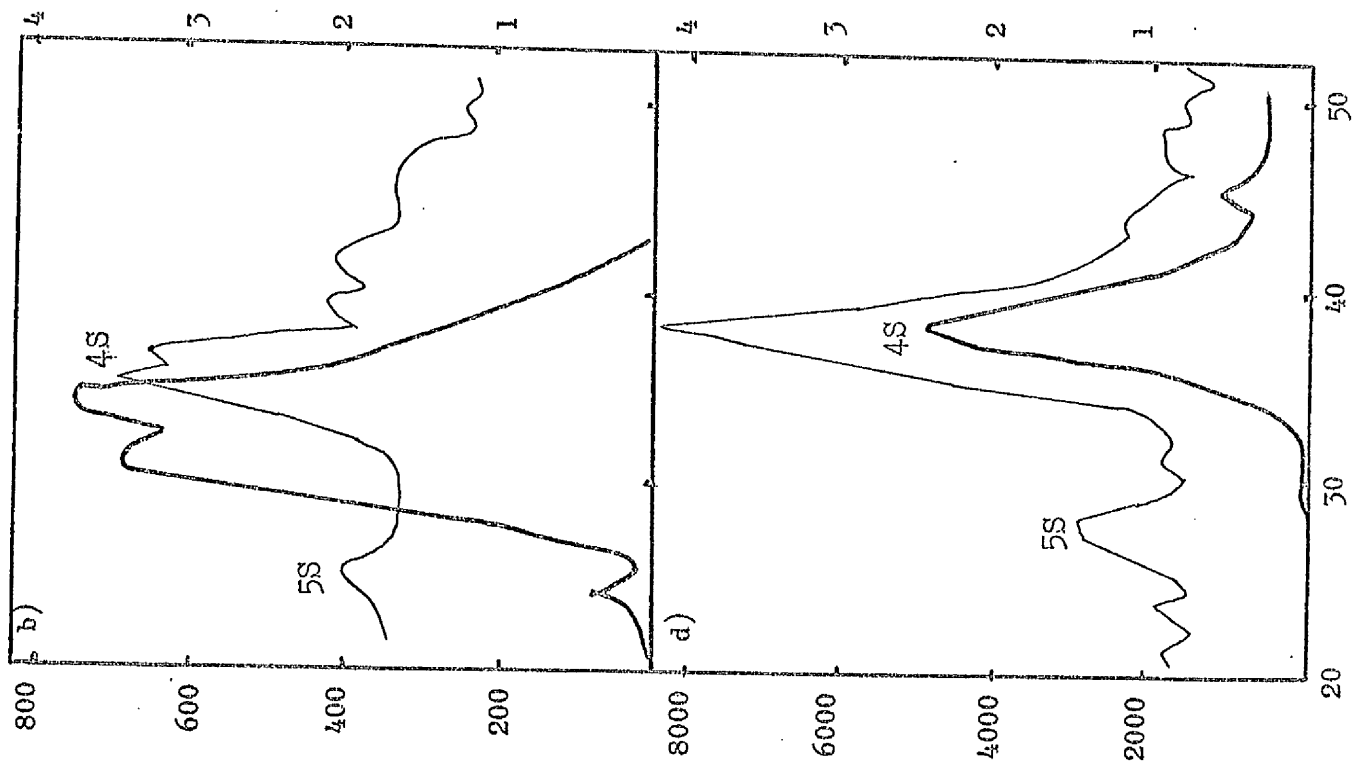
Figure 34.

Effect on 5S RNA, RNA species I and II, and 4S RNA (from uninfected cells) of elution from a 10% polyacrylamide gel

Aliquots of the RNA species (a) 5S, (b) I, (c) II and (d) 4S, eluted from the gel of uninfected cell RNA as described in Fig. 33 were co-electrophoresed on 10% polyacrylamide gels with 10 μ l ^{14}C -marker RNA; the gels were sliced and counted by liquid scintillation spectrometry, as described in "Methods", II.3.(ii).

—————, ^3H d.p.m. from ^3H -RNA
—————, ^{14}C d.p.m. from ^{14}C -marker RNA.

Figure 34

 ^{14}C -activity (d.p.m. $\times 10^{-2}$ /slice)

Slice number

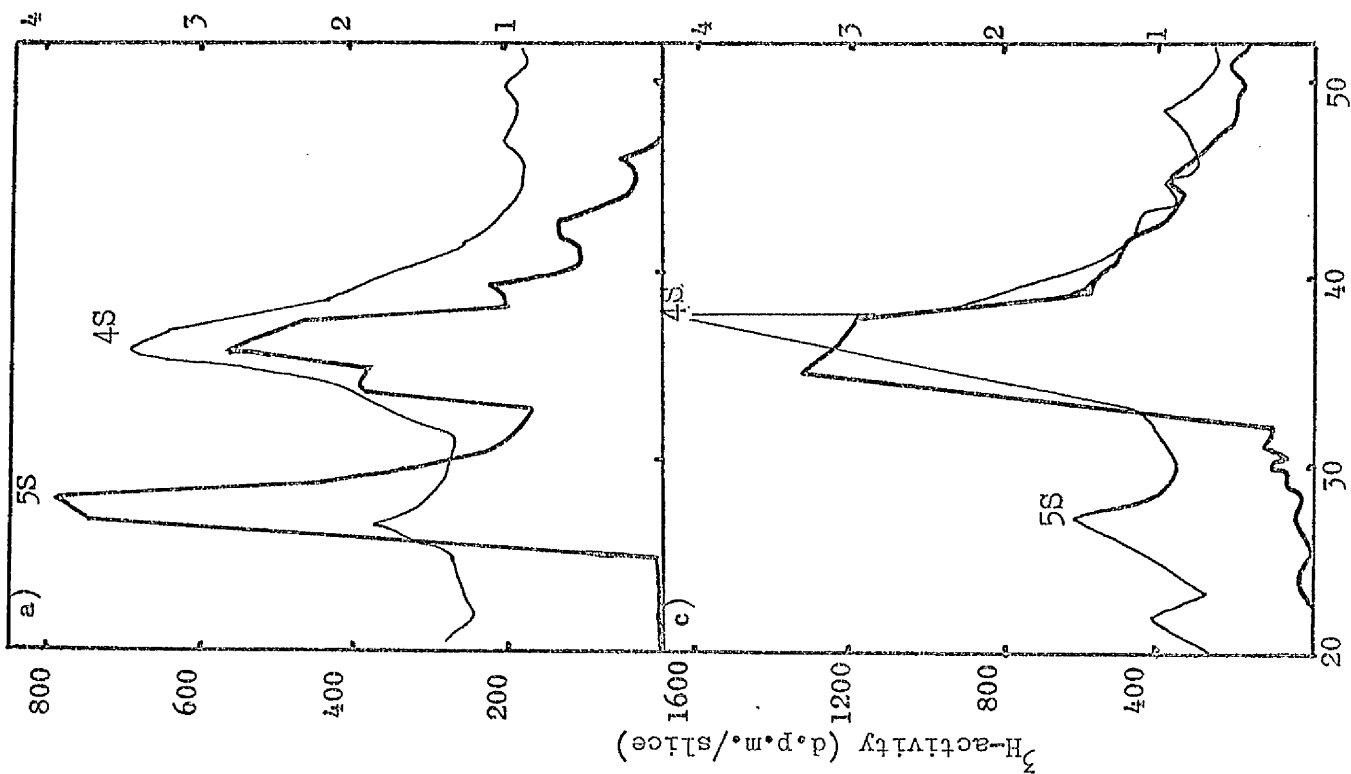


Figure 35.

Effect on 5S RNA, RNA species I and II, and 4S RNA (from PRV-infected cells) of elution from a 10% polyacrylamide gel

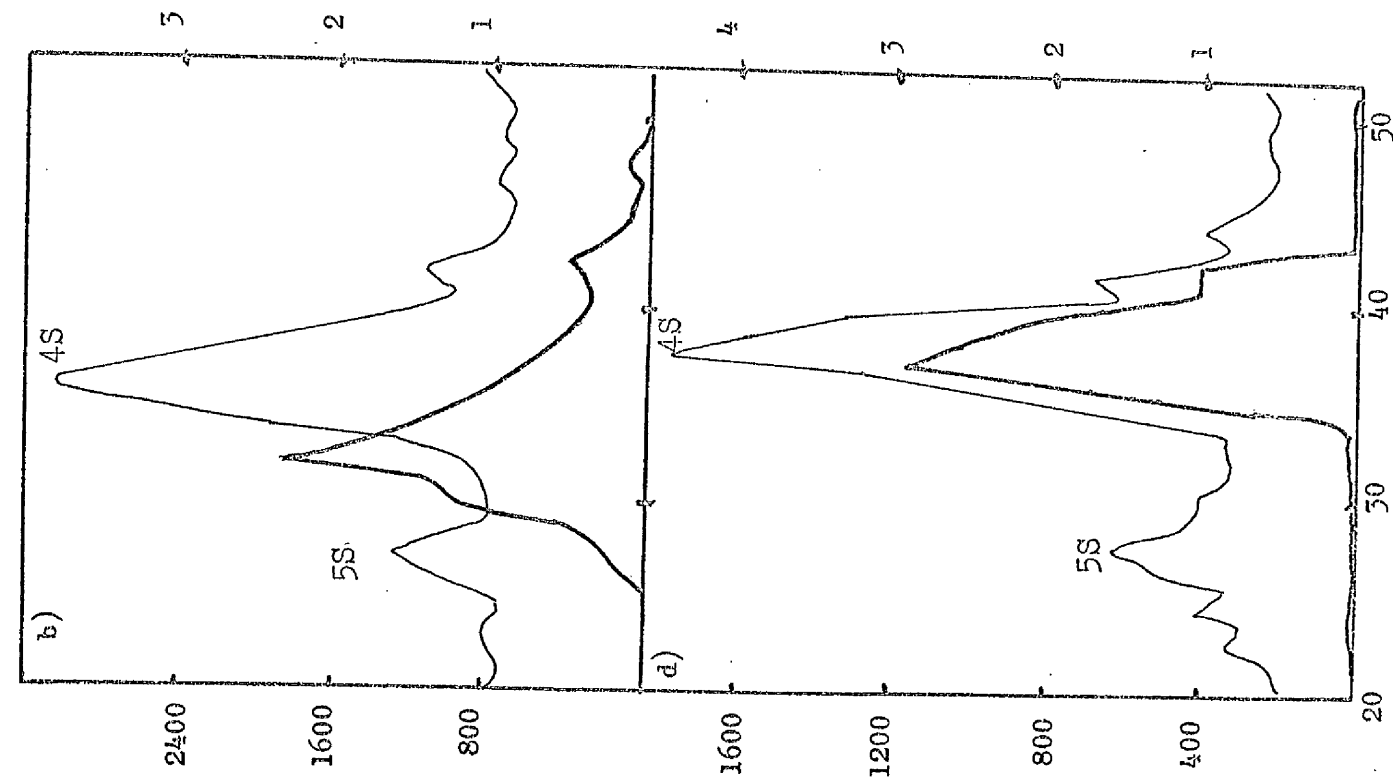
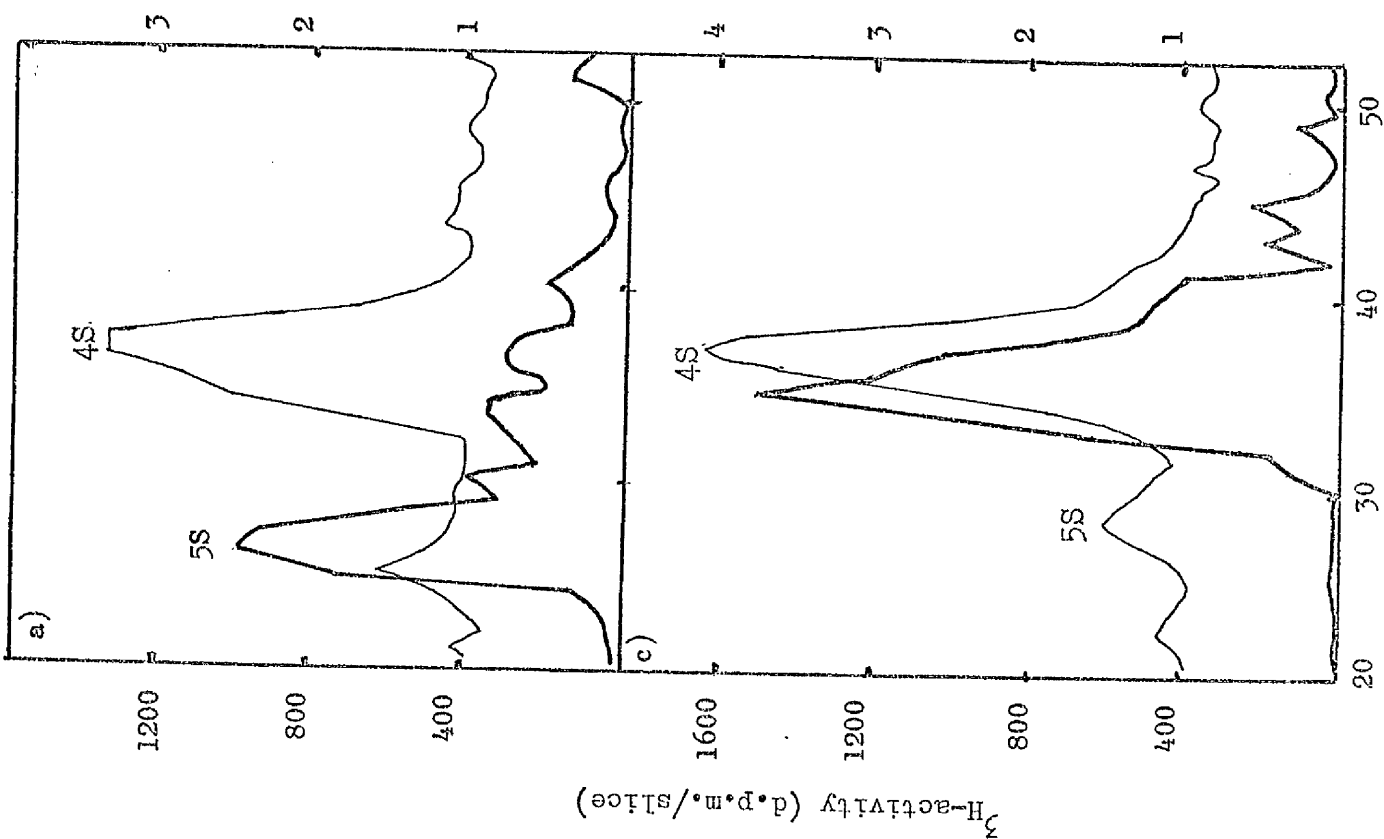
Aliquots of the RNA species (a) 5S, (b) I, (c) II and (d) 4S, eluted from the gel of PRV-infected cell RNA as described in Fig. 33 were electrophoresed on 10% polyacrylamide gels as described in Fig. 34.

—————, ^3H d.p.m. from ^3H -RNA
—————, ^{14}C d.p.m. from ^{14}C -marker RNA.

Figure 35

^{14}C -activity (d.p.m. $\times 10^{-2}$ / slice)

Slice number



much material was found in the 4S RNA position as was found in the uninfected eluted "5S RNA".

Thus elution from the gel slice has no effect on the electrophoretic properties of species I, II and 4S RNA; 5S RNA may undergo a slight amount of degradation. The next part of the experiment, viz., incubation of these RNA species with cell extract could now be carried out.

The eluted RNA species were now incubated with either buffer or cell extract. The incubation conditions were as follows. 1 ml of reaction mixture contained :-

5 μ moles 2'-mercaptoethanol

20 μ moles tris-HCl pH = 8.0

20 μ moles $MgCl_2$

3H -RNA (negligible absorbancy)

Crude cell extract containing about 2.0 mg protein.

The cell extract was omitted from the "buffer only" incubations.

The results of incubating the eluted 3H -RNA with the buffer for 1 hour are shown in Fig. 36 and 37 for uninfected cell RNA and infected cell RNA respectively. These results show that, under the sterile conditions used, the incubation with buffer has very little effect; the RNA species I and II possibly move very slightly towards the 4S position; this is in agreement with the in vitro experiments shown in Fig. 28.

The remaining portion of the eluted 3H -RNA was then incubated with uninfected cell extract in the conditions described above; infected cell extract was not used since the in vitro experiments of section II.2 showed clearly that the "maturing ability" of the extracts is identical.

Figure 36.

Effect on eluted 5S RNA, RNA species I and II, and 4S RNA (from uninfected cells) of incubation with buffer at 37° for 1 hour.

Aliquots of the RNA species - (a) 5S, (b) I, (c) II and (d) 4S, eluted from the gel of uninfected cell RNA as described in Fig. 33 were incubated for 1 hour at 37° with buffer as described in the text. After the incubation period the RNA was precipitated with 2% : sodium acetate/67% ethanol and electrophoresed on a 10% polyacrylamide gel with ^{14}C -marker RNA; the gel was sliced and counted by liquid scintillation spectrometry ("Methods. II.3.(ii)).

—————, ^3H d.p.m. from ^3H -RNA

—————, ^{14}C d.p.m. from ^{14}C -marker RNA.

^{14}C -activity (d.p.m. $\times 10^{-2}$ / slice)

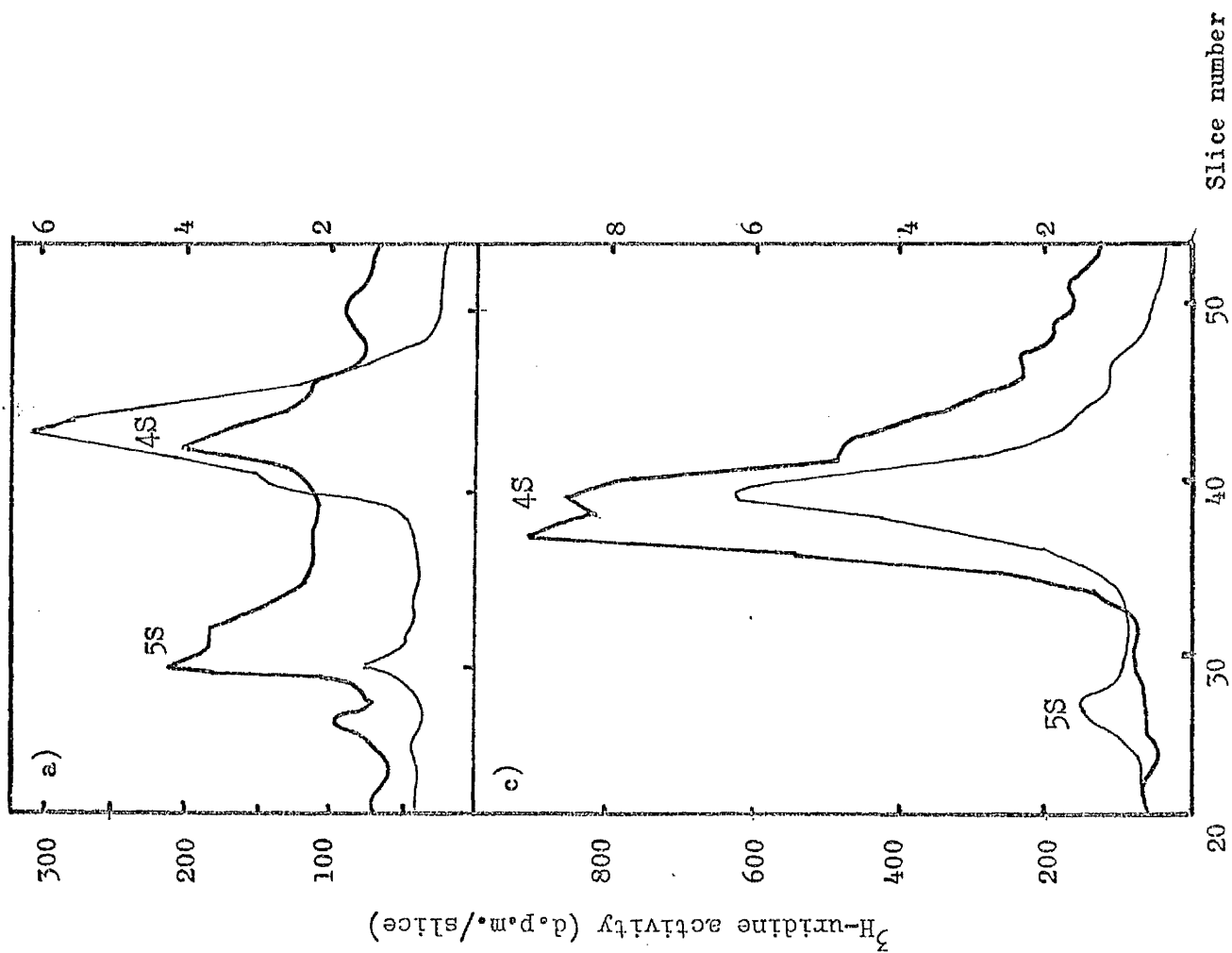
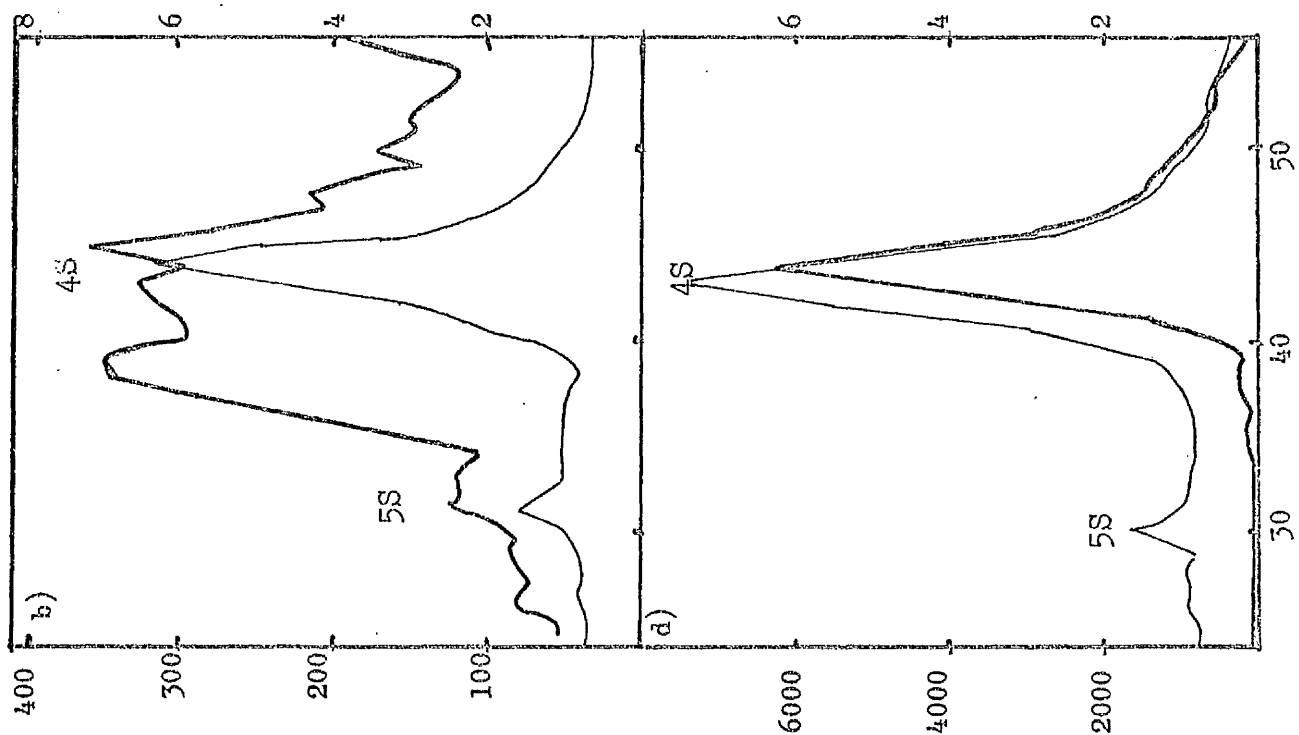


Figure 37.

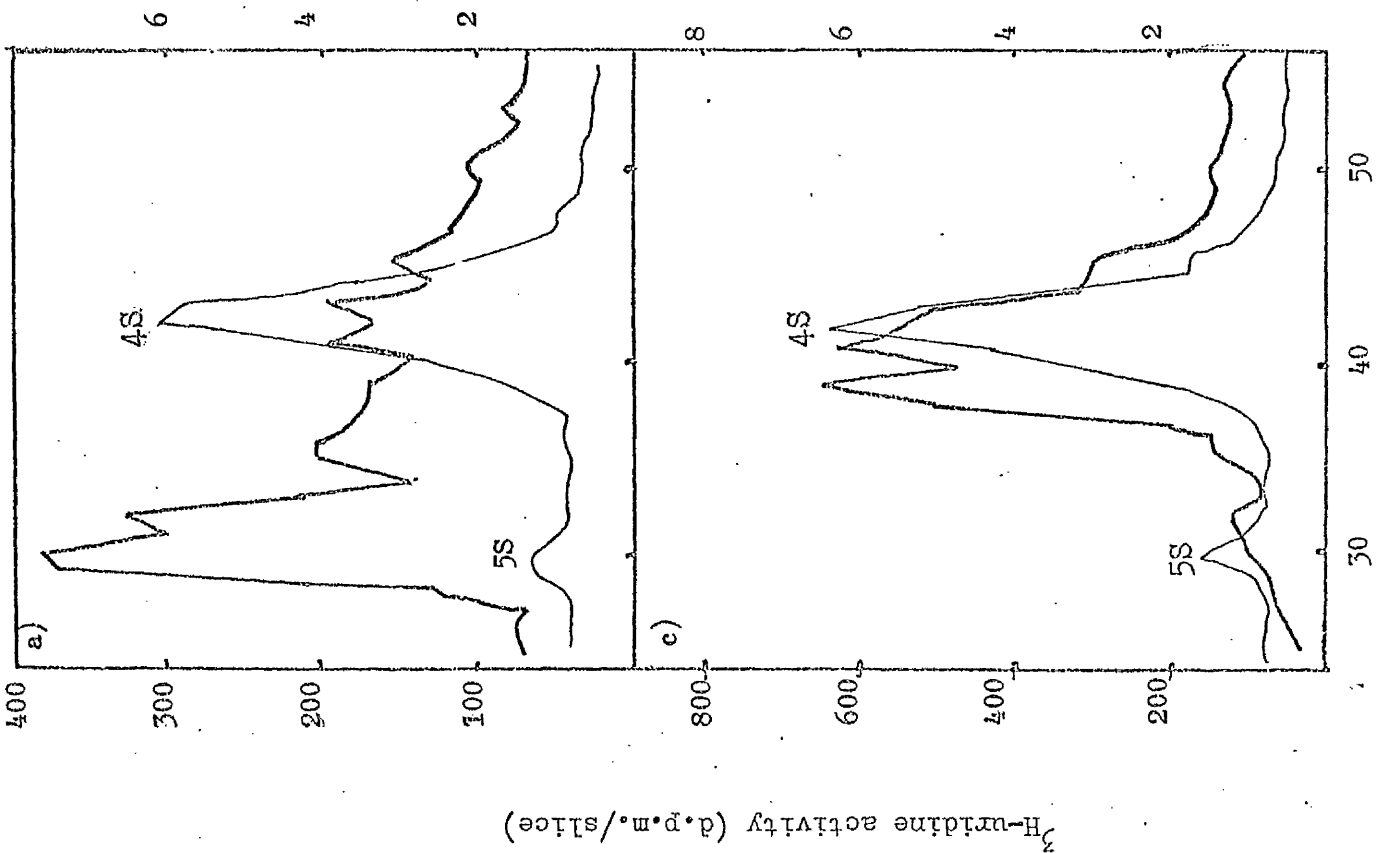
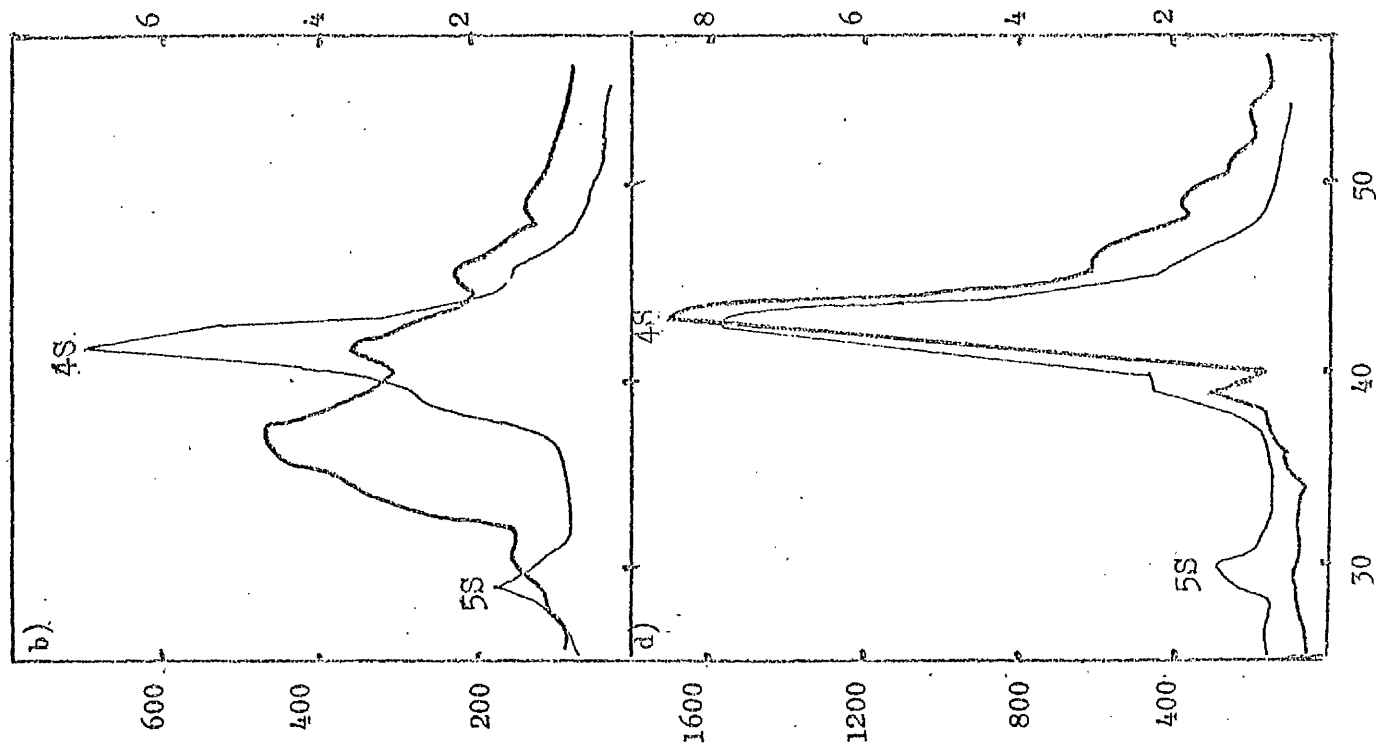
Effect on eluted 5S RNA, RNA species I and II, and 4S RNA (from PRV-infected cells) of incubation with buffer at 37° for 1 hour

Aliquots of the RNA species - (a) 5S, (b) I, (c) II and (d) 4S, eluted from the gel of PRV-infected cell RNA as described in Fig. 33 were incubated for 1 hour at 37° with buffer as described in the text.

After the incubation period, the RNA was precipitated and electrophoresed on 10% polyacrylamide gels as described in Fig. 36.

—————, ^3H d.p.m. from ^3H -RNA
—————, ^{14}C d.p.m. from ^{14}C -marker RNA.

^{14}C -activity (d.p.m. $\times 10^{-2}$ /slice)



The results of incubating the eluted ^3H -RNA with cell extract for 1 hour are shown in Fig. 38 (uninfected cell RNA) and Fig. 39 (infected cell RNA). The extract has no effect on the 4S RNA -- whether from uninfected or infected cells (Figs. 38d and 39d). Species II from the uninfected cell (Fig. 38c) has moved almost completely into the 4S position (it was mostly 4S anyway); species II from the infected cell (Fig. 39c) almost, but not quite, co-electrophoreses with the ^{14}C -marker 4S RNA after incubation with cell extract. Species I from the uninfected cell is very nearly in the 4S position (Fig. 38b); in fact it electrophoreses in a very similar fashion to the uninfected species II before incubation with extract (Fig. 36c). Species I from the infected cell (Fig. 39b) co-electrophoreses with 4S RNA except for a shoulder in the position of species II. The incubation of species I, II and 4S RNA with cell extract causes no degradation producing material smaller than 4S RNA and in all cases the leading edge of the peak is very sharp. The results from this experiment seem to suggest that species I may move to the position of species II before moving to the 4S position, as previously suggested (P. 121).

The effect of incubation with cell extract on 5S RNA is more difficult to interpret, partly because of the double peak effect (5S + 4S) obtained as a result of elution and also because the ^3H -d.p.m. in the 5S RNA sample was lower than that in the other RNA species. The effect of incubation with cell extract on uninfected cell 5S (Fig. 38a) shows a clear peak at 4S and a diffuse, smaller peak in the 5S-II region. Since 4S RNA is now known to be stable on incubation, this peak may represent true 4S RNA which could be contaminating this sample while 5S RNA itself has been partially degraded.

The effect of extract on the infected cell 5S RNA is somewhat

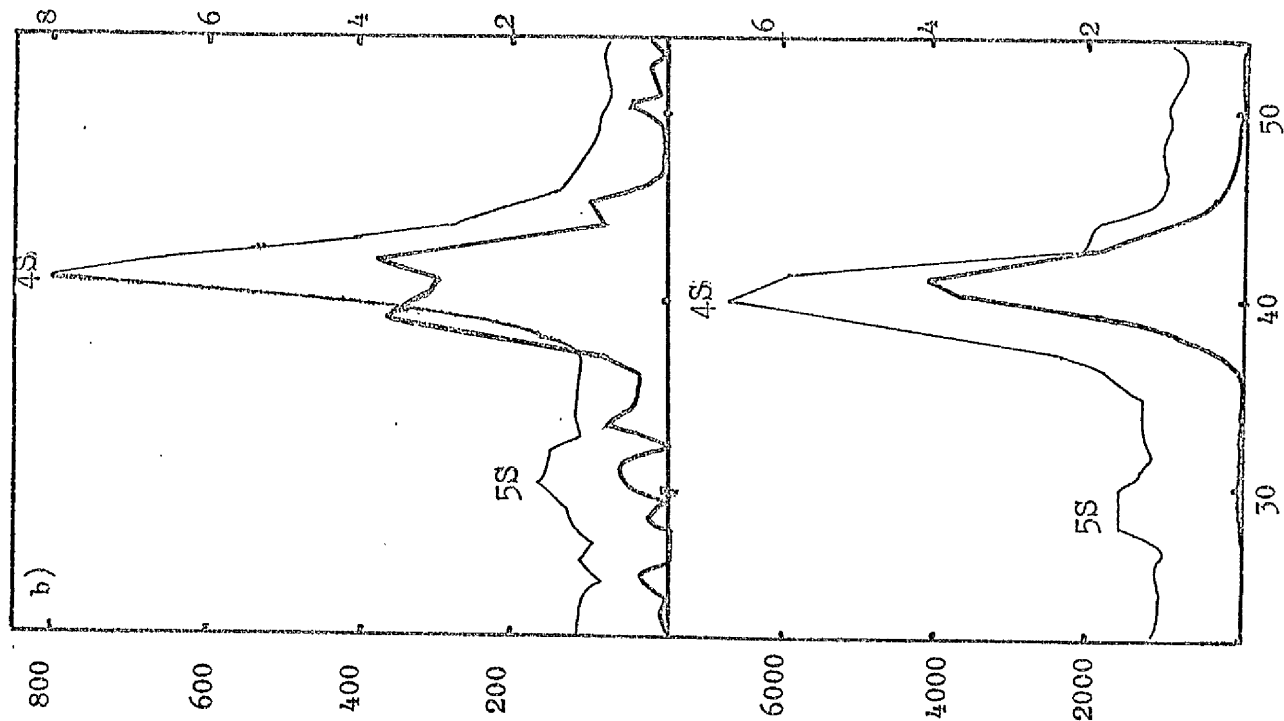
Figure 38.

Effect on eluted 5S RNA, species I and II, and 4S RNA (from uninfected cells) of incubation with cell extract at 37° for 1 hour.

Aliquots of the RNA species - (a) 5S, (b) I, (c) II and (d) 4S, eluted from the gel of uninfected cell RNA as described in Fig. 33 were incubated for 1 hour at 37° with cell cytoplasmic extract ("Methods", II.6.(i)), as described in the text. After the incubation period the RNA was phenol-extracted ("Methods", II.6.(ii)) and co-electrophoresed with 20 μ l ^{14}C -marker RNA on 10% polyacrylamide gels. The gels were then sliced and counted by liquid scintillation spectrometry ("Methods", II.3.(ii)).

—————, ^3H d.p.m. from ^3H -RNA
—————, ^{14}C d.p.m. from ^{14}C -marker RNA.

^{14}C -activity (d.p.m. $\times 10^{-2}$ /slice)



Slice number

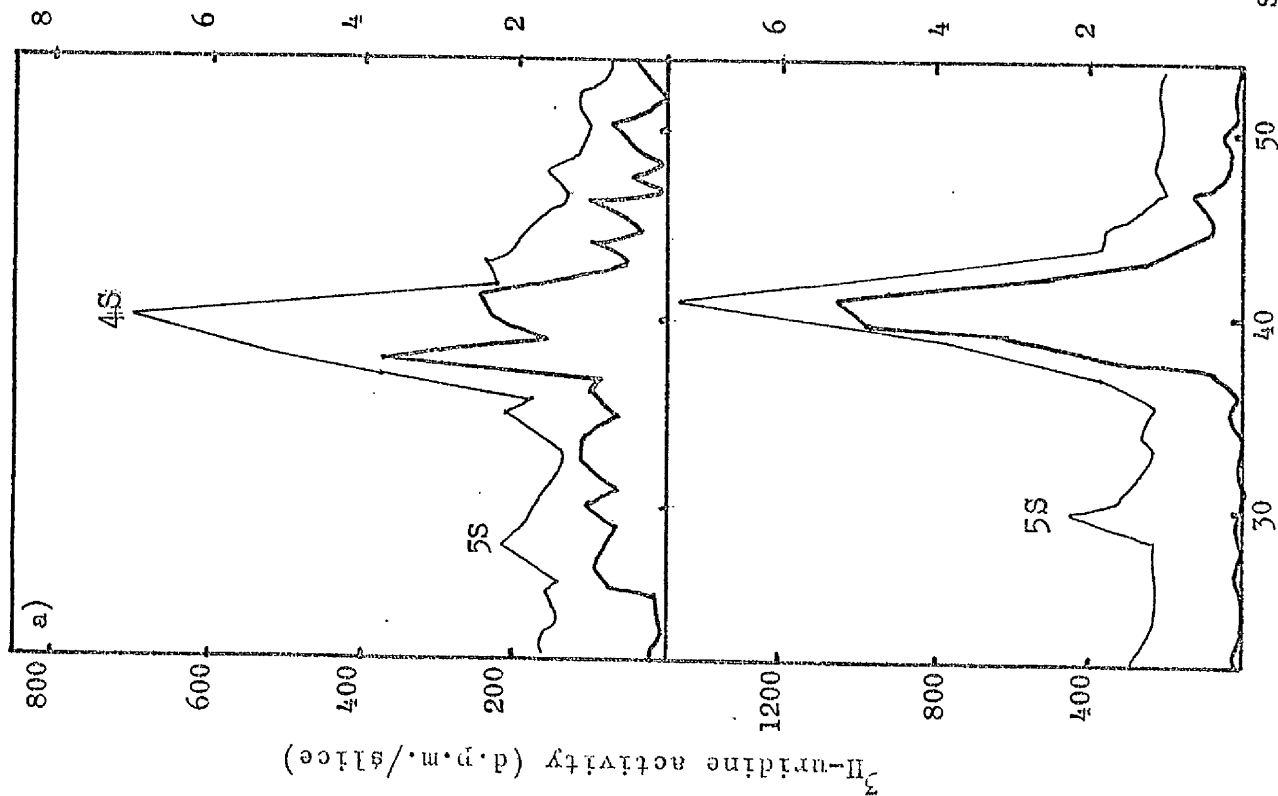
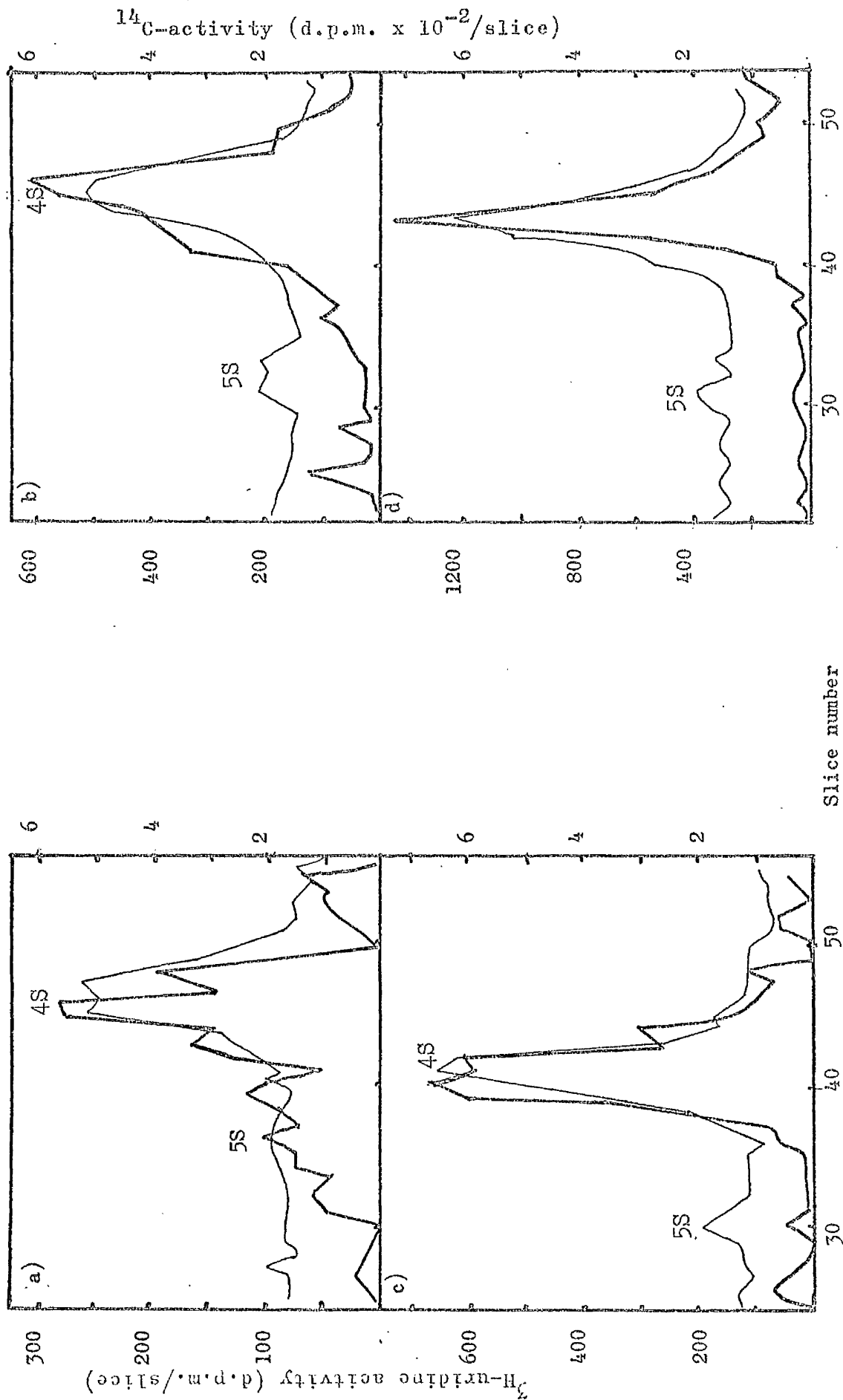


Figure 39.

Effect on eluted 5S RNA, species I and II, and 4S RNA (from PRV-infected cells) of incubation with cell extract at 37° for 1 hour.

Aliquots of the RNA species - (a) 5S, (b) I, (c) II and (d) 4S, eluted from the gel of PRV-infected cell RNA as described in Fig. 33 were incubated for 1 hour at 37° with cell cytoplasmic extract as described in the text. The resulting RNA was extracted and electrophoresed on 10% polyacrylamide gels as described in Fig. 38.

_____, ^3H d.p.m. from ^3H -RNA
_____, ^{14}C d.p.m. from ^{14}C -marker RNA.



different (Fig. 39a). Before incubation, this species appeared in the 5S region of the gel, with only a small amount of material in the peak I-4S region. After incubation with extract there is a sizeable peak in the 4S region. Although calculations of recoveries are difficult to assess owing to the many manipulations involved in working-up this series of experiments, it can be calculated that the d.p.m. in the 4S region can not be accounted for by the small amount of "4S" in the infected cell 5S RNA preparation present before incubation, and therefore some at least must have come from the 5S peak. This suggests that the infected cell contains a 5S RNA species which specifically produces a 4S RNA species on incubation with cell extract and not on incubation with buffer. We cannot rule out that a similar situation occurs in the uninfected cell.

IV. "Resting" cell experiments.

One of the difficulties experienced when investigating virus function in exponentially-growing cells is the interference of remaining cell metabolism with the pattern of virus metabolism. Therefore, a series of experiments in the "resting" cell ("Methods", I.1.(ii)) was performed, as it was thought that the depression of host function which occurs in these cells would allow the effects of the virus to be observed more clearly.

IV. 1. Calibration of the "resting" cell system.

The "resting" cell system used was that described by Fried and Pitts, 1968, as modified for Cl3 cells by Nicholas and Pitts, 1971. Thinly-seeded exponentially-growing cells are put to "rest" by replacing the EC10 medium with EC1. The measure of the degree of "resting" achieved was incorporation of ^3H -thymidine. To investigate the length of time, after the

change of medium, taken by Cl3 cells to reach the "resting" state, plates of cells were put to "rest" and pulse-labelled for 24 hour periods. The results of such an experiment are shown in Fig. 40, where the incorporation of ^3H -thymidine into DNA in cells going to "rest" is compared with the incorporation into exponentially-growing cells, and into "resting" cells to which serum was added back 6 hours previously. It is shown that when cells are put to "rest", incorporation of ^3H -thymidine decreases steadily until by the 5th day the incorporation is 1.3% and by the 6th day 0.4% of the exponential level. In fact, the decrease in incorporation is even greater than these results indicate. Measurement was by incorporation into the cells in a given area (a coverslip); after putting the cells to "rest" they doubled approximately once and hence the number of cells after 5 - 6 days in a given area was about twice the exponential number. Hence the level of incorporation per cell at 5 and 6 days can be divided by about two. Thus the DNA synthesis in these cells by day 5 is less than 1% of the exponential level, and is even lower by day 6. Yet these cells are not dead; replacement of the serum to 10% results in a renewal of their ability to synthesise DNA. Thus in all subsequent experiments 5 - 6 day "resting" cells were used.

The ability of these "resting" cells to support virus growth was tested by infecting "resting" cells at 20 p.f.u./cell and by calculating the total p.f.u. produced 30 hours later, by plaque assay. The yield was 110 p.f.u./cell which is of the same order as the level produced in exponentially-growing cells; it was also shown that the growth curve of PRV in "resting" and exponentially-growing cells is similar.

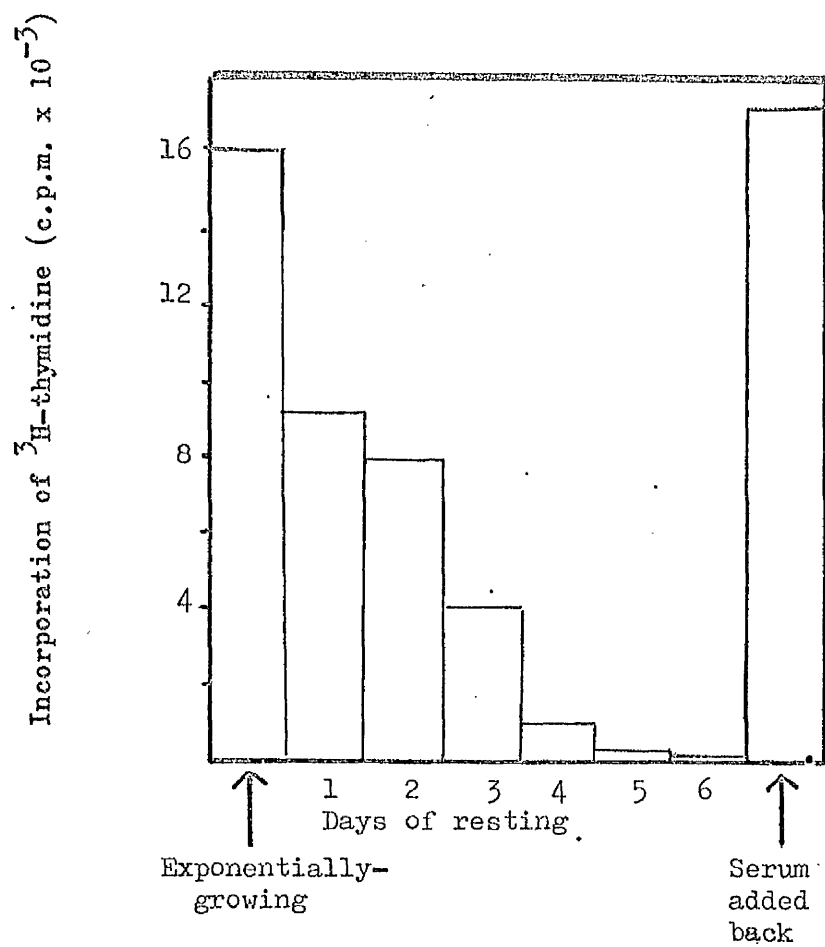
Since the level of DNA synthesis in the "resting" cells is so low,

Figure 40.

DNA synthesis in exponentially growing Cl3 cells going
to "rest"

50 mm plastic Petri dishes were seeded in EC10 at 1.3×10^6 cells/dish over 3 x 13 mm glass coverslips ("Methods", II.1.(ii) and II.4.(ii)). 18 hours later, the medium was changed to EC1 and the cells were incubated for 6 days in EC1. Two dishes were labelled every 24 hours for a 24 hour labelling period with 3.3 μ Ci/ml of 3 H-thymidine (concentration of thymidine in medium = 2.5 μ M). On the 6th day serum was added back to two Petri dishes (to raise the serum concentration to 10%) and these two Petri dishes were also labelled for 24 hours. The coverslips were removed and fixed immediately after the labelling period as described in "Methods", II.4.(ii), and counted by liquid scintillation spectrometry. Since the experiment was carried out in duplicate, there were 6 coverslips for each time point; the average value of the 4 median counts was graphed.

Figure 40



it was thought that virus DNA synthesis may be distinguished by an increase in overall DNA synthesis as occurs in stationary RK cells (Ben-Porat & Kaplan, 1963); in the exponentially-growing cell, infection produces a decrease in total DNA synthesis (Kaplan & Ben-Porat, 1963). Thus plates of "resting" cells were infected and then different plates were pulse-labelled with ^3H -thymidine for 1 hour periods and harvested immediately after labelling. The experiment was carried out using both medium containing full phosphate and also containing 10% phosphate; this was because some experiments using the "resting" system were carried out in low phosphate medium when $^{32}\text{PO}_4^{=}$ was used as RNA precursor. The results are shown in Fig. 41. In both sets of results the DNA synthesis increases after infection, reaching a peak between 6 - 7 hours P.I. In the 10% phosphate medium the increase is only to about half of the level attained in the full phosphate medium. However, the p.f.u. produced per cell in either medium is always about 100.

IV. 2. Infectivity of PRV--confirmation using autoradiography.

This "resting" system is an excellent system with which to study the infectivity of PRV and investigate the percentage of cells infected, since "resting" cells should not be synthesising DNA while "resting" cells infected with PRV synthesise DNA; this can be estimated by autoradiography.

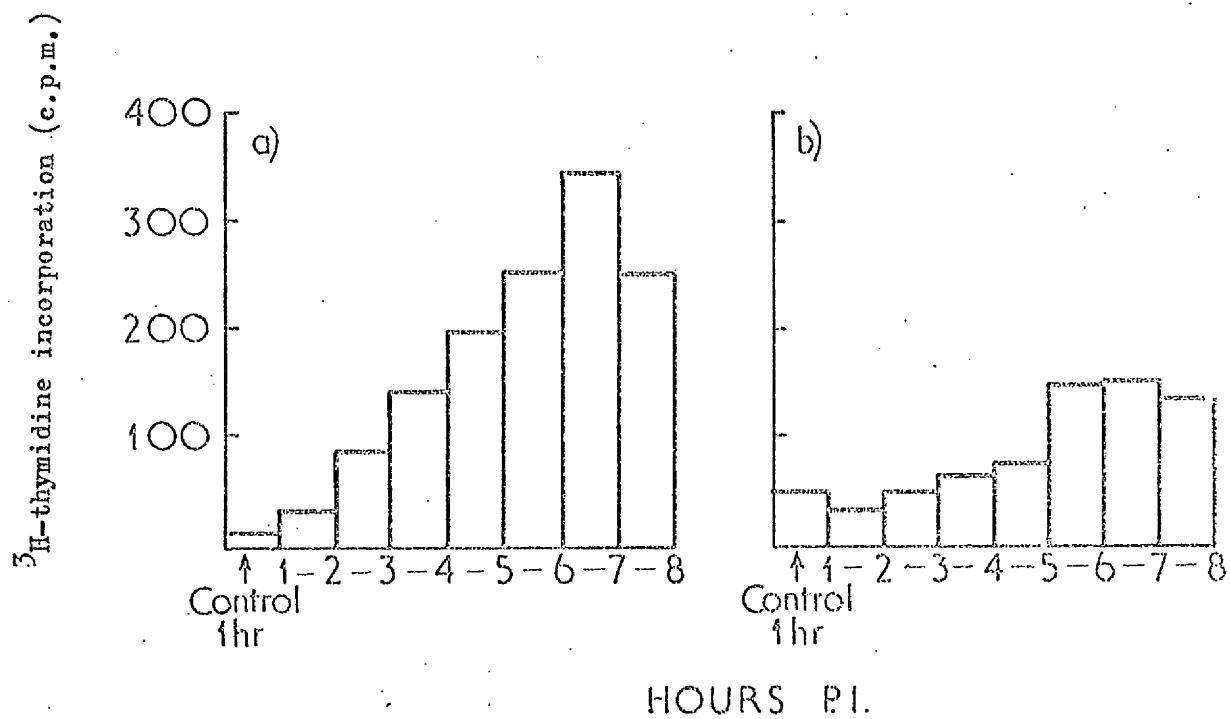
Thus Cl3 cells were seeded over glass coverslips ("Methods", II.9) and put to "rest". On the sixth day of "resting", some plates were infected with PRV at 20 p.f.u./cell while the rest were mock-infected. Five hours P.I. or after mock-infection, the plates were labelled with ^3H -thymidine for 1 hour periods. At the end of the labelling period the coverslips were

Figure 41.

DNA synthesis in "resting" cells infected with pseudorabies virus

50 mm plastic Petri dishes were seeded in EC10 at 1.3×10^6 cells/dish over 3 x 13 mm glass coverslips ("Methods", I.1.(ii) and II.4.(ii)). 18 hours later the medium was changed to EC1, half the plates receiving EC1 containing the full amount of phosphate (a), while the other half received EC1 containing 10% the normal level of phosphate (b). The cells were used on the 6th day of "resting". They were infected with PRV at 20 p.f.u./cell and labelled in duplicate for 1 hour periods from 1 - 7 hours P.I. with 3.0 μ Ci/ml of ^3H -thymidine (concentration of thymidine in medium = 0.25 μM). Uninfected cells were also labelled for a 1 hour period. The coverslips were removed and counted as described in Fig. 40.

Figure 41



prepared for autoradiography as described in "Methods", II.9.

Microscopic examination revealed that $\ll 1\%$ of the "resting" mock-infected cells were making DNA, while 100% of the PRV-infected cells were making DNA, and thus 100% of the cells were infected at 5 hours P.I. These results are consistent with the results described in section IV.1, which demonstrate that "resting" cells incorporate $\ll 1\%$ of the ^3H -thymidine incorporated by exponentially-growing cells.

IV.3. In vivo labelling studies of "resting" cells.

The synthesis of low molecular weight cell sap RNA in the "resting" cells was now investigated. Since the metabolism of these cells is lower than that of exponentially-growing cells, the synthesis of pulse-labelled RNA throughout the infectious cycle was investigated. Thus infected "resting" cells were pulse-labelled with ^3H -uridine for 30 min periods every two hours from 1 hour P.I. until 7 hours P.I. Uninfected "resting" cells were also pulse-labelled for 30 min, as were uninfected exponentially-growing cells. The effect of adding serum back to "resting" cells was examined by pulse-labelling cells for 30 min, 48 hours after the addition of serum (to 10%) to resting cells. This showed an increase in RNA synthesis to levels approaching the pre-"resting" levels and also confirmed that the cells are resting and not dead or dying.

The results of 10% gel electrophoresis of the resulting "cell sap" RNA are shown in Fig. 42. In this experiment, ^{14}C -marker RNA was not added because of the low level of ^3H -d.p.m., but the positions of 5S RNA and 4S RNA were obtained from the absorbancy trace.

Fig. 42 shows that there is a large decrease in the synthesis of

Figure 42.

30 min labelled cell sap RNA in "resting" cells at various times
after PRV-infection.

Roux bottles, seeded with Cl3 cells, were put to "rest" as described in "Methods", I.1.(ii) and were used on the 6th day. An additional Roux bottle was used in the exponential phase of growth when nearly confluent ("high serum" control). One "resting" Roux bottle had serum replaced (to 10% level) and was used two days later ("serum back" control). The uninfected "control" Roux bottles were all labelled for 30 min periods with 12 μ Ci/ml of 3 H-uridine:

(a) uninfected "resting" cells, (b) "high serum" exponentially-growing cells, and (c) "serum back" cells. "Resting" Roux cells were infected with PRV and were also labelled for 30 min periods: (d) 1 - 1.5 hours, (e) 3 - 3.5 hours (f) 5 - 5.5 hours and (g) 7 - 7.5 hours P.I.

At the end of the labelling period, the cells were harvested, mechanically disrupted and subjected to differential centrifugation to obtain the cell sap fraction ("Methods", II.1.(ii)) from which the cell sap RNA was obtained by phenol extraction. (Commercial 4S RNA was added as carrier). The cell sap RNA was electrophoresed on 10% polyacrylamide gels, which were scanned on the Gilford spectrophotometer, sliced and counted by liquid scintillation spectrometry as described in "Methods", II.3.(ii).

Figure 42

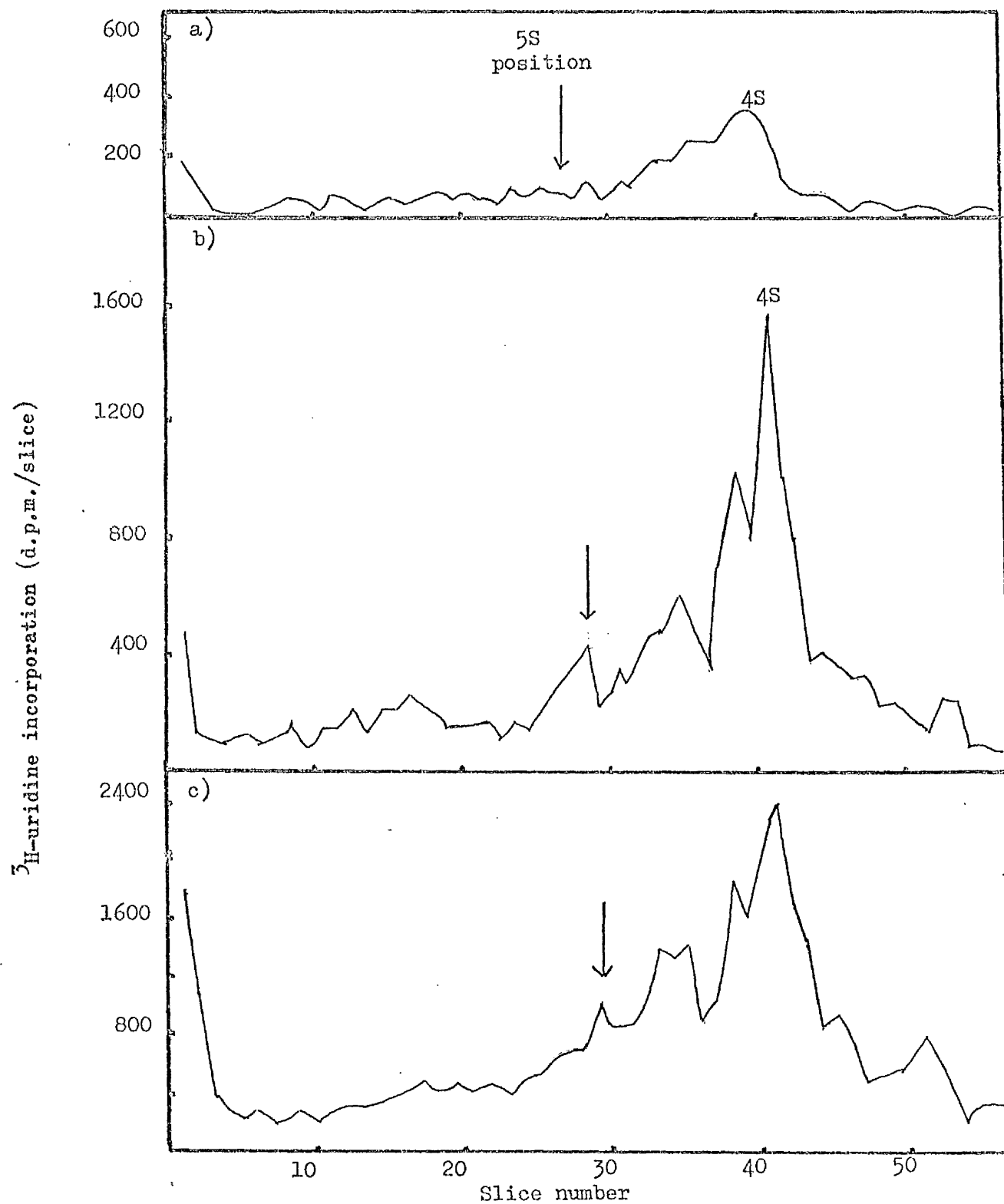
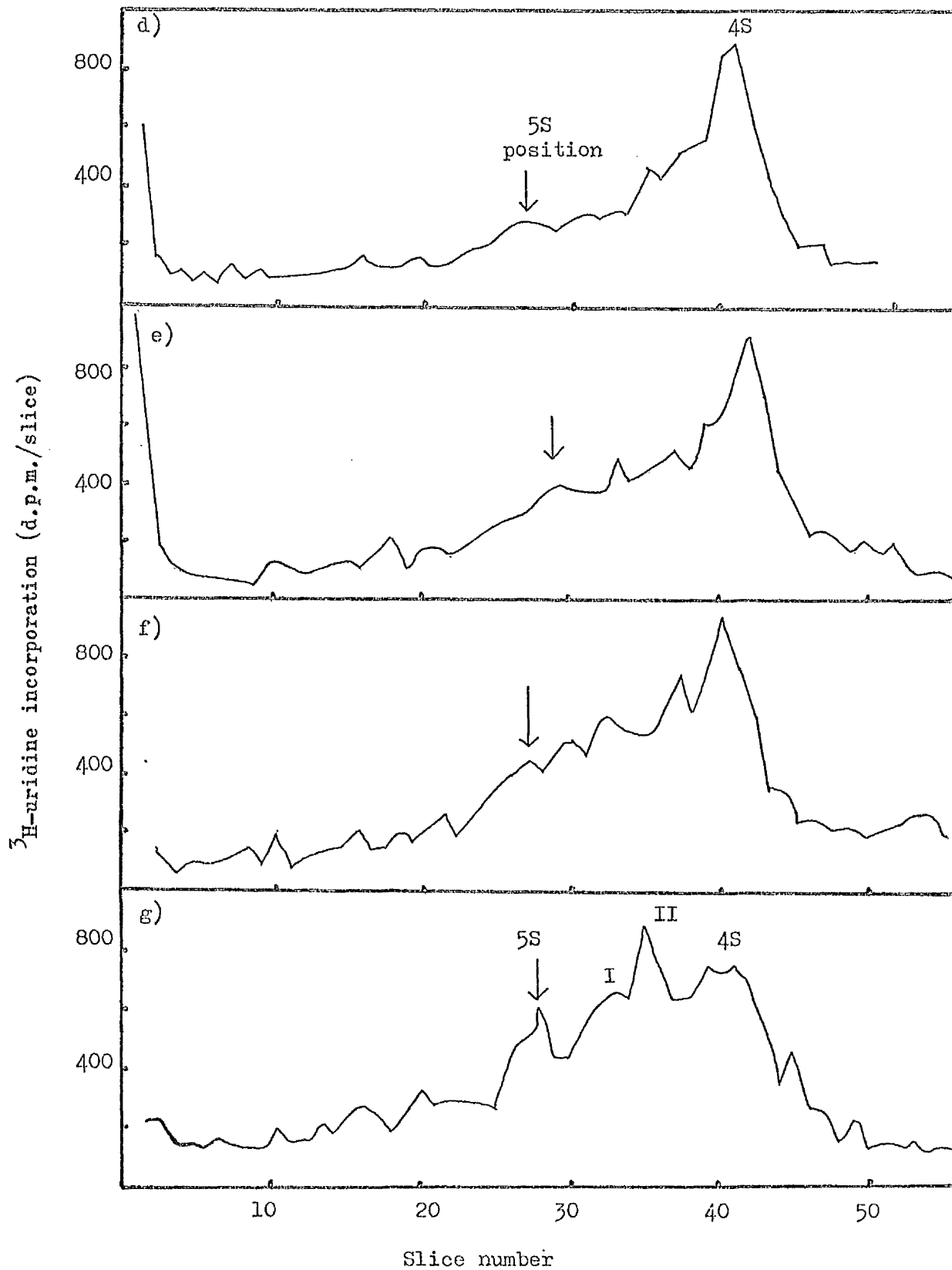


Fig. 42, d-g: overleaf

Figure 42 (continued)



4S RNA in "resting" cells to about 25% of the level of synthesis observed in exponentially-growing cells. However, the pattern is very similar - mostly 4S RNA, a little material between 5S and 4S and even less in the 5S position. The "serum-back" 30 min pulse-labelled RNA also shows a similar pattern of incorporation, and has an even higher level of incorporation of isotope than the exponential sample; this can be accounted for by the fact that the cell numbers had approximately doubled in the 48 hours between addition of serum and the pulse-labelling period. The low level of 5S RNA in all three uninfected samples is probably due to the almost complete precipitation of ribosomes in the preparation of the cell sap.

The series of infected "resting" cell sap RNA profiles can now be examined (Fig. 42, d-g). At 1 - 1.5 hours P.I. there is mostly 4S RNA, a small amount of 5S-4S material and a very little 5S RNA. The amount of material between 5S and 4S relative to 4S increases as infection proceeds, as does the amount of 5S RNA, so that by 7 - 7.5 hours P.I. there is a very distinct 5S peak, and a considerable amount of material between 5S and 4S in two peaks, and a relatively smaller 4S RNA peak. The profile of cell sap RNA pulse-labelled from 5 - 5.5 hours P.I. from "resting" cells is very similar to the pattern from exponentially-growing cells for the same period of time.

An unexpected finding in this experiment is the increase in 4S RNA synthesis after infection to about three times the "resting" level. In this experiment the whole amount of cell sap RNA from each sample was added to the gels and hence the recovery may vary slightly, although it is clear that at any rate there is no decrease in 4S RNA synthesis comparable to that observed in the exponentially-growing cells on infection.

To investigate this particular point further, "resting" cells were infected and labelled for 3 hour periods to determine the levels of mature 4S RNA produced, since after a 3 hour pulse-label most of the radioactivity in the low molecular weight RNA species is in 4S RNA (in exponential cells, at any rate, e.g. Fig. 31d).

Thus "resting" cells were infected and pulse-labelled with ^3H -uridine for 1 - 4 hours P.I. and 4 - 7 hours P.I. As controls, uninfected "resting" cells, exponentially-growing cells and "resting" cells to which serum had been replaced 48 hours earlier were also pulse-labelled for 3 hours. The results are shown in Fig. 43. In all cases there is only a very small amount of 5S RNA, consistent with the very small amount of ribosomal RNA present; no material electrophoreses between 5S and 4S in any of the three control samples (Fig. 43, a-c). However, there is a small amount in the sample pulse-labelled from 1 - 4 hours P.I. and even more at 4 - 7 hours P.I. (Fig. 43d, e); in both cases the material is predominantly in the position of species II. This appearance of species II agrees with the results obtained in exponentially-growing cells (e.g. Fig. 31d).

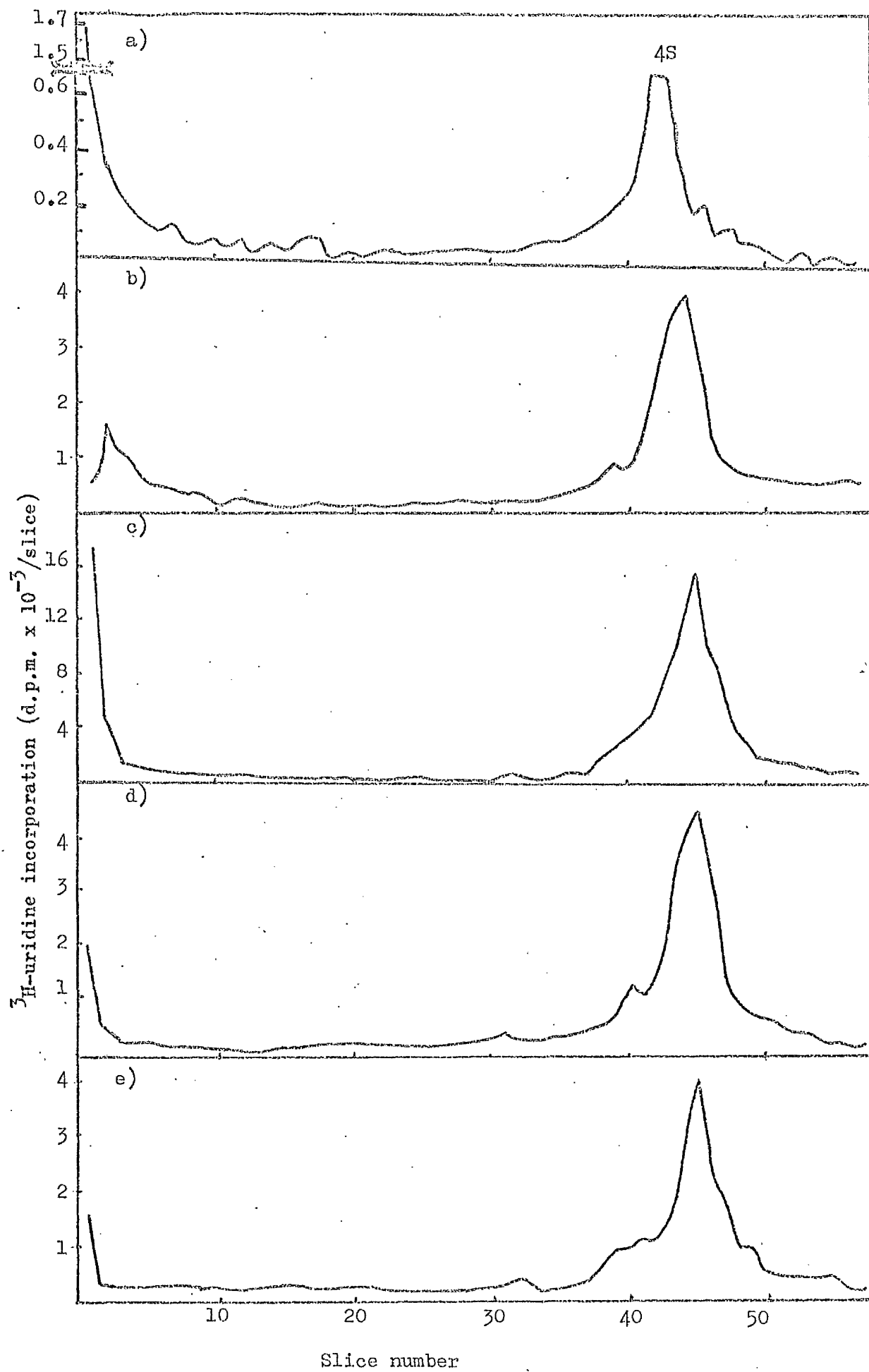
Comparison of the actual amounts of 4S RNA synthesised shows that, as in the 30 min pulse-labelled material, the "resting" level is about 20% of the exponential level. The "serum-back" RNA again shows a big increase in 4S RNA synthesis over the original exponential value. On infection of "resting" cells, the level of 4S RNA synthesis increases about 5-fold to approximately the exponential level.

Thus Fig. 43 confirms the results of Fig. 42 that, on infecting "resting" cells, PRV causes an increase in the synthesis of 4S RNA.

Figure 43.

3 hour labelled cell sap RNA in "resting" cells at various times
after PRV infection

Cell sap RNA was prepared from PRV-infected "resting" cells and uninfected controls as described in Figure 42, except that in all cases the labelling period was for 3 hours with 4 μ Ci/ml of ^3H -uridine. As in Fig. 42 the controls were - (a) uninfected "resting" cells, (b) "high serum" exponentially-growing cells, and (c) "serum back" cells; "resting" Roux bottles were infected with PRV and were also labelled for 3 hour periods: (d) 1 - 4 hours and (e) 4 - 7 hours P.I.



One further point of interest emerges on comparison of Figs. 42 and 43. In the 30 min labelled cell sap there is a significant amount of 5S material, while there is very little in the 3 hour labelled material. This occurs in both infected and uninfected samples and tends to suggest the possibility (discussed earlier) that at least some material in the 5S RNA position becomes 4S RNA.

IV. 4. Studies on methylation of 4S RNA before and after PRV-infection.

PRV-infection of "resting" cells has been shown to cause an increase in the level of 4S RNA synthesis. Thus there may be changes in the population of tRNA after infection; since tRNA is methylated, it seemed worthwhile to examine the methylation of uninfected and PRV-infected "resting" cells to discover if infection causes changes in the degree of methylation of the 4S RNA.

The methylation of cell sap RNA in "resting" cells before and after PRV-infection was thus examined; exponentially-growing cells and "resting" cells to which serum had been replaced 48 hours previously were used as controls. The cells were put to "rest" in ECl containing 25% phosphate, 50% methionine and infected on the 6th day, while the control cells were changed to EC10 containing 25% phosphate, 50% methionine 15 hours prior to labelling; thus care should be taken in comparing the control results to those obtained in the "resting" cells because of possible differences in the specific activity of methionine and phosphate.

$^{32}\text{PO}_4$ and ^3H -(methyl)-methionine were used to pulse-label the cells. 10 mM sodium formate was added with the "label" to prevent the ^3H -methyl group of methionine labelling the intracellular one-carbon pools; this

level of sodium formate does not affect cell or virus growth (Low, 1970).

The three uninfected samples ("resting", exponentially-growing and "serum-back") were pulse-labelled for 3 hours, while the "resting" infected cells were pulse-labelled for 1 - 4 hours, 4 - 7 hours and 7 - 10 hours after infection. The cell sap RNA was prepared ("Methods", II.1.(i)a and II.1.(ii)) and electrophoresed on 10% polyacrylamide gels; the results are shown in Fig. 44. The ^{32}P -incorporation represents RNA synthesis, while the ^3H -incorporation represents methylation. The small amount of ribosomal RNA on top of the gel and the 4S RNA peak are methylated in all samples. In the three uninfected samples (Fig. 44, a-c) the very small amount of RNA present in the 5S position is not methylated; neither is the relatively larger amount of 5S RNA present in the infected cell sap at all periods after infection (Fig. 44, d-f) and present in a large amount at 4 - 7 hours P.I. Note also that, in the cell sap RNA at 4 - 7 hours and 7 - 10 hours after infection, the 4S RNA on the precursor side of the 4S RNA peak seems to be submethylated, relative to mature 4S RNA.

The amount of 4S RNA produced after infection does not show the large increase obtained previously (Figs. 42 and 43); however, the level of 4S RNA synthesis remains constant after infection until 7 - 10 hours P.I. when a decrease in 4S RNA synthesis occurs. A likely explanation for this result is the variation in the degree of "resting" of the cells; if the "resting" cells are more active metabolically than in previous experiments then there is more 4S RNA being synthesised, which will mask any virus-induced synthesis.

The degree of methylation of the 4S RNA in all the gels is very similar, except in the case of 4S RNA from the "serum back" cell sap; this difference is probably due to the specific activity problems discussed above,

Figure 44.

Methylation of cell sap RNA before and after PRV-infection of
"resting" cells.

Roux bottles, seeded with Cl3 cells were put to "rest" as described in "Methods", I.1.(ii) except the EC1 contained 25% normal phosphate and 50% normal methionine. The cells were used on the 6th day of "resting". Additional Roux bottles were used in the exponential phase of growth when nearly confluent ("high serum" control); some "resting" Roux bottles had their medium replaced to normal EC10 and were used two days later. The "high serum" and "serum back" control Roux bottles had their medium changed to EC10 containing 25% phosphate, 50% methionine, 15 hours prior to labelling.

The uninfected "control" Roux were all labelled for 3 hour periods with 100 $\mu\text{Ci/ml}$ of $^{32}\text{PO}_4^=$ and 5 $\mu\text{Ci/ml}$ of ^3H -(methyl)-methionine: (a) uninfected "resting" cells, (b) "high serum" exponentially-growing cells, and (c) "serum back" cells. "Resting" cells were infected with PRV at 20 p.f.u./cell and were also labelled for 3 hour periods: (d) 1 - 4 hours, (e) 4 - 7 hours, and (f) 7 - 10 hours P.I. The cell sap RNA was prepared and electrophoresed as described in Fig. 42.

—————, ^{32}P c.p.m. from $^{32}\text{PO}_4^=$
 ————, ^3H c.p.m. from ^3H -(methyl)-methionine.

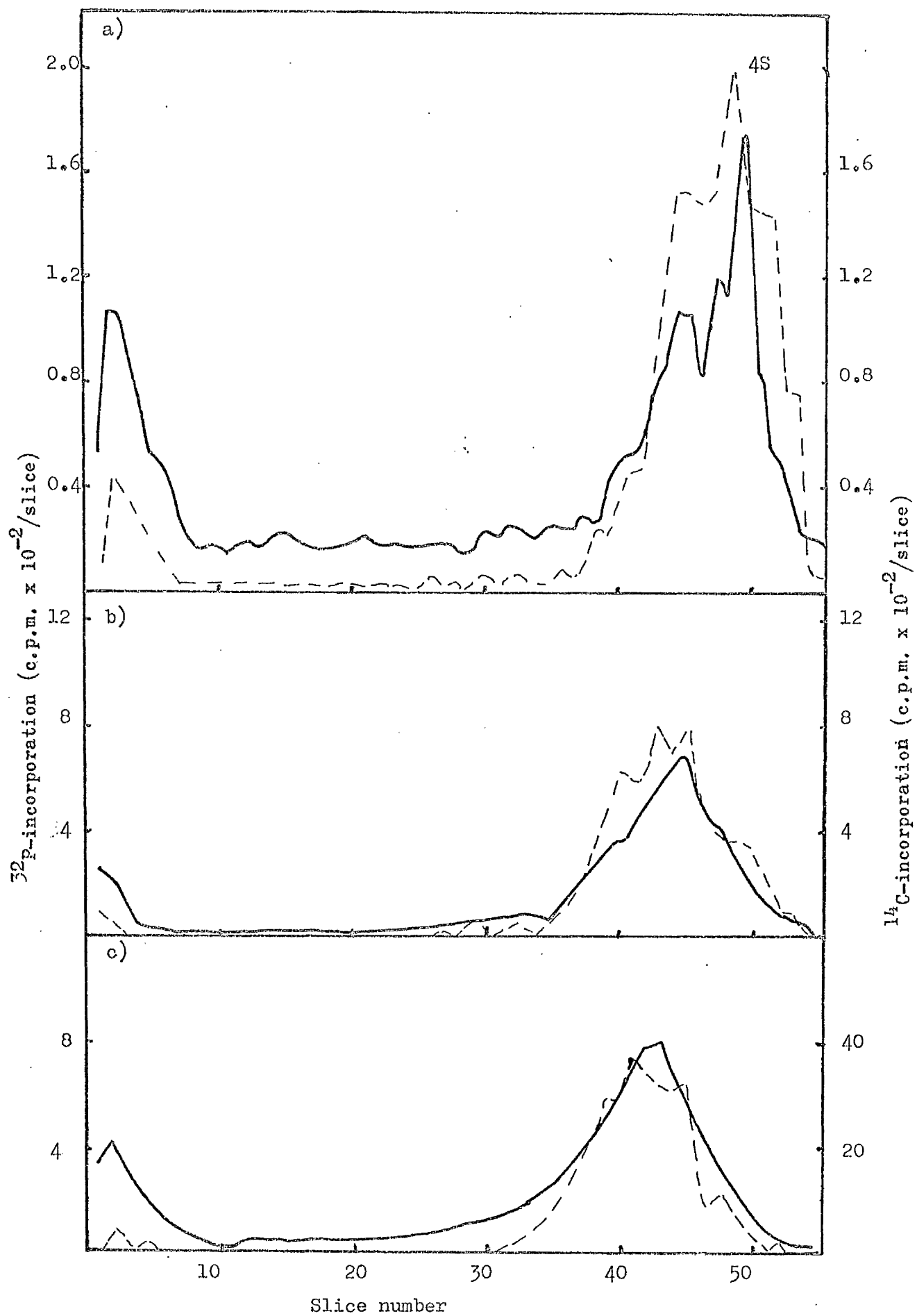
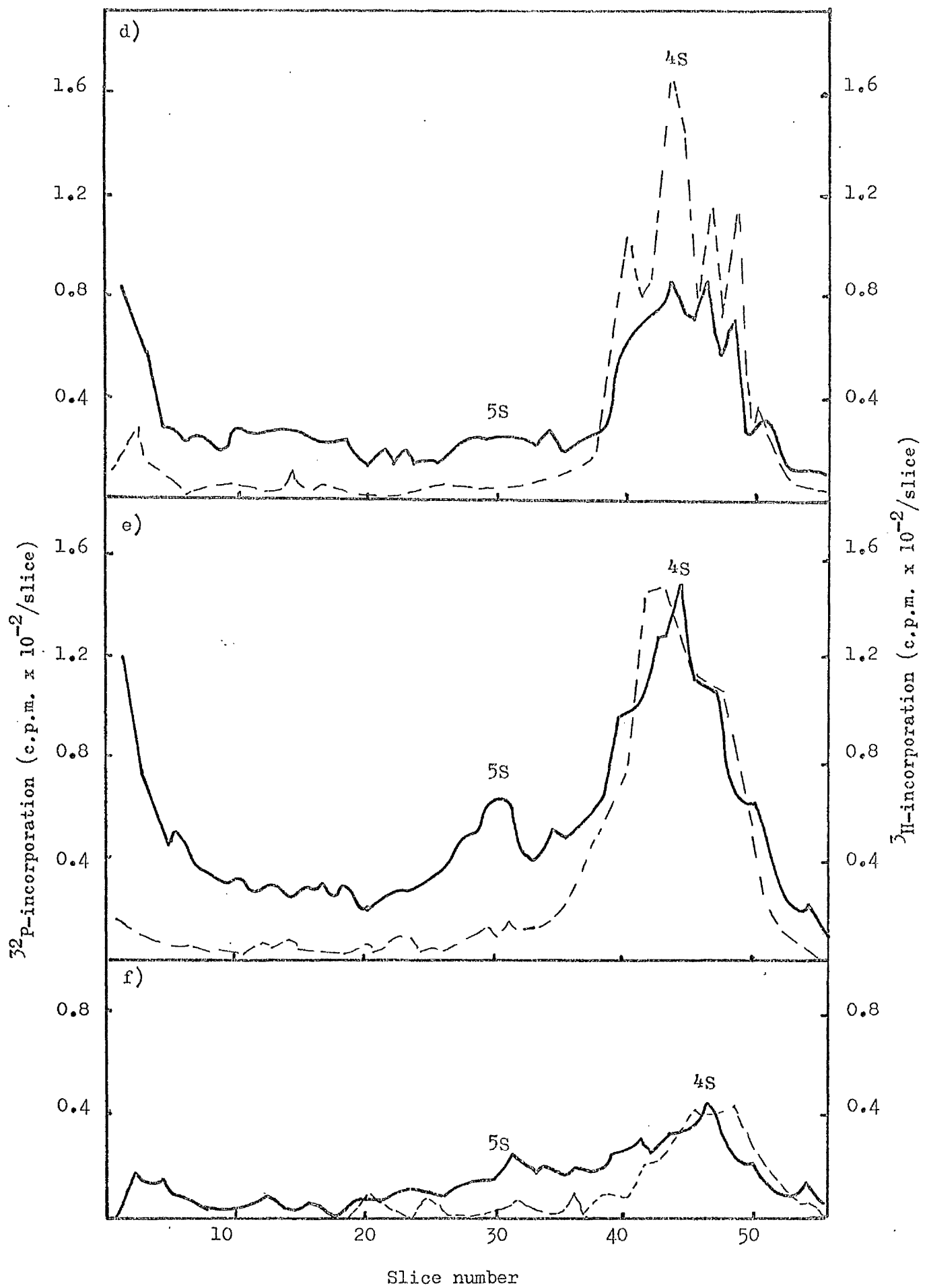


Fig. 44, d-f: overleaf



and also to possible alterations in pool size as the cells change from the "resting" state to growing exponentially.

Hence Fig. 44 demonstrates that there are no gross changes in the degree of methylation of mature 4S RNA after PRV-infection.

V. Studies on the nucleotide composition and sequence of 4S RNA and 5S RNA in PRV-infected cells.

The results in section IV have shown that when "resting" cells are infected with PRV, 4S RNA synthesis does not decrease (as occurs when exponentially-growing cells are infected) and may even increase. Thus, as mentioned in section IV.4, there may be changes in the population of tRNA after PRV-infection. It was also considered that there may be changes in the 5S RNA or even a new 5S RNA after PRV-infection as has been discussed earlier (section III.1). If such changes do take place they may perhaps be reflected in changes in nucleotide composition or in the nature of the oligonucleotides produced after enzymic digestion of the RNA, and thus the following experiments were undertaken.

V.1. Determination of the nucleotide composition of 4S RNA and 5S RNA in uninfected and PRV-infected cells.

The nucleotide composition of 4S RNA and 5S RNA before and after PRV-infection was determined. The method used to obtain nucleotide composition after infection involves labelling the RNA with $^{32}\text{PO}_4^{\equiv}$. The RNA samples were those prepared as described in the legend to Fig. 45; thus "resting" cells in low phosphate ECl were infected and labelled for 3 hour periods with $^{32}\text{PO}_4^{\equiv}$ (1 - 4, 4 - 7 and 7 - 10 hours after infection); uninfected "resting" cells were also labelled for a 3 hour period, as were

(as controls) uninfected exponentially-growing cells and "resting" cells to which serum had been replaced 48 hours previously; these controls had low phosphate EC10 added to them 15 hours prior to labelling. The 3 hour pulse-label of $^{32}\text{PO}_4^{\text{P}}$ was chosen in the hope of achieving the necessary equilibration of label in the intracellular triphosphate pools.

The results obtained by 10% gel electrophoresis of the cell sap RNA are shown in Fig. 45. In this experiment the gel slices were counted in a gas-flow counter to record the ^{32}P -emissions. The RNA samples are those electrophoresed in Fig. 44 and the fact that in this experiment the "resting" levels of 4S RNA remained constant after infection until a decrease at 7 - 10 hours P.I. has already been discussed. The amount of 5S RNA present in the different samples is now examined. In all the infected cell samples (Fig. 45, d-f) there is more 5S RNA than is present in any of the three uninfected samples (Fig. 45, a-c); the increase in synthesis of 5S RNA relative to 4S RNA after infection is always obtained on short pulse-labels but only occasionally on longer labels. It is not clear if this material is due to the presence of a small number of ribosomes in the cell sap fraction. However, it would be surprising if the ribosomes which precipitated almost completely in the three uninfected samples had not in the three infected samples; also, the small amount of ribosomal RNA on top of the gel is much lower in the "resting" cells than in the exponentially-growing cells, and further decreases as infection proceeds.

To obtain the nucleotide composition of 4S RNA, the slices of RNA indicated in Fig. 45, a-f were eluted. The nucleotide composition of 5S RNA was obtained by elution of the slices from those gels which contained 5S RNA sufficiently highly labelled; these were the gels of "serum added

Figure 45.

Cell sap RNA in resting cells labelled with $^{32}\text{PO}_4$ for nucleotide analysis.

The ^{32}P -RNA used in this experiment was that obtained as described in Fig. 4. The RNA samples were electrophoresed on 10% polyacrylamide gels, sliced and counted on a gas-flow counter to record the counts from ^{32}P only. The slices indicated by hatching were combined and the RNA was eluted from them as described in "Methods", II.7 for nucleotide analysis.

- (a) Uninfected "resting": 4S RNA
- (b) "High serum" exponentially growing : 4S RNA
- (c) "Serum back" : 4S RNA and 5S RNA
- (d) 1 - 4 hours P.I. : 4S RNA
- (e) 4 - 7 hours P.I. : 4S RNA and 5S RNA
- (f) 7 - 10 hours P.I. : 4S RNA.

Figure 45

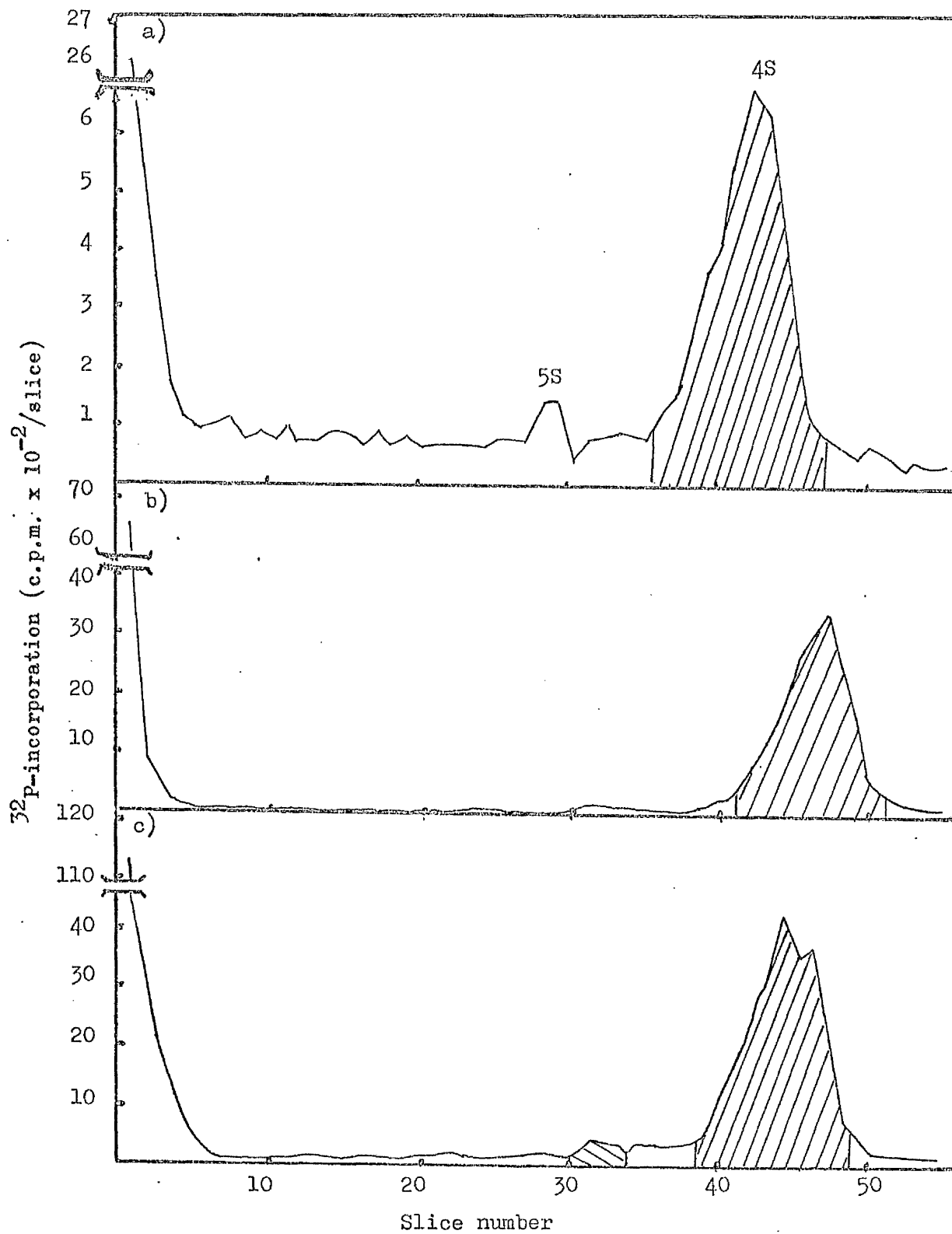


Fig. 45, d-f: overleaf

Figure 45 (continued)

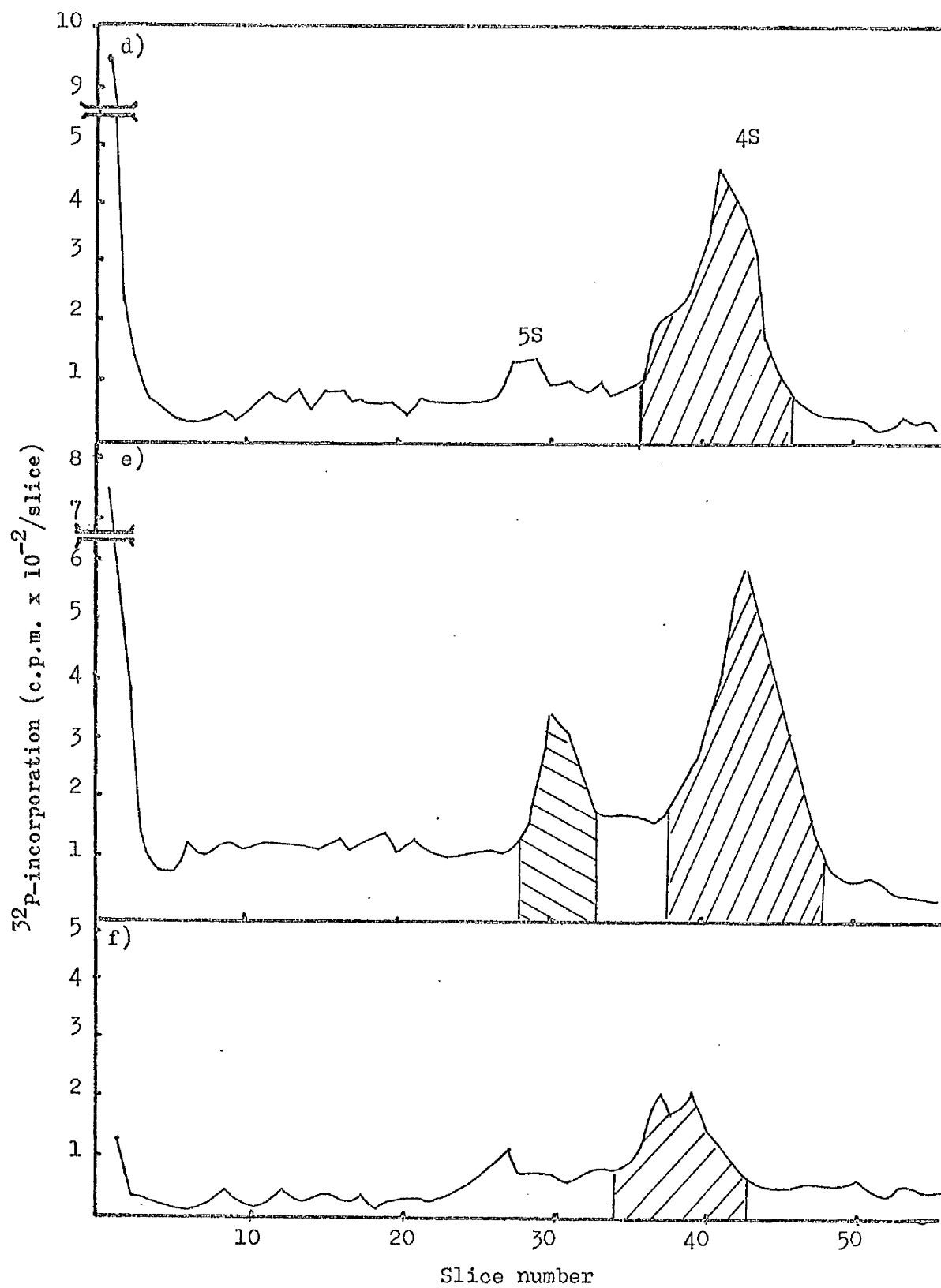


Table 7

Nucleotide composition of 4S RNA and 5S RNA from uninfected and PRV-infected cells

	C	A	G	U	% G + C
Low serum "resting" 4S RNA	27.0	19.4	31.4	22.2	58.4
1-4 hour P.I. 4S RNA	27.4	18.7	31.8	22.1	59.2
4-7 hour P.I. 4S RNA	27.1	18.7	32.1	22.0	59.2
7-10 hour P.I. 4S RNA	27.0	18.9	32.9	21.1	59.9
High serum 4S RNA	27.9	18.9	32.5	20.7	60.4
"Serum back" 4S RNA	27.9	18.6	32.1	21.4	60.0
"Serum back" 5S RNA	24.7	19.3	31.9	24.1	56.6
4-7 hour P.I. 5S RNA	21.3	18.3	38.7	21.7	60.0
4S RNA - by chromatography*	28.3	21.8	31.6	18.3	59.9

³²P-labelled RNA species were eluted from gels as described in Fig. 45 and their nucleotide composition was determined as described in "Methods", II.8. The percentage of each of the four main nucleotides is tabulated.

back" cells (Fig. 45c) and PRV-infected cells at 4 - 7 hours P.I. (Fig. 45e). The nucleotide composition of the eluted RNAs was obtained by KOH hydrolysis and electrophoresis (as described in "Methods", II.8) and the results are shown in Table 7.

There is no significant difference among any of the samples of 4S RNA and the results agree well with those obtained by paper chromatography (see Table) as well as published results (Jarrett et al., 1971) using the same method. However, this does not mean that there are no alterations in the population of 4S RNA species after infection, for the changes would have to be quite dramatic to affect the overall base ratio.

The two sets of 5S RNA results obtained differ slightly; 5S RNA obtained 4 - 7 hours P.I. is more like 4S RNA than is the 5S RNA from the "serum back" cells.

V.2. "Fingerprints" of 4S RNA and 5S RNA in uninfected and PRV-infected cells.

It was decided, in view of the slight change in nucleotide composition of 5S RNA after PRV-infection to examine the sequence of 5S RNA before and after infection by the method of "fingerprinting" T_1 ribonuclease digests of RNA, described by Sanger et al., 1965 ("Methods", II.10). 4S RNA was also examined in view of the postulated changes in the population of tRNA after infection discussed in section V.1.

In order to obtain sufficient quantities of the very highly labelled RNA needed for this technique, a large amount of cells and isotope ($^{32}\text{PO}_4$) were used, as described in the legend to Fig. 46. The labelling period was for 3 hours in the case of the uninfected cells and from 4 - 7

hours P.I. in the case of the PRV-infected cells. The ribosomal pellet was obtained by a 3 hour x 105,000 g centrifugation; the RNA extracted from the cell sap (not shown) contained negligible 5S RNA on 10% gel electrophoretic separation. The low molecular weight ribosome-associated RNA was obtained (as described in the legend to Fig. 46), and electrophoresed on 10% polyacrylamide gels. The absorbancy trace was obtained of both gels, and were identical qualitatively, the only difference being in the amount of RNA present. The trace (from uninfected cells) is shown in Fig. 46a; the ratio of 5S RNA to 4S RNA in the ribosome-associated fraction will be discussed in more detail in relation to Fig. 51a.

The gels were sliced and counted on a gas-flow counter and the results from the uninfected and infected cells are shown in Fig. 46b and c respectively. The 5S RNA slices marked were eluted ("Methods", II.8; the 4 x SSC was removed by dialysis and no commercial 4S RNA was added as the sample must be ≤ 20 μ g RNA for "fingerprinting"). "Fingerprints" were obtained of a T_1 ribonuclease digest of this 5S RNA ("Methods", II.10) and are shown in Fig. 47a and b - uninfected and infected cell 5S RNA respectively. The "fingerprints" obtained lacked the resolution of larger fragments (at the top of the "fingerprint") which is expected from this method; this "smudged" effect was obtained in every one of the several RNA preparations which were "fingerprinted", and is possibly due to a contaminating component of the gel. However, the lower portion of the "fingerprints" (9 spots) is well resolved and, on comparison, the two fingerprints appear identical. 8 of these spots seem to be identical to those from a T_1 ribonuclease digest of 5S RNA from Landschutz ascites cells (Williamson and Brownlee, 1969) and have been numbered as such using the numbering system of Forget and Weissman, 1967b. The 9th resolved spot is very faint in both cases and corresponds to

Figure 46.

10% polyacrylamide gel electrophoretic separation of low
molecular weight ribosome-associated RNA for "finger-printing"

Cl3 cells in 9 Roux bottles were put to rest in EC1 containing 10% phosphate ("Methods", I.1.(ii)) and on the 6th day were infected with PRV at 20 p.f.u./cell. The cells were labelled with 150 μCi $^{32}\text{PO}_4^{\equiv}/\text{ml}$ from 4 - 7 hours P.I. As controls, 4 Roux flasks containing exponentially-growing Cl3 cells in EC10 (10% phosphate) were labelled for 3 hours with 150 μCi $^{32}\text{PO}_4^{\equiv}/\text{ml}$. After labelling the cells were disrupted mechanically ("Methods", II.1.(i)a) and the nuclei, mitochondria and ribosomes were pelleted by centrifugation; the final centrifugation was 105,000 g for 3 hours ("Methods", II.1.(ii)). RNA was prepared from the ribosomal pellet, and the low molecular weight RNA was extracted from it by 1M NaCl ("Methods", II.2.(iii)). This RNA was then electrophoresed on 10% polyacrylamide gels which were scanned at 260 nm on the Gilford (a); the gels were then sliced and the slices counted on a gas flow counter.

The slices indicated by hatching were combined and the RNA was eluted from them without addition of commercial 4S RNA ("Methods", II.7) for "finger-printing".

(b) Uninfected ribosome-associated 5S RNA

(c) PRV-infected ribosome-associated 5S RNA.

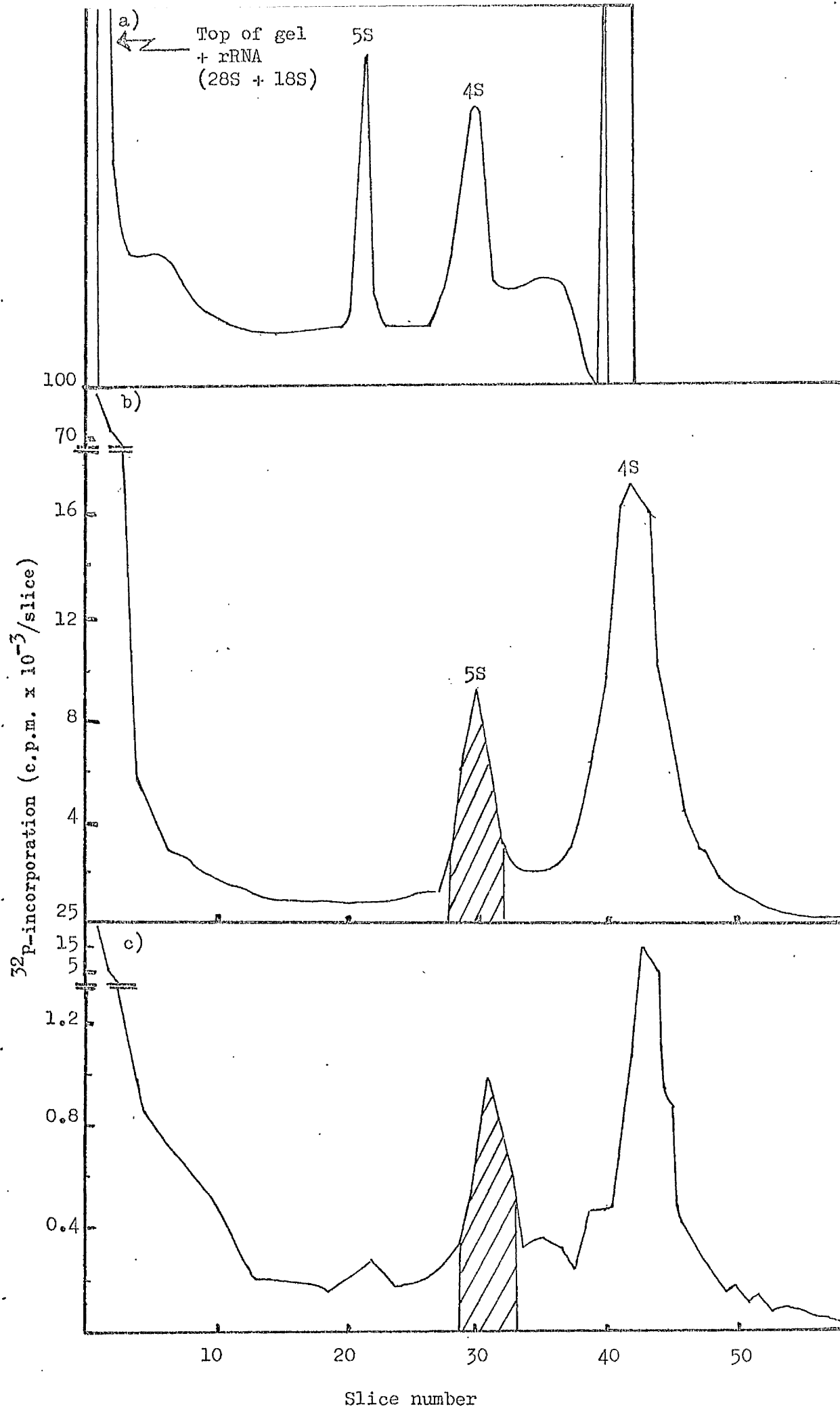


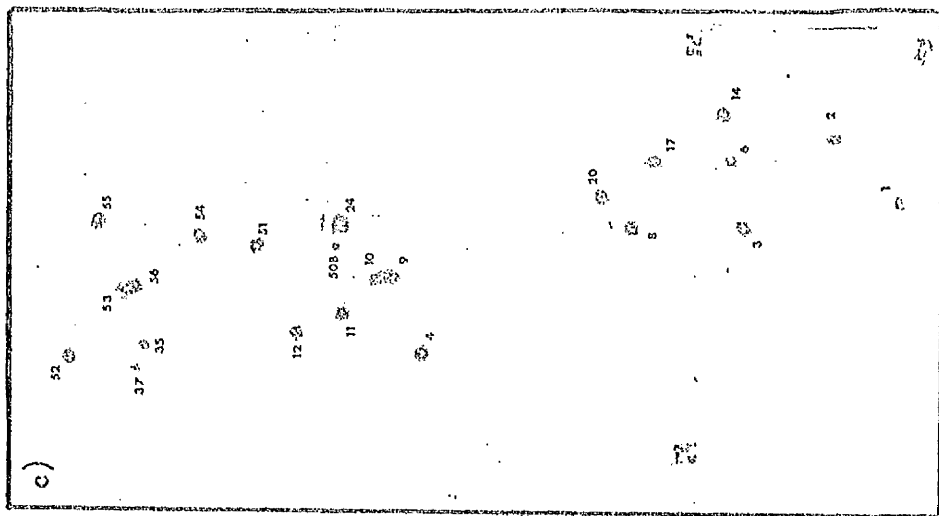
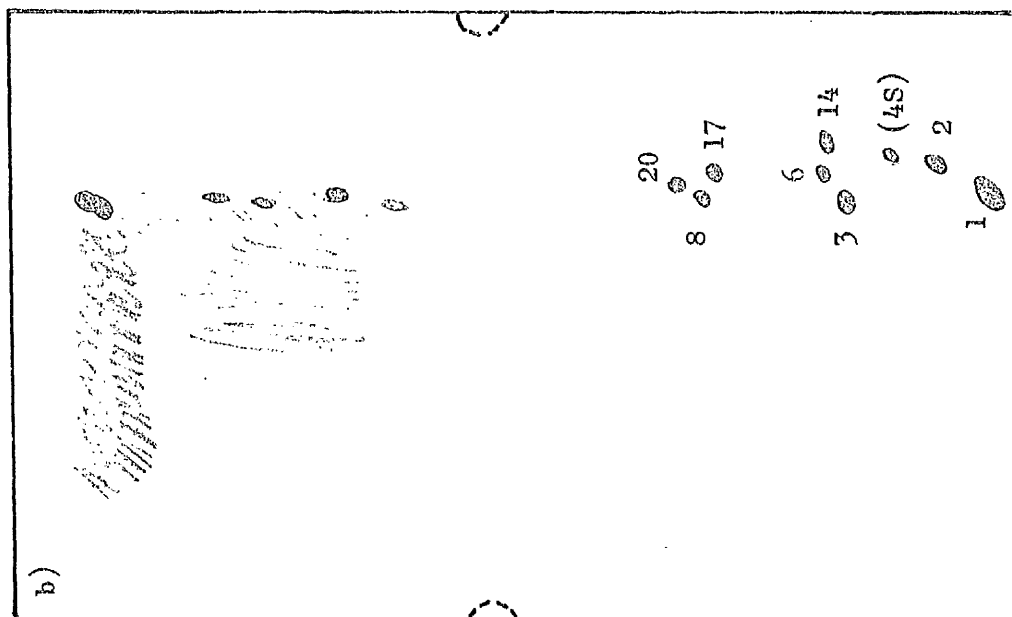
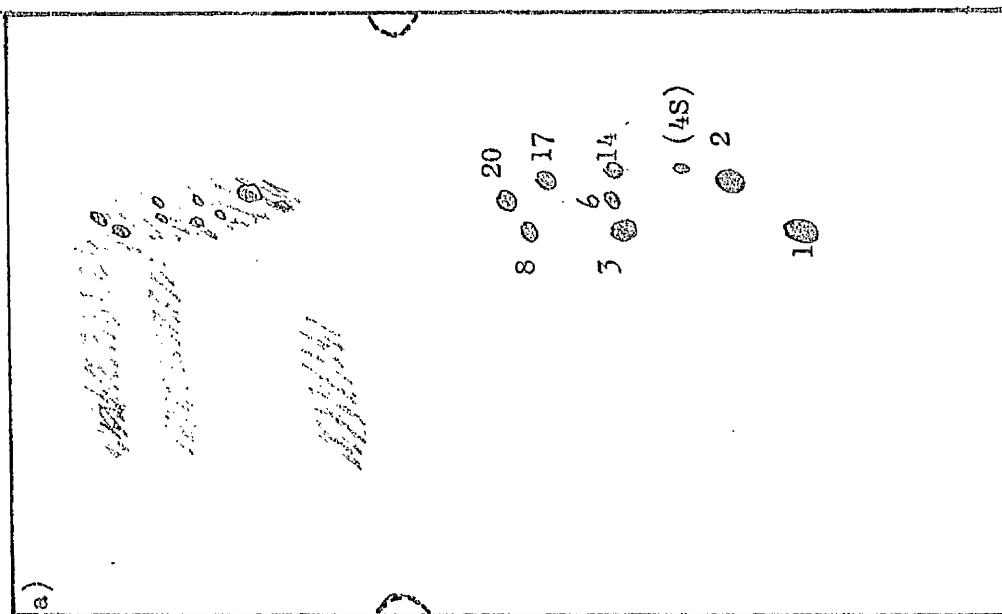
Figure 47.

"Fingerprints" of T_1 digests of 5S RNA in the uninfected and
PRV-infected Cl3 cell

5S ribosome-associated 32 P-RNA samples from (a) uninfected cells and (b) PRV-infected Cl3 cells, prepared as described in Fig. 46, were enzymatically digested with T_1 and "finger-printed" as described in "Methods", II.10. The resulting fingerprints are compared with (c) a fingerprint of 5S RNA from Landschutz ascites cells (Williamson and Brownlee, 1969).

(The position of the marker dye in the second dimension is indicated by ).

← Cellulose acetate pH 3.5



a strong spot in a similar fingerprint of 4S RNA; it therefore is probably due to slight contamination of the 5S RNA with 4S RNA. Thus it appears that ribosome-associated 5S RNA is probably not a different species after PRV-infection.

"Fingerprints" were also made of the ribosome-associated 4S RNA (Fig. 46) and of the 4S RNA from the corresponding cell sap (see legend to Fig. 46). They were all found to be identical, but the heterogeneity of 4S RNA means that all possible spots should be present and hence comparative conclusions from ^{32}P -fingerprints of mixed 4S RNA can not be drawn.

VI. Studies on ribosomal RNA synthesis after PRV-infection.

The effect of PRV on ribosomal RNA synthesis was investigated. This would determine if the effect on 5S RNA synthesis is the same as the effect on the larger rRNA species and would act as a useful comparison for 4S RNA synthesis.

VI.1. Appearance of rRNA in the cell cytoplasm.

Short labelling periods mostly of 30 min and 45 min have so far been used to study the production of 4S RNA and 5S RNA. However, as discussed in "Introduction", III.2.(ii), the appearance of label in cytoplasmic RNA is slow, taking about 20 - 30 min for cytoplasmic 18S RNA to become labelled and about 1 hour for cytoplasmic 28S RNA to become labelled in HeLa cells (Penman et al., 1966; Penman, 1966); the kinetics of appearance of ribosomal RNA in Cl3 cells has not been reported and it was decided to check briefly if short labels such as were used to study 4S and 5S RNA would be suitable for labelling ribosomal RNA, or if longer labels would be necessary.

Thus two Roux flasks, one containing exponentially-growing and one containing "resting" cells were pulse-labelled with ^3H -uridine for 30 min, and cytoplasmic ribosomal RNA was prepared from the ribosomal pellet ("Methods", II.2.(iii)). Since the specific activity of the rRNA was very low it was decided to fractionate these samples on 5 - 20% sucrose gradients (Britten and Roberts, 1960), as described in "Methods", II.3.(iii), as these gradients have a large capacity. The results of this experiment are shown in Fig. 48.

Three absorbancy peaks are shown, designated as 28S, 18S and low molecular weight RNA (4S + 5S RNA). In neither the exponentially-growing nor in the "resting" cells is there a 28S RNA peak of radioactivity, but there is a significant 18S peak. This suggests that the kinetics of maturation of rRNA in Cl3 cells is similar to that of HeLa cells and that pulse-labelling periods of greater than 1 hour must be used to get both cytoplasmic high molecular weight rRNA species labelled, and three or four hour labels were used thereafter. Fig. 48 also demonstrates the decrease in rRNA synthesis in "resting" cells, and this will be investigated more fully later.

VI.2. Calibration of a 2.5% polyacrylamide gel system.

Since the problem of low specific activity rRNA was not expected to arise again because of the longer labels to be employed, it was decided to use a polyacrylamide gel system to separate the rRNA. This does not have the capacity of the sucrose gradients, but it was shown to give better separation of the RNA species - especially between 18S RNA and the low molecular weight peak (4S + 5S RNA).

Figure 48.

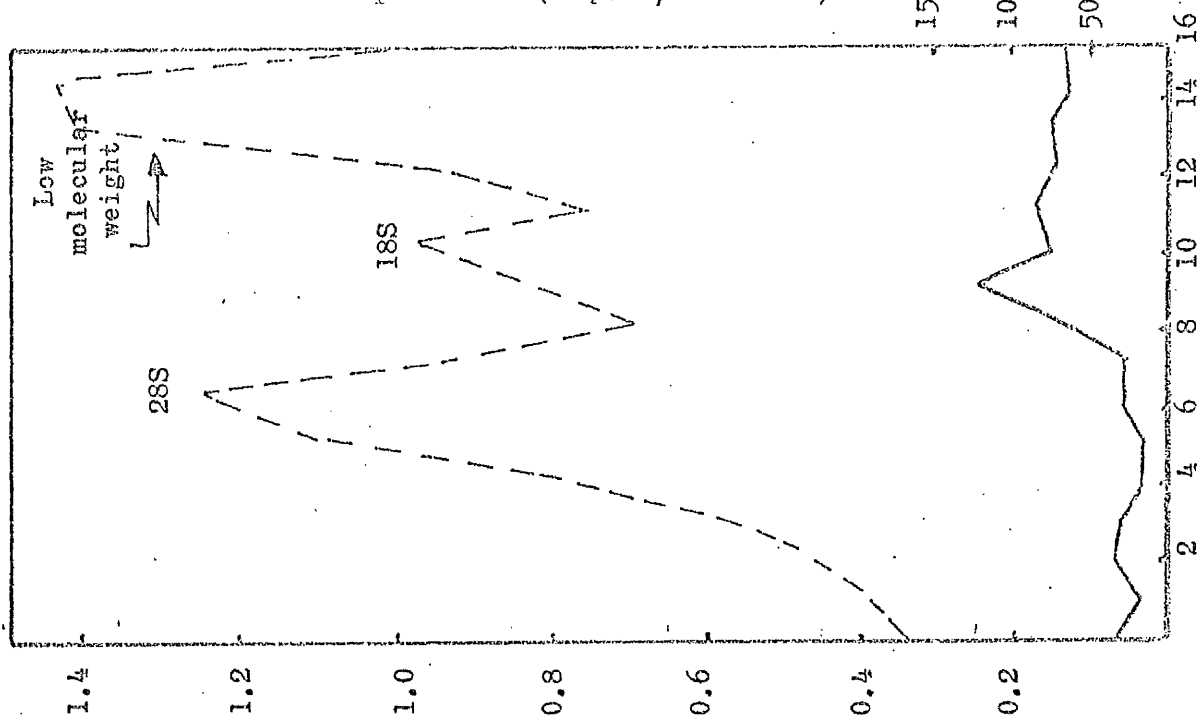
Sucrose gradients of cytoplasmic ribosomal RNA pulse-labelled
for 30 mins with ^3H -uridine

- a) Nearly-confluent Cl3 cells in a Roux bottle were labelled for 30 min with 12 $\mu\text{Ci/ml}$ of ^3H -uridine.
- (b) "Resting" Cl3 cells ("Methods", I.1.(ii)) in a Roux bottle were used on the sixth day of "resting" and were labelled for 30 min with 12 $\mu\text{Ci/ml}$ of ^3H -uridine.

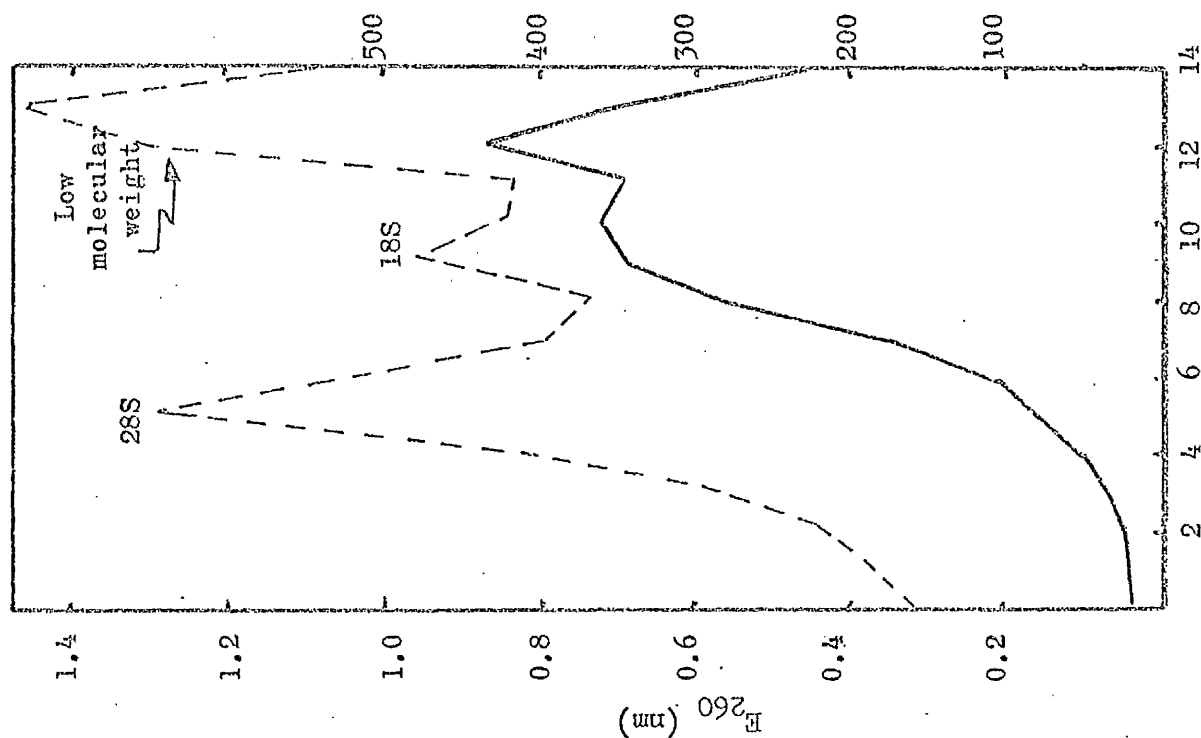
The cells from a) and b) were harvested and the ribosomal pellets were prepared by homogenisation of the cells and differential centrifugation ("Methods", II.1.(ii)). RNA was extracted from these pellets ("Methods", II.2.(iii)) and the total RNA obtained was fractionated (along with 120 μg of unlabelled "cold-phenol" extracted cytoplasmic RNA) on 4 ml 5 - 20% sucrose gradients as described in "Methods", II.3.(iii), and the absorbancy of each fraction at 260 nm and the d.p.m. in each fraction were obtained as described.

— — — — —, absorbancy at 260 nm
—————, ^3H -d.p.m. from ^3H -uridine.

³H- uridine incorporation (d.p.m./fraction)



Fraction number



E_{260} (nm)

170

The gel system used was the 2.5% polyacrylamide gel ("Methods", II. 3.(ii)). The absorbancy trace of fractionation of ribosomal RNA on these gels is shown in Fig. 49a, and the radioactive profile obtained by fractionation of the ^{14}C -internal marker on these gels is in Fig. 49b. Good separation between 28S and 18S RNA species is achieved, and the low molecular weight species (4S RNA and 5S RNA) co-electrophorese but are well-separated from 18S RNA; the peaks were identified by reference to Loening, 1967.

VI.3. Ribosomal RNA synthesis after PRV-infection in exponentially-growing cells.

To determine as exactly as possible the effect of the virus on rRNA synthesis, infected and uninfected cells were labelled with ^3H -uridine and ^{14}C -uridine respectively. This was found difficult for examination of the low molecular weight RNA because of the short pulse-labelling times necessary (section II.1.(ii)). However, to examine cytoplasmic ribosomal RNA long labelling times are not only possible but necessary because of the relatively slow processing of the rRNA.

Thus exponentially-growing cells were mock-infected or infected with pseudorabies virus. ^{14}C -uridine was used to pulse-label the uninfected cells and ^3H -uridine was used to label the infected cells from 2 - 6 hours P.I. Unlabelled uridine was added to the medium containing ^3H -uridine to bring the level of uridine up to that of the medium containing ^{14}C -uridine so that in both cases the molarity was the same ($3.3 \mu\text{molar}$); at this level slight differences in molarity of uridine in the medium do not affect incorporation of radioisotope (Fig.10). The number of curies of ^3H -uridine used was nine times the number of curies of ^{14}C used; this was arranged partly because the efficiency of counting ^3H is much less than that of ^{14}C ,

Figure 49a

Absorbancy trace at 260 nm of a 2.5% polyacrylamide gel
electrophoretic separation of
ribosomal RNA

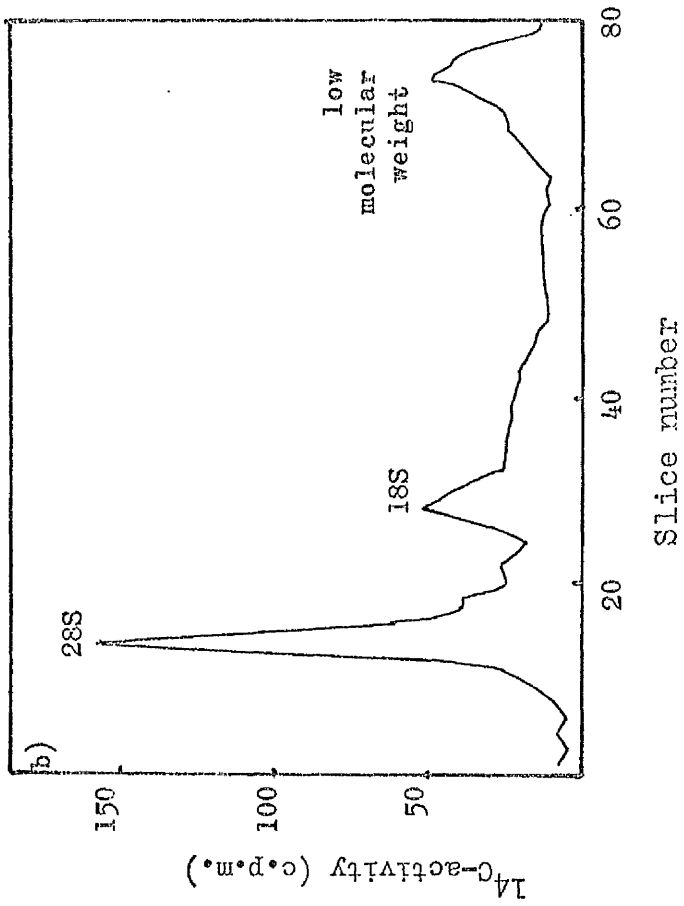
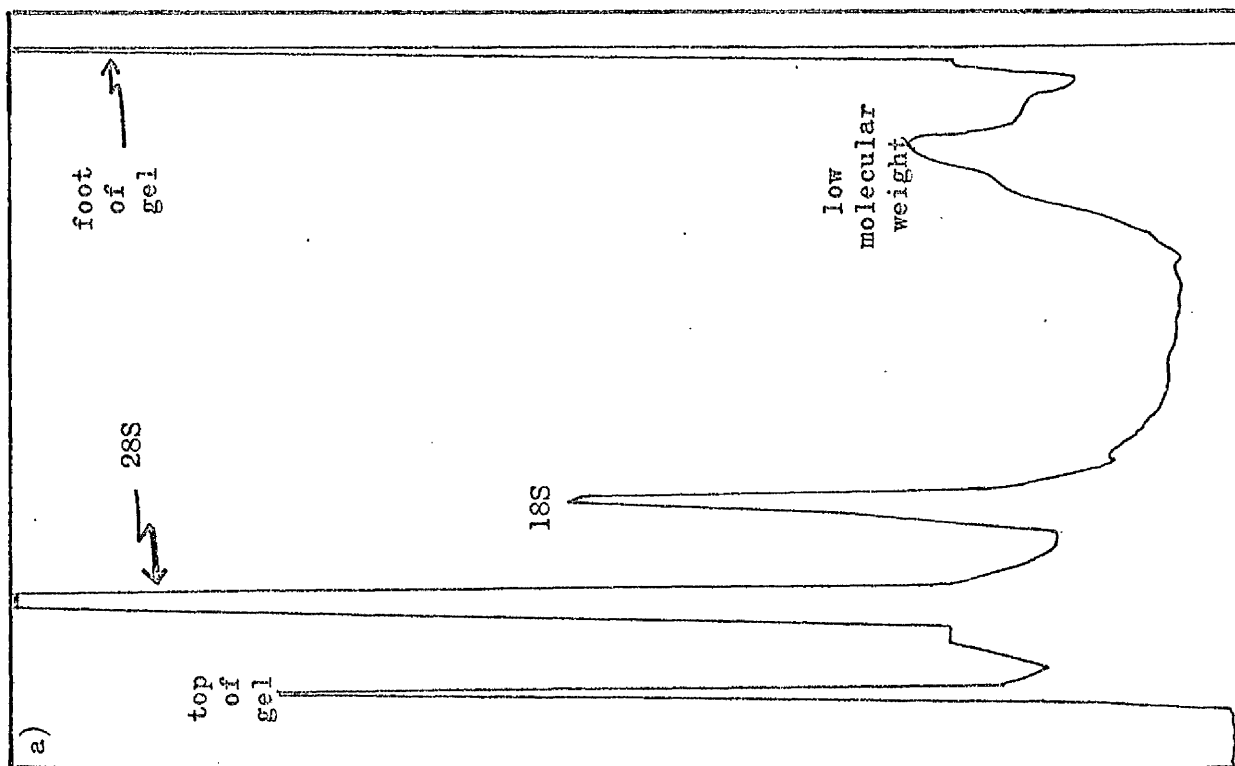
Ribosomal RNA was prepared by phenol-extraction of the ribosomal fraction of Cl3 cells ("Methods", II.2.(iii)) and was electrophoresed on a 2.5% polyacrylamide gel ("Methods", II.3.(ii)). The absorbancy trace at 260 nm was obtained using a Gilford model 240 spectrophotometer with a linear transport attachment (model 2410).

Figure 49b

¹⁴C-labelled cytoplasmic RNA electrophoresed on a 2.5% polyacrylamide gel

40 µl of the ¹⁴C-labelled cytoplasmic marker RNA, prepared as described in Fig. 15, was electrophoresed on a 2.5% polyacrylamide gel, which was sliced and counted on a gas flow counter ("Methods", II.3.(ii)).

Figure 49



and also because of the expected decrease in incorporation of radioisotope in the virus-infected cells.

After the four-hour pulse-label period the infected and uninfected cells were harvested together, the ribosomal RNA pellet was prepared and the ribosomal RNA was extracted ("Methods", II.2.(iii)) and electrophoresed on a 2.5% gel. The result is shown in Fig. 50. The ratios of 28S to 18S RNA are very similar in the infected and uninfected cells (1.2 and 1.5 respectively), but the ratio of either of the larger rRNA species to the low molecular weight RNA is very different in the infected and the uninfected cell. It is clear that there is relatively much more low molecular weight RNA being synthesised in the infected than in the uninfected cell. However, when the 9-fold excess of d.p.m. added to the infected cell has been accounted for, there is still an absolute decrease in the low molecular weight synthesis after infection. In Table 8 the incorporation into each of these species is tabulated. 28S and 18S RNA synthesis in the infected cell is strongly decreased to a very similar extent (to 16% and 19% respectively). The difference in decrease between 28S and 18S RNA is very slight; a similar degree of inhibition for these two species is expected since they have a common precursor (45S RNA). The low molecular weight RNA from the ribosomal pellet consists of 5S:4S in the molecular ratio of 1:2. The decrease in synthesis of this ribosome-associated low molecular weight RNA after infection is much less than the ribosomal RNA (to 34%), and is of the same order as previously noted for 4S RNA and less than that previously noted for 5S RNA (e.g. Table 4). Thus the decrease in the synthesis of the larger rRNAs P.I. is greater than the decrease in synthesis of low molecular weight RNA.

Figure 50.

2.5% polyacrylamide gel of ribosomal RNA from exponentially-
growing uninfected and PRV-infected cells

Nearly-confluent burlers of Cl3 cells were infected or mock-infected with PRV. From 2 - 6 hours P.I. the infected cells were labelled with 1.8 $\mu\text{Ci/ml}$ of ^3H -uridine while the mock-infected cells were labelled with 0.2 $\mu\text{Ci/ml}$ of ^{14}C -uridine; the concentration of uridine in the medium was 3.3 μM in both cases. The cells were harvested together, mechanically disrupted and after pelleting nuclei and mitochondria, the ribosomes were precipitated by a 16 hour x 105,000 g centrifugation. ("Methods", II.1.(ii)). (The cell sap fraction was retained and analysed as described in Fig. 52). Ribosomal RNA was extracted from the ribosomal pellet ("Methods", II.2.(iii)) and was electrophoresed on a 2.5% polyacrylamide gel; the gel was scanned at 260 nm in a Gilford spectrophotometer, sliced and counted by liquid scintillation spectrometry as described in "Methods", II.3.(ii).

—————, ^3H -d.p.m. from ^3H -uridine (PRV-infected cells)
-----, ^{14}C -d.p.m. from ^{14}C -uridine (mock-infected cells)

Figure 50

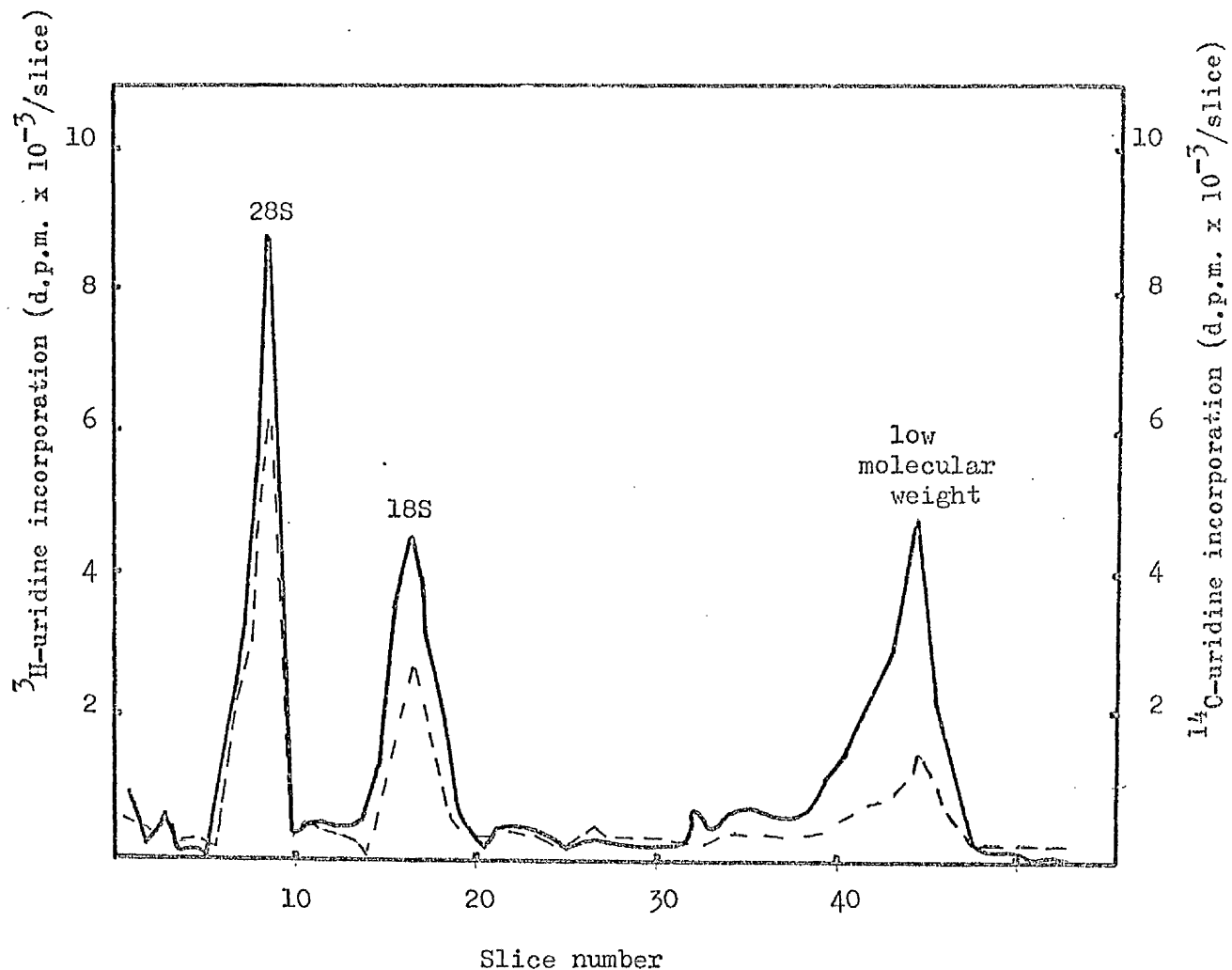


Table 8

Incorporation of labelled uridine into ribosomal RNA species
in uninfected and PRV-infected cells

	Uninfected cell (^{14}C -d.p.m.)	Infected cell (^3H -d.p.m.)	Infected cell incor- poration as percentage of uninfected cell x
28S	10,900	15,300	16
18S	7,500	12,800	19
Low molecular weight	6,700	20,800	34

Data taken from Fig. 50.

(x Corrected for 9-fold excess curies added to infected cell medium --
 see legend to Fig. 50).

To investigate this further, a portion of the sample from Fig. 50 was electrophoresed on 10% polyacrylamide gels to obtain the separation of the 5S and 4S RNA species. The trace of absorbancy at 260 nm is shown in Fig. 51a. Since there are two 4S RNA molecules each about 80 nucleotides long to every 5S RNA molecule 120 nucleotides long, then the expected ratio of the optical densities of 4S RNA to 5S RNA is 1.3; this is in fact the ratio obtained here on comparison of the areas under the peaks. Note that the 4S RNA peak, due to its heterogeneity, is wider than the 5S RNA peak. The high background across the gel is due to the large amount of ribosomal RNA necessary to have sufficient low molecular weight species to be able to obtain an absorbancy trace. The radioactive gel profile is shown in Fig. 51b. The calculation of the activity in each peak is shown in Table 9, although these calculations must be treated with reservation because of the high background across the gel. However, the decrease in 4S RNA synthesis after infection is about 36% while the decrease in 5S RNA synthesis is only about 54%; note that in this case 4S RNA is almost certainly tRNA since it is ribosome-associated and similarly the 5S RNA is ribosomal 5S RNA. These results are extremely similar to the decrease found in 4S and 5S RNA synthesis after infection in cytoplasmic RNA (e.g. Table 4).

In the above experiment, the effect of PRV-infection on the production of the larger ribosomal species and on the smaller ribosomal species was examined (Figs. 50 and 51 respectively). We considered that even although we had already accumulated a considerable amount of data on the effect of PRV-infection on cell sap RNA synthesis, it would be worthwhile to examine the cell sap RNA obtained from the above experiment to allow a more valid comparison of relative rates of synthesis of all cytoplasmic RNA species. An especial point of comparison is the rate of synthesis of

Figure 51

10% polyacrylamide gels of low molecular weight ribosomal RNA

Ribosomal RNA from mock-infected or PRV-infected C13 cells, prepared as described in Fig. 50, was electrophoresed on a 10% polyacrylamide ("Methods", II.3.(ii)). The gel was scanned at 260 nm using a Gilford model 240 spectrophotometer and the trace shown in (a) was obtained. The gel was then sliced and counted by liquid scintillation spectrometry (b).

—————, ^3H d.p.m. from ^3H -uridine (PRV-infected cells)
—————, ^{14}C d.p.m. from ^{14}C -uridine (mock-infected cells).

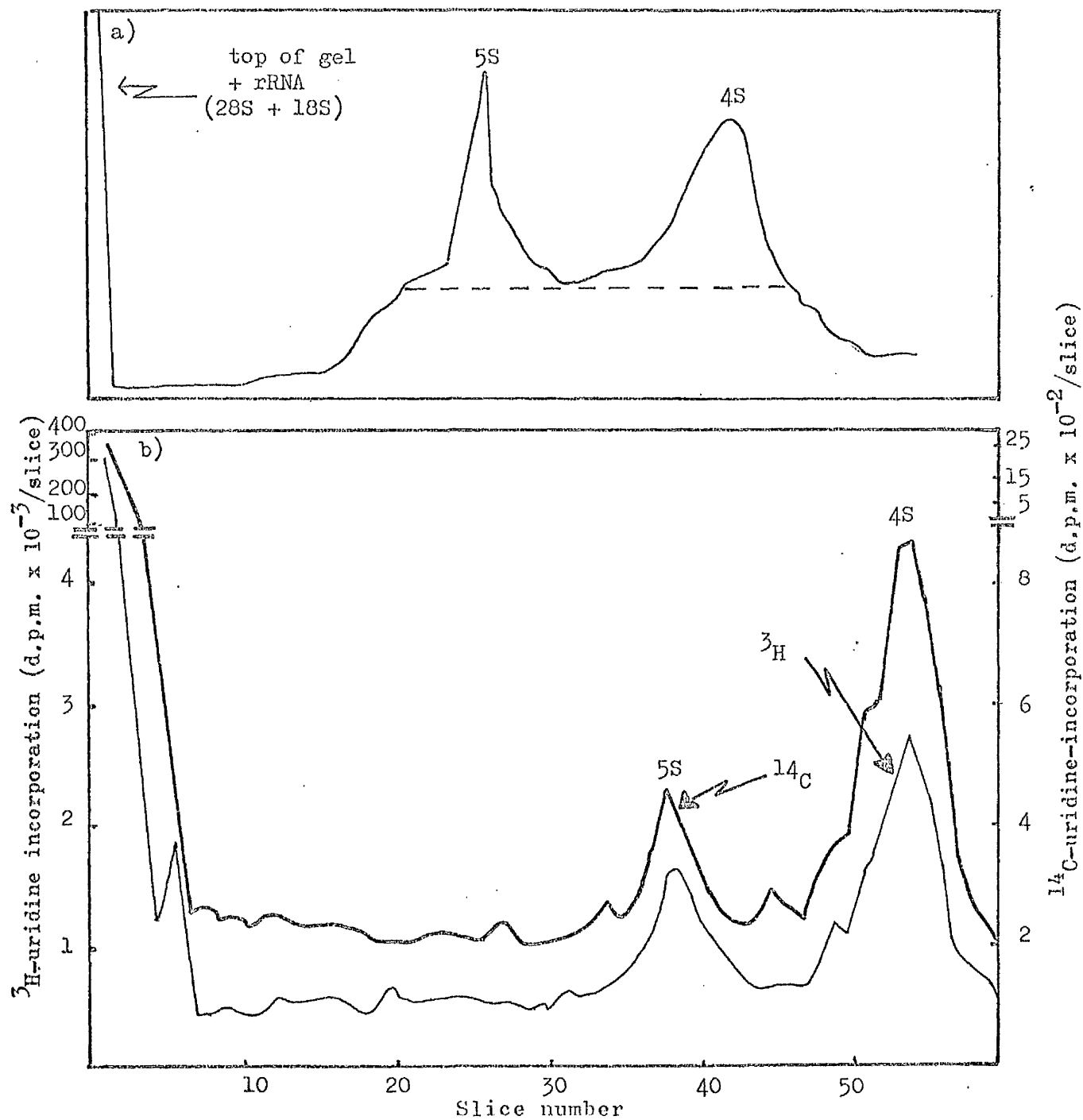


Table 9

Incorporation of labelled uridine into low molecular weight
ribosome-associated RNA species in uninfected and PRV-infected cells

	Uninfected cell (^{14}C -d.p.m.)	Infected cell (^3H -d.p.m.)	Infected cell incorporation as percentage of uninfected cell *
5S	740	3,600	54
4S	3,580	11,700	36

Data taken from Fig. 51b.

(* Corrected for 9-fold excess curies added to infected cell medium --
see legend to Fig. 50).

ribosome-associated 4S RNA and cell sap 4S RNA, before and after PRV-infection.

Thus the cell sap before and after PRV-infection was prepared as described in the legend to Fig. 50, and the RNA was extracted and electrophoresed on a 10% polyacrylamide gel. The resulting radioactive profile is shown in Fig. 52. In this case there is no ribosomal RNA or 5S RNA in the cell sap fraction; this is because a 16 hour x 105,000 g centrifugation was used to precipitate the ribosomes. This was possible as the pulse-label period had been too long to label unstable precursor species and hence the care shown previously to prevent maturation was unnecessary (see P. 117). In the infected cell sap there is a small amount of heterodisperse material >5S. The d.p.m. incorporated into the cell sap 4S RNA is shown in Table 10, and indicates that cell sap RNA decreases to 35% after infection.

VI.4. Ribosomal RNA synthesis after PRV-infection in "resting" cells.

The relative effects of infection on ribosomal RNA, 4S RNA and 5S RNA in "resting" cells was examined. Comparison was also made between exponentially-growing cells, "resting" cells and "resting" cells to which serum had been replaced 48 hours previously. ³H-uridine was used to pulse-label all the samples; a 3 hour pulse-label was used throughout, and in the infected cells two labelling periods were used, 1 - 4 hours and 4 - 7 hours after infection. The ribosomal pellet was precipitated by centrifugation and the RNA extracted from it was electrophoresed on 10% polyacrylamide gels to obtain separation of the ribosome-associated 5S and 4S RNA species (Fig. 53). In order to obtain a sufficient number of counts in the low molecular weight species, the gels were heavily overloaded; hence there

Figure 52.

10% polyacrylamide gel of cell sap RNA from exponentially-
growing mock-infected and PRV-infected cells.

Cell sap RNA from mock-infected and PRV-infected cells was prepared by phenol-extraction of the cell sap fraction described in Fig. 50, and was electrophoresed on a 10% polyacrylamide gel, which was sliced and counted by liquid scintillation spectrometry ("Methods", II.3. (ii)).

—————, ^3H d.p.m. from ^3H -uridine (PRV-infected cells)
-----, ^{14}C d.p.m. from ^{14}C -uridine (mock-infected cells).

Figure 52

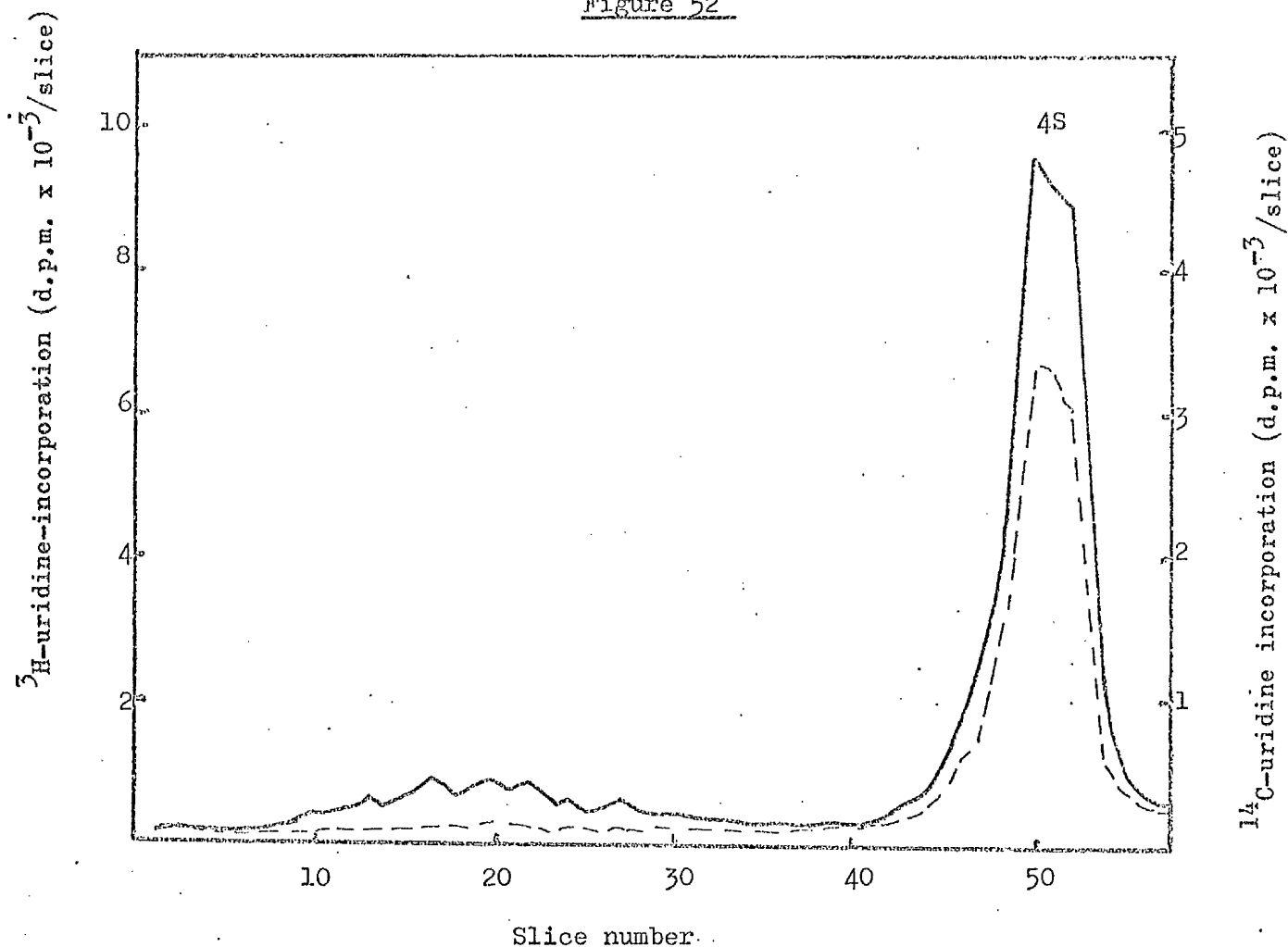


Table 10

Incorporation of labelled uridine into cell sap 4S RNA in
uninfected and PRV-infected cells

	Uninfected cell ^{14}C -d.p.m.	Infected cell ^3H -d.p.m.	Infected cell incorporation as percentage of uninfected cell*
4S	15,650	49,300	35%

Data taken from Fig. 52.

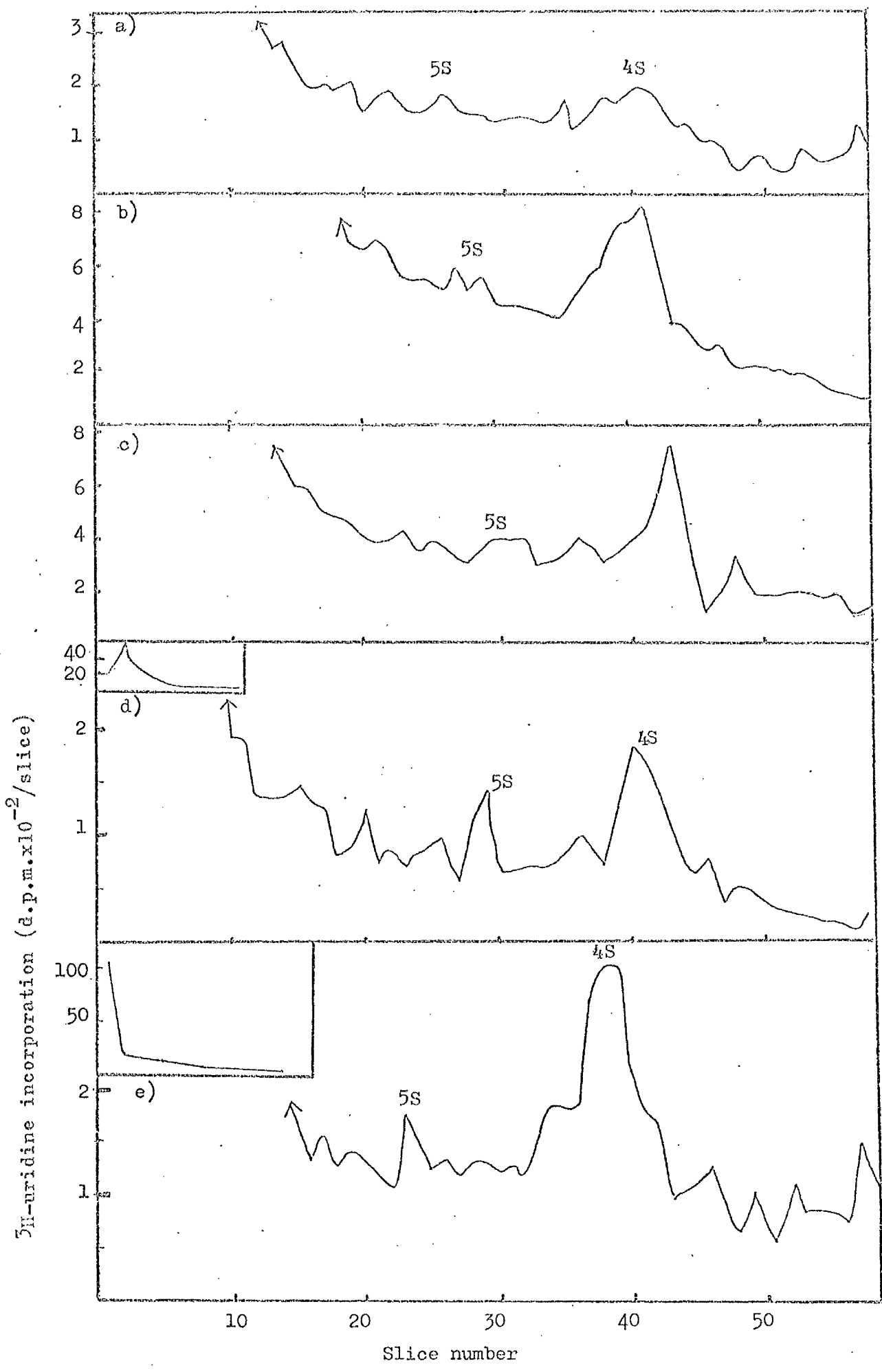
(* Corrected for 9-fold excess curies added to infected cell medium—
see legend to Fig. 50).

Figure 53.

Ribosomal RNA from uninfected and PRV-infected cells electrophoresed
on 10% polyacrylamide gels

Uninfected "resting" (a), "high serum" (b) and "serum back" (c) "control" cells were obtained as described in Fig. 42, and were labelled for 3 hour periods with 4 μ Ci/ml of ^3H -uridine. "Resting" cells were also infected with PRV and labelled for 3 hour periods: (d) 1 - 4 hours and (e) 4 - 7 hours P.I.

At the end of the labelling period the cells were harvested, mechanically disrupted and subjected to differential centrifugation to obtain the ribosomal pellet, from which the ribosomal RNA was extracted ("Methods", II.2.(iii)). Aliquots of this RNA were electrophoresed on 10% polyacrylamide gels which were sliced and counted by liquid scintillation spectrometry ("Methods", II.3.(ii)).



is a high background which makes a few of the 5S RNA peaks difficult to quantitate precisely. Once again it can be seen that the incorporation of labelled precursor into the "serum back" RNA is higher than into the exponentially-growing cells -- probably once more due to the larger number of cells. The level of 4S RNA synthesis in the "resting" cells is about 25 - 30% of the exponentially-growing level, but on infection this level does not fall further and may even rise. This agrees with results for infected cell sap 4S RNA in "resting" cells (Figs. 42 - 45). 5S RNA synthesis seems less affected on going to "rest" than is 4S RNA synthesis; its "resting" level is about 50% of its exponentially-growing level, and on infection 5S RNA synthesis does not decrease very much further.

Although 4S and 5S RNA are resolved on 10% gels, the synthesis of the larger ribosomal RNAs can not be measured on these gels. Hence, the ribosomal RNA samples used in the above 10% gels were electrophoresed on 2.5% polyacrylamide gels to resolve 28S and 18S RNAs. The radioactive profiles obtained are given in Fig. 54, and the counts in each RNA species were calculated and are tabulated in Table 11. Note that in these gels there is a relatively high background across the gel and the low molecular weight RNA is present in such small amounts in these "resting" cells that calculation of total counts, especially the low molecular weight RNA, is liable to error. The significant findings are the following:

The synthesis of 28S and 18S RNA in resting cells is about 40% of the level in exponentially-growing cells. The level of rRNA synthesis in the "serum back" cells is about 1.5 times that of the exponentially-growing level, probably due to the increase in cell numbers. Infection of the "resting" cells causes a further decrease in the level of 28S and 18S RNA

Figure 54.

Ribosomal RNA from uninfected and PRV-infected cells electrophoresed
on 2.5% polyacrylamide gels

Aliquots of the ribosomal RNA fractions prepared as described in Fig. 53 were electrophoresed on 10% polyacrylamide gels, which were sliced and counted by liquid scintillation spectrometry ("Methods", II. 3.(ii)).

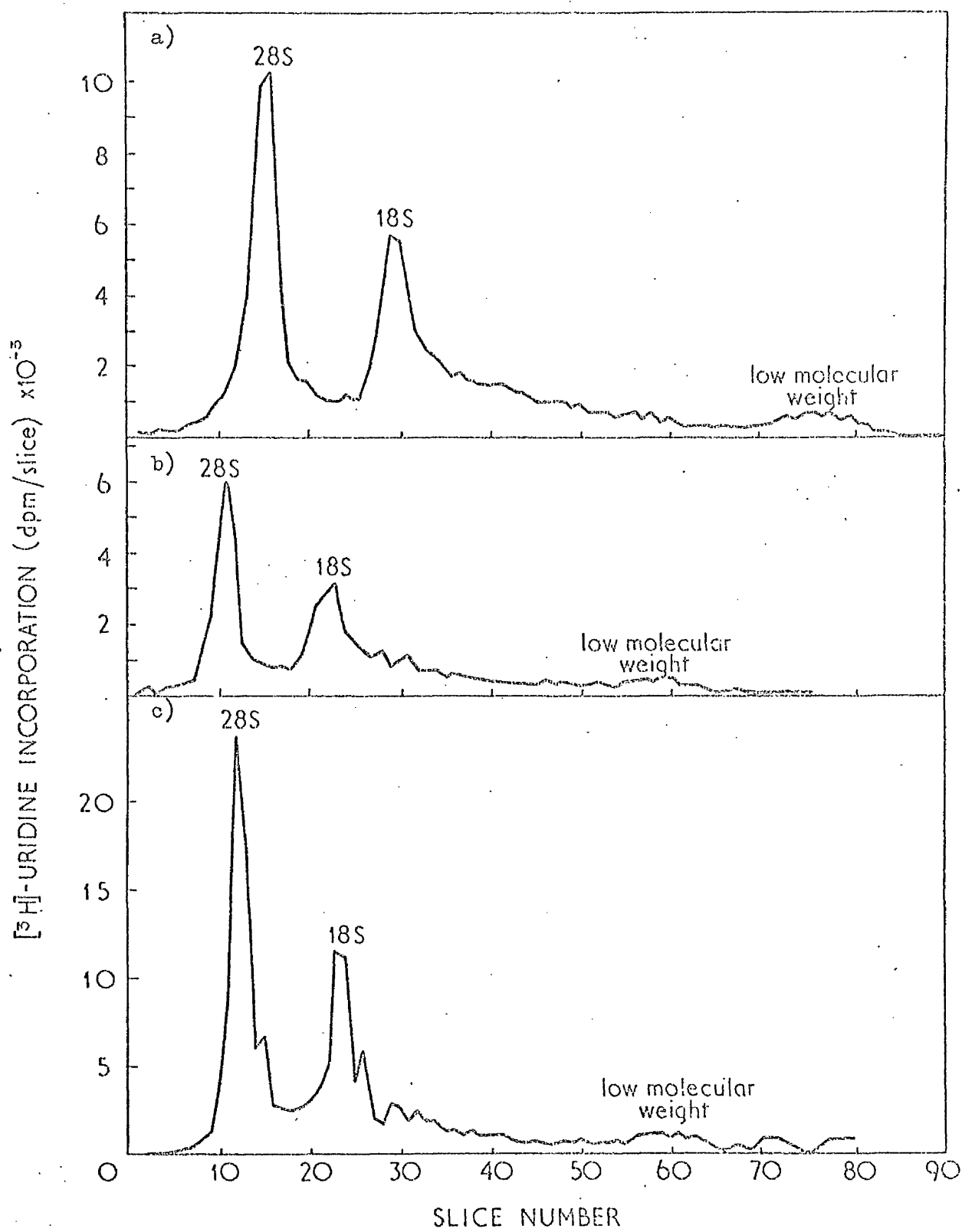


Fig. 54, d-e: overleaf

Figure 54 (continued)

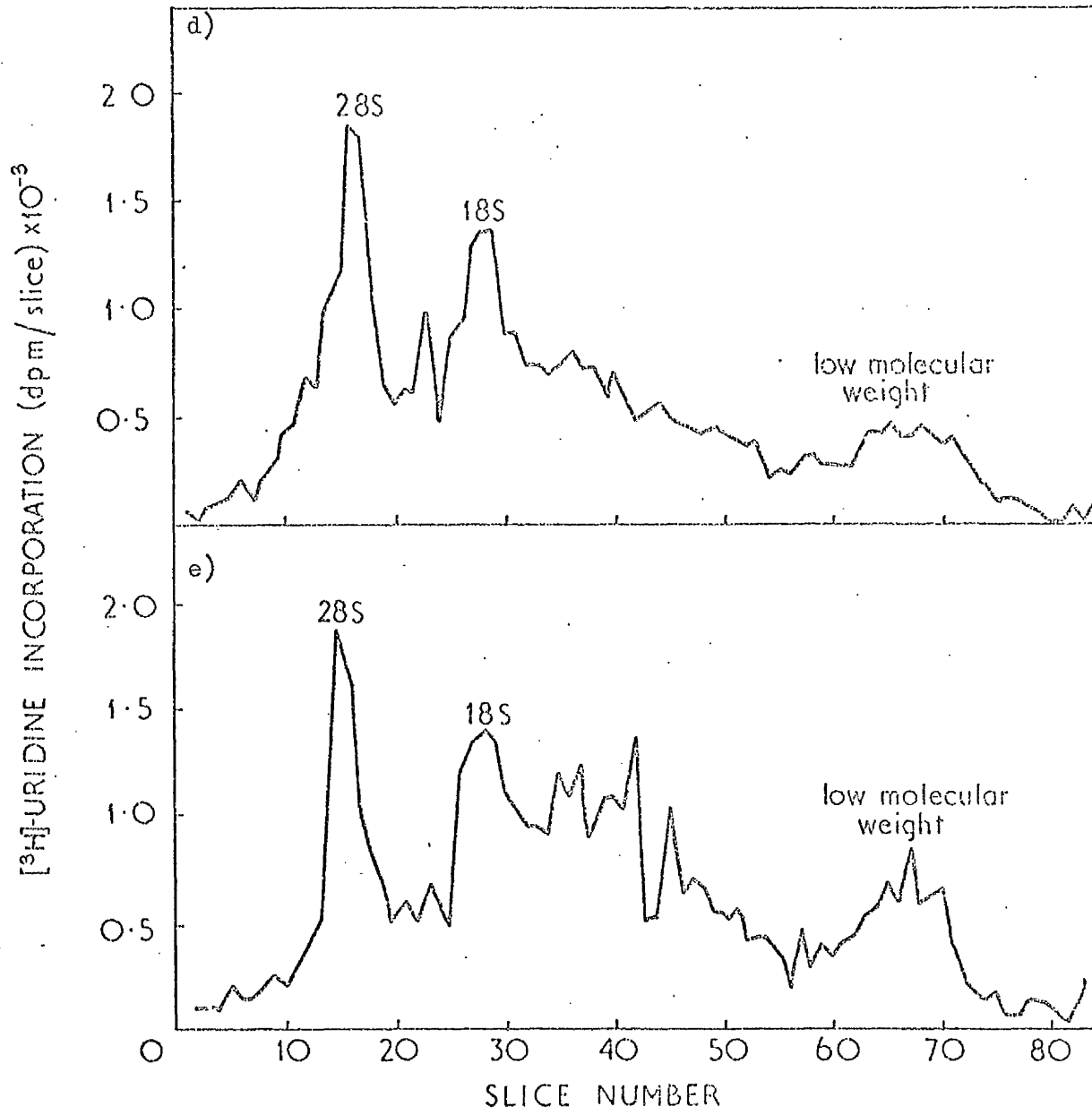


Table 11

Incorporation of ^3H -uridine into ribosomal RNA species into PRV-infected "resting" cells
and into uninfected cells during various growth conditions

	Uninfected exponentially- growing (d.p.m.)	Uninfected "serum back" (d.p.m.)	Uninfected "resting" (d.p.m.)	1-4 hours P.I. (d.p.m.)	4-7 hours P.I. (d.p.m.)
28S	41,700	72,300	16,900	8,830	7,170
18S	30,600	44,800	15,500	8,260	8,490
Low molecular weight	6,900	11,300	4,900	4,490	7,160

Data taken from Fig. 54.

synthesis and the level of 28S RNA synthesis reaches a level of 42% of the "resting" level, and 17% of the exponentially-growing level, at 4 - 7 hours P.I. The level of decrease in the synthesis of 18S RNA after infection is impossible to calculate with any degree of accuracy because of the appearance of a large amount of heterodisperse RNA electrophoresing between 18S RNA and the low molecular weight RNA; thus the apparent incorporation of ^3H -uridine into 18S RNA is artificially elevated. The "resting" cell levels of low molecular weight RNA do not seem to decrease and may be increased after PRV-infection, in agreement with the results obtained when ribosomal RNA was fractionated on 10% polyacrylamide gels (Fig. 53).

An interesting correlation can be made between ribosome-associated 5S RNA synthesis and the synthesis of the larger ribosomal RNAs. Calculation from Table 11 shows that 28S + 18S RNA synthesis in "resting" cells is 45% of the exponentially-growing level, and this level decreases a further 50% after PRV-infection, whereas 5S RNA synthesis (calculated from Fig. 53) has a "resting" level of about 50% of its exponential level but shows no further decrease on PRV-infection. Thus 5S RNA synthesis seems to decrease in step with the larger ribosomal RNAs on going to "rest", but on infection this correlation does not hold, 28S and 18S RNA synthesis being much more decreased than is 5S RNA synthesis.

DISCUSSION

I. Incorporation of ^3H -uridine into total cell RNA

Conditions for optimal incorporation of ^3H -uridine into total cell RNA were first determined. It was found that a medium concentration of 1.0 - 6.0 μM uridine gives maximal incorporation of ^3H -uridine into RNA and that ^{14}C -uridine behaves similarly (Fig. 10). Above the level of 6.0 μM there is a strong decrease in incorporation, probably simply due to dilution of the radioisotope; below the level of 1.0 μM there is a slight decrease in incorporation and this is probably due to a minimum medium concentration of uridine being necessary to facilitate its own transport into the cell.

It was also found that at the "plateau" level of 1.0 - 6.0 μM uridine, the effect of intracellular pool size is negligible compared to the concentration of uridine in the medium (Table 3).

Uptake of ^3H -uridine into PRV-infected cells was then studied and it was found that PRV-infection of exponentially-growing cells causes a steep decrease in total RNA synthesis (Fig. 11). As already discussed ("Introduction", IV. 2.(ii)), this effect has been reported by Kaplan & Ben-Porat, 1960, and very recently by a report from the same laboratory (Rakusanova et al., 1971), and is in agreement with data for HSV-I (Roizman et al., 1965; Hay et al., 1966; Flanagan, 1967). The decrease in RNA synthesis in PRV-infected cells is not due to changes in the intracellular pool of uridine and its derivatives (Rakusanova et al., 1971).

II. The "resting" cell system

In several of the experiments described in this report a "resting"

cell system was utilised as it was considered that the depression of host RNA synthesis which occurs in these cells would allow the positive effects of the virus on RNA synthesis to be seen more clearly. Cl3 cells are not strongly contact-inhibited, and the serum-depletion system used was described. Several investigators have reported experiments with "resting" cells which are contact-inhibited; in many ways these two types of "resting" cells (obtained by contact-inhibition and serum-depletion) are similar — e.g. Clarke et al., 1970 showed that even Cl3 cells exhibit density-dependent inhibition in low concentrations of serum; also fresh serum can reverse the effects of inhibition in contact-inhibited 3T3 cells (e.g. Cunningham & Pardee, 1969; Emerson, 1971).

The low levels of DNA synthesis characteristic of the "resting" system were observed (Fig. 40); the low enzyme levels in this serum-depleted system and the increase in enzyme activity which occurs after restoring serum have been reported by Howard et al., 1972.

Since this Cl3 cell system had not been used for infection by herpesviruses, the ability of PRV to infect these cells, and the yield of PRV produced was examined; such studies, using autoradiographic techniques, have confirmed that the level of PRV used (20 p.f.u./cell) results in 100% infection by 5 hours P.I. ("Results", IV.1 and IV.2).

Care must be taken in comparison of radioactivity incorporated into "resting" and exponentially-growing cells because of possible variation in the size of the nucleotide pools and of variation in the rate of transport of nucleotides. Contact-inhibited cells transport exogenously-labelled nucleosides and phosphate more slowly than do rapidly-growing cells, suggesting that rate of incorporation of isotopically-labelled uridine or

phosphate into total cellular RNA may be a poor measure of the absolute rate of RNA synthesis by these cells (Cunningham & Pardee, 1969; Weber & Rubin, 1971). Thus, too much emphasis is not laid on the measured decrease of incorporation of radioisotope into RNA in "resting" cells compared to exponentially-growing cells. However, comparison of rates of labelling in "resting" cells before and after infection is as valid as similar comparisons in exponentially-growing cells.

III. Choice of a fractionation system for low molecular weight RNA

The work reported describes for the most part low molecular weight RNA species after PRV-infection. Some preliminary work using PRV had been reported (Shepherd, 1969) but the fractionation method used, Sephadex G100 column chromatography, whilst having the advantage of a large capacity, did not have as good resolution as was desired.

Thus the method of fractionation of RNA on 10% polyacrylamide gels (Loening, 1967) was examined. It was found that excellent separation of 4S and 5S RNAs from each other and from the larger ribosomal RNAs could be obtained. Disadvantages to the system were (a) the low capacity of the gels -- about 100 μ g RNA was maximal, (b) the difficulty of relating absorbancy to radioactivity, and (c) the loss of ribosomal RNA from the top of the gel.

Point (a) was resolved by the use of highly labelled RNA, and for most of the studies in this report ^3H -uridine was the isotope of choice, instead of ^{14}C -uridine, since ^{14}C -uridine is of such low specific activity that the 6.0 μM level of uridine considered optimal contains too few d.p.m. Point (b) was of especial importance because of the necessity of measuring

small differences in electrophoretic mobility. This was resolved by the routine co-electrophoresis of ^{14}C -cytoplasmic RNA with ^3H -labelled sample, and the calibration of this marker was described (Fig. 16). Point (c), the loss of ribosomal RNA from the top of the gel, was unavoidable, and the loss was particularly severe when the gels were stained with toluidine blue (probably due to the lengthy destaining process). Hence any value for "rRNA" from the top of a 10% polyacrylamide gel must be treated with reservation; however, the Sephadex G100 system is not much more satisfactory in this respect since it does not separate the larger rRNAs from each other nor from any mRNA.

IV. The synthesis and fate of RNA species I and II from PRV-infected exponentially-growing cells

The radioactive profile of cytoplasmic RNA labelled for 30 min with ^3H -uridine after PRV-infection differs markedly from a similar analysis of RNA from uninfected cells (Shepherd, 1969); the particularly striking features are a sharper 5S RNA peak, relatively more material between 5S and 4S RNA, and relatively much less 4S RNA than in uninfected cells. These results were confirmed both by Sephadex G100 column chromatography (Fig. 13) and on the 10% polyacrylamide gel system (e.g., Fig. 17) and differences due to isotope effects or variation in preparation were ruled out ("Results", II.1.(i),(iii)). The 10% polyacrylamide gel system, apart from the clear resolution of 5S RNA from the larger rRNAs, also resolved the material between 5S RNA and 4S RNA into two peaks, the one nearer 5S being designated I and the other II. It is the metabolism of these two RNA species which is now to be discussed.

The origin of this material, species I and II, between 5S and 4S RNA was considered first. Reference to the literature indicated that examples of RNA eluting or electrophoresing between 4S and 5S have been diversely identified as (a) stable dimers of tRNA molecules (Loehr & Keller, 1968), (b) new mature species of tRNA after T4 infection of E. coli (Weiss et al., 1968), (c) mitochondrial-specific 4S RNA as described in "Introduction", III.7 (Knight & Sugiyama, 1969; Knight, 1969), (d) some of the low molecular weight nuclear species described by Penman's group, and others ("Introduction", III.6), and (e) unstable precursors to tRNA found in a wide variety of organisms, as described in "Introduction", III.3. (ii); it is also possible that these could be new virus-coded species as described in adenovirus-infected cells by Weissman's group ("Introduction", IV.2.(ii)d).

The fact that, in our hands, species I and II are not stable, e.g. they proved unstable to actinomycin D "chase", means that postulates (a), (b), (c) and (d) are untenable. (If (d) were the case, it would mean leakage of nuclear species to the cytoplasm in the infected cell - but Weinberg & Penman, 1968 and 1969 have shown that these low molecular weight nuclear species are all stable or have long half-lives). Thus the suggestion (e) that species I and II are precursors to tRNA was investigated, and the kinetics of synthesis and specificity of metabolism both in vitro and in vivo have served to discount any suggestion that they are merely products of degradation of cellular RNA produced by PRV-infection.

Precursors to tRNA appear in uninfected mammalian cells labelled with radioactive nucleosides for short periods of time (e.g. 10 min), as material eluting in the " $4\frac{1}{2}$ S" position rather than in the 4S RNA position;

longer periods of labelling gradually produce more 4S RNA and less $4\frac{1}{2}$ S RNA. This $4\frac{1}{2}$ S RNA has been designated "pre-tRNA". As discussed in "Results", II.(iii), if species I and II in the PRV-infected cell are indeed precursors to tRNA then their maturation process must be retarded in the PRV-infected cell, since relatively long pulse-labels of up to 1 hour in PRV-infected cells still label species I and II rather than 4S RNA.

In an attempt to establish if species I and II are precursors to tRNA three main lines of approach were used and have been discussed in this report ("Results", II.1.(iii) and II.2):

1. Comparison of the gel electrophoretic properties of species I and II with those of RNA (from uninfected cells) known to be pre-tRNA or to contain a high proportion of pre-tRNA.

2. Kinetic experiments in which the fate of species I and II was followed during both longer labelling times and in the presence of actinomycin D.

3. In vitro incubation experiments in which cytoplasmic RNA containing species I and II and also partially-purified (eluted) species I and II were incubated with cell extracts.

These three methods of approach will now be discussed.

1. Comparison of gel electrophoretic properties of species I and II to those of pre-tRNA. It has been shown that in uninfected Cl3 cells a 5 min or a 10 min pulse-label of ^3H -nucleoside produces cytoplasmic RNA which on fractionation on a Sephadex G100 column elutes predominantly between 5S and 4S RNA in one wide peak; this RNA has been shown to be pre-tRNA (Smillie, 1970; Smillie & Burdon, 1970). When such RNA was prepared by labelling

with ^3H -uridine and was electrophoresed on 10% polyacrylamide gels (Fig. 18) between 5S and 4S RNA two peaks appeared in similar positions to peaks I and II of the PRV-infected cell; the 5 min label produced, relative to 4S, much more label in species I and II than did the 10 min label in which 4S RNA was quite heavily labelled, but the position of the two peaks in both species was similar to each other and to species I and II from the infected cell.

Pre-tRNA which had been column-purified by Dr. J. Smillie and had been established to be pre-tRNA (Smillie & Burdon, 1970) was electrophoresed on 10% gels (Fig. 19) and also eluted between 5S and 4S RNA, with virtually all its radioactivity in two peaks in the position of infected cell species I and II.

Thus, as far as polyacrylamide gel electrophoretic characteristics are concerned, species I and II from PRV-infected cells behave as does pre-tRNA from the uninfected Cl3 cell. The appearance of two peaks of pre-tRNA is novel; other studies of mammalian pre-tRNA, even those using gels, have shown only one peak, although always a diffuse one (e.g. Burdon & Clason, 1969; Bernhardt & Darnell, 1969). However, although the precursor of tRNA in Cl3 cells has been studied using Sephadex G100 columns (Smillie & Burdon, 1970; Smillie, 1970), polyacrylamide gels have not previously been used in the study of Cl3 low molecular weight RNA.

2. Kinetic experiments in the study of species I and II. The kinetic experiments performed fell into two classes, (i) those in which a time-course of labelling with ^3H -uridine was carried out, and (ii) those in which the fate of labelled RNA was followed by means of an actinomycin D "chase".

(i) Time-courses were performed to follow the production of cytoplasmic RNA and cell sap RNA. A time-course of cytoplasmic RNA synthesis showed that in the PRV-infected cell after a 10 min pulse-label there was virtually no radioactivity in 4S RNA whereas peaks I and II were labelled; these continued to be more heavily labelled than 4S RNA even after a 45 min pulse-label. After a 2 hour label most of the radioactivity was found in 4S RNA although there was still a little in species I and II. A composite graph of uninfected and PRV-infected cell cytoplasmic RNA was constructed (Fig. 23), which, although it must be treated with reservation because of overlap between the species, indicates that I and II are precursors to 4S RNA, since the proportion of radioactivity in 4S RNA rises as that in I + II falls. The composite graph also indicates that the relative amounts of the various cytoplasmic RNA species in the infected cell at about 100 min of labelling is similar to those in the uninfected cell at about 15 min indicating that the process of production of 4S RNA is that much slower. Similar criteria have been described by Kay & Cooper, 1969 for their claim that the rate of conversion of pre-tRNA to mature tRNA is accelerated after lymphocytes have been stimulated with phytohaemagglutinin.

Similar conclusions as to the kinetics of species I and II were obtained after a time-course of production of uninfected and PRV-infected cell sap RNA. The problems of preparing ribosome-free cell sap will be discussed in section VII; suffice to say here that species I and II were found in the cell sap to the extent that would be expected if they were precursors to tRNA.

An attempt was made to gauge if the flow of material is $I \longrightarrow II \longrightarrow 4S$ or if each species matures separately to 4S RNA. Species I may be labelled

faster than species II, even in the uninfected cell (Fig. 18a) which at 5 min had most of its radioactivity nearer to 5S. However, even if species I is labelled more rapidly than II it does not necessarily mean that it is a precursor to II. These kinetic experiments do, however, indicate that species I and II may be precursors to 4S RNA.

(ii) Actinomycin D "chase" experiments were the second kinetic line of approach. In these experiments PRV-infected cells were labelled with ^3H -uridine to produce RNA species I and II, and the fate of the infected cell cytoplasmic RNA was followed after an actinomycin D "chase". Actinomycin D inhibits new RNA synthesis; 4S RNA (and 5S RNA) are more resistant to this drug than are the larger RNAs, but, at the level used (5 $\mu\text{g/ml}$), synthesis of these species too is almost completely suppressed (Burdon *et al.*, 1967; Perry & Kelley, 1968, 1970). The results obtained (Figs. 21 and 22) showed an increase in the amount of radioactivity in 4S and a concomitant decrease in the radioactivity in species I and II. The 1 hour "chase" resulted in a decrease in RNA species I + II of 14,100 d.p.m. and an increase in 4S RNA of 13,500 d.p.m., while 87% of the original low molecular weight radioactivity was recovered (Table 6). Smillie & Burdon, 1970, showed that 50% of uridine residues were lost in conversion of C13 pre-tRNA to mature tRNA in an in vitro study. Thus the observed conversion of radioactivity to the 4S region seems to be rather more efficient than would be expected, and could be explained by the following factors:

- (a) Owing to the difficulty in determining the exact radioactivity in a peak, the relatively small amount of radioactivity in the 4S region before the "chase" may have been under-estimated,
- or (b) Some of the radioactivity in 4S RNA could have come from the 5S RNA region which lost 8,350 d.p.m. during the same "chase". This could either

be because species I may overlap into 5S RNA or because some of 5S RNA itself may be a pre-tRNA precursor; this latter point will be discussed in section VII.

In any case there is an excellent correlation between the number of d.p.m. lost from species I and II and those appearing in the 4S RNA position, and thus the actinomycin D "chase" experiments confirm the suggestion that species I and II are precursors to tRNA.

3. In vitro incubation experiments in the studies of species I

and II. The in vitro incubation experiments fell into two sections, firstly, whole cytoplasmic RNA incubated with uninfected or infected cell extracts, and secondly, partially-purified (eluted) RNA species incubated with uninfected cell extracts only. The experiments in which whole cell cytoplasmic RNA was incubated will be discussed first. These experiments were primarily designed to show a postulated difference in "maturing ability" between extracts of uninfected and PRV-infected cells. The rationale for such experiments has already been discussed ("Results", II.2) and will be expanded here. There are three stages in the production of mature 4S RNA (i) the transcriptional stage (ii) the transporting stage and (iii) the maturing stage, and effects caused by PRV-infection at any one (or more) of these stages could produce the observed result of inhibition of 4S RNA synthesis.

(i) An inhibitor at the transcriptional level (i.e. of the RNA polymerase) after virus-infection has been reported in two mammalian systems; mengovirus-infected L cells (Balandin & Kastrikin, 1967) and recently in poliovirus-infected HeLa cells (Ho & Washington, 1971). Even if in the

PRV-infected cell the counts in species I and II are added to those of 4S RNA, there is still less than in the uninfected cell (Table 4); thus it seems clear that the synthesis of 4S RNA is affected, at least to some extent, by inhibition of transcription. This inhibition may be due to an inhibitor of the polymerase, as mentioned above, or, alternatively, retardation of maturation (stage (iii)) may cause a build-up of pre-tRNA which could act to inhibit transcription by some negative-feedback process.

(ii) Inhibition of 4S RNA by decrease in the rate of transport of RNA from nucleus to cytoplasm would be practically impossible to study, since the use of aqueous media for cell fractionation may mean leakage of low molecular weight RNA, and even enzymes, from the nuclei. Thus, although it is generally believed that the maturing enzyme for pre-tRNA and the methylating enzymes are cytoplasmic (e.g. Burdon et al., 1967), micro-dissection techniques using non-aqueous analytical methods in insects have shown that, in that system at least, some formation of 4S RNA is a nuclear event (Egyhazi et al., 1969). Thus, owing to these technical considerations, the aspect of transport has not been considered.

(iii) Inhibition of 4S RNA production after PRV-infection by retardation of maturation of the precursor could be achieved by various means, and it is this aspect which the in vitro experiments (Figs. 24-28) were designed to investigate. As described in "Introduction", III.3.(ii)b), in bacterial cells an endonuclease removes a long sequence of 38 nucleotides at the 5'-terminus of tyrosine pre-tRNA and at least 3 nucleotides are removed (in a manner not clarified) from the 3'-terminus (Altman & Smith, 1971). There is present in Cl3 cell extracts a factor which will remove an average of about 31 nucleotides from pre-tRNA releasing them as nucleosides and nucleotides (Smillie & Burdon, 1970); these may have arisen

by subsequent degradation of a larger oligonucleotide produced by endonucleolytic attack as in the bacterial system. Thus it was reasoned that in PRV-infected cells this "maturing" nuclease may be missing (by suppression of its synthesis) or an inhibitor of this nuclease may be produced; if either of these situations holds the maturing ability of uninfected and infected cell extracts should differ, maturing ability being measured in an in vitro incubation (as reported by Smillie & Burdon, 1970; Mowshowitz, 1970). In the event, extracts from uninfected and PRV-infected cells both caused maturation (as assayed by movement towards 4S) and no difference in maturing ability was observed. The assay system of polyacrylamide gels using the ^{14}C -internal marker would have detected very small differences if any had existed.

There are several explanations for this inability to reproduce in vitro the inhibition observed in vivo. The inhibitor may be very labile, even although the cell extract was prepared shortly before use and used without being frozen; the disruption of the intracellular structure may damage the inhibitor in some way; or the correct cofactors for the inhibitor may not have been found. The maturation of pre-tRNA itself needs no cofactors and without S-adenosyl methionine produces submethylated RNA in the 4S RNA position (Smillie & Burdon, 1970; Mowshowitz, 1970); the removal of the extra sequence seems to be sufficient to allow the remaining portion to form the correct (or almost correct) tertiary structure. However, the inhibitor itself may require some cofactors, the determination of which could only be carried out on a trial-and-error basis.

However, from the point of view of comparing the behaviour of infected cell species I and II to that of pre-tRNA from the uninfected cell,

these in vitro incubations were most successful, for the same conditions which caused the maturation of pre-tRNA (or species I and II) in the uninfected cell (labelled for 10 min) caused the maturation of species I and II in the PRV-infected cell (labelled for 30 or 45 min). The movement in the two systems was exactly analagous, producing little material beyond 4S RNA, indicating little non-specific degradation. Even 10 min incubation produced some movement of pre-tRNA to tRNA, and a decrease of substrate:protein ratio increased the rate of movement. This is characteristic of an enzyme-catalysed reaction.

These cell extracts caused maturation of pre-tRNA whether the nuclei were removed or not. As mentioned earlier, aqueous methods of purification allow leakage of material from the nucleus and thus, although the maturing enzyme is believed to be cytoplasmic, no firm conclusions about this can be drawn from these experiments.

In these experiments, even very short incubation times caused the almost complete disappearance of species I. It is difficult to tell if this species matures to 4S directly or via species II, and in an attempt to clarify this and other issues it was decided to incubate with cell extract RNA species after partial purification (Figs. 33-39).

The RNA species I and II were eluted from PRV-infected cells labelled with ^3H -uridine for 1 hour. Eluted species I and II from the PRV-infected cells were re-electrophoresed and ran exactly in the position from which they had come. Not only does this show that these species are stable to the effects of elution but also that they genuinely do represent distinct, albeit heterogeneous, species. Incubation with buffer had very little effect. Incubation with cell extract - only uninfected cell extract

was used since the "maturing ability" of the two extracts had been shown to be identical -- caused both species I and II to move towards the 4S RNA position. Similar movement of species I and II from the uninfected cell also occurred, but since these species came from 1 hour-labelled uninfected cells they were in any case mostly 4S RNA.

Species II from the infected cell moved to co-electrophorese almost exactly with 4S RNA; species I also moved mainly into the 4S RNA position, with a distinct shoulder in the position of species II and only a tiny amount of material in the position from which it came. In both cases there was a sharp leading edge indicating negligible non-specific breakdown to material smaller than 4S RNA. The shoulder in the position of species II appearing after incubation of species I may well indicate that at least some species I passes through a stage as species II, and this is in agreement with previous findings from the kinetic data. A likely reason for such a two-stage process is that removal of the extra sequences at the 5' and 3'-termini (as described by Altman & Smith, 1971) could occur in two stages, removal at first one end and then the other, corresponding to the transition $I \longrightarrow II$ followed by the $II \longrightarrow 4S$ RNA shift. However, none of these results conclusively shows that species II is such an intermediate in pre-tRNA maturation and it is still possible, or even probable, that I and II are simply different groups of precursor tRNAs which separate in the polyacrylamide gel system.

Thus these three separate lines of investigation (1. - 3.) of species I and II have demonstrated that infected cell species I and II are precursors to 4S RNA and that the process whereby this occurs is very similar to the maturation of pre-tRNA in the uninfected cell; this must account, at least in part, for the decrease in the level of synthesis of

4S RNA in the PRV-infected cell. It should be noted that the question of whether the 4S RNA produced has any of the functions of tRNA such as amino acid-accepting ability has not been investigated in this report, but this aspect has also not been investigated in any studies of pre-tRNA in uninfected mammalian cells; this is because of the difficulty in preparing enough purified pre-tRNA. Thus all evidence that "4¹/₂S" RNA in the uninfected mammalian cell is pre-tRNA has resulted from kinetic studies and in vitro studies, as described here for species I and II. A preliminary report of these findings has been published (Abrahams & Hay, 1972).

V. Quantitation of 4S RNA synthesis after PRV-infection of exponentially-growing cells

As has been established, PRV-infection of exponentially-growing cells causes a decrease in the rate of maturation of pre-tRNA. Thus, calculation of the absolute level of 4S RNA after infection should best be done after a relatively long pulse-label such as 2 hours or, alternatively, by the summation of radioactivity in species I, II and 4S RNA; this latter method must be an estimate at best since, even if all of species I and II is pre-tRNA, material must be lost in processing; also some 5S RNA may also be a precursor species (see section VII).

Values of the level of 4S RNA synthesis after infection compared with that before infection were obtained from various experiments: total cytoplasmic 4S RNA labelled for 45 min (by addition of I + II to 4S RNA - Table 4), from cell sap RNA (Table 10) or from ribosome-associated 4S RNA (Table 9). These results all show a remarkably similar decrease in 4S RNA synthesis to 33-36% of the uninfected level; this suggests that when exponentially-growing cells are infected there is no selection of 4S RNA

for translational purposes since, if only newly-synthesised 4S RNA were used, the measured decrease in ribosome-associated 4S RNA after infection would be expected to be less than that in the cell sap.

VI. 4S RNA synthesis and synthesis of species I and II in PRV-infected "resting" cells

In "resting" cells the synthesis of all RNA species was found to be decreased relative to exponentially-growing cells. On infection with PRV the level of synthesis of 28S and 18S rRNAs decreased even further whereas the level of 4S RNA synthesis, both in cell sap and associated with ribosomes, either stayed constant or even rose (Figs. 42 - 45, 53); late in infection (7 - 10 hours) it finally fell (Fig. 44f). The fact that sometimes the level of 4S RNA synthesis remained constant while sometimes it rose was attributed to variation in the degree of "resting" of the cells. Values reported in the literature comparing levels of RNA synthesis in exponentially-growing and "resting" cells often vary, within the same report, by a factor of 2 or 3 (e.g. Emerson, 1971; Weber, 1971).

It should be noted that the decrease in 4S RNA synthesis, when cells go to "rest", cannot be attributed to a decrease in rate of maturation of the pre-tRNA as reported by Kay & Cooper, 1969 for unstimulated lymphocytes, for a 30 min pulse-label of uninfected "resting" cells produced no more pre-tRNA (in the position of species I and II) than did a 30 min pulse-label of uninfected exponentially-growing cells (Fig. 42). Thus a decrease in the level of transcription seems to be the probable explanation for the decrease in 4S RNA synthesis in "resting" Cl3 cells.

After PRV-infection, this level of 4S RNA synthesis is maintained

and probably rises. 30 min pulse-labels at various stages after infection (Fig. 42) show that species I and II begin to appear slightly at 3 hours, and clearly by 5 hours, after infection, even although there is no decrease in the amount of 4S RNA produced at these times. It is possible that when PRV infects "resting" cells it does not further depress transcription because the level of transcription is already depressed; thus the level of 4S RNA synthesis does not fall. However, the virus may still allow a postulated inhibitor of the tRNA maturing process to be formed (or prevent the synthesis of the maturing nuclease). As infection proceeds, inhibitor levels rise (or nuclease levels fall) and there is a gradual build-up of pre-tRNA (species I and II) which eventually results, much later in infection, in a decrease in 4S RNA synthesis. Thus these "resting" cell experiments once again suggest that 4S RNA production can be affected at two levels (a) transcription and (b) maturation.

Studies on the composition of 4S RNA in "resting" cells. In view of the continuation of 4S RNA synthesis after PRV-infection of "resting" cells, it was clearly of importance to determine whether the 4S RNA synthesised after PRV-infection could be distinguished from the 4S RNA of the uninfected cell. Subak-Sharpe *et al.*, 1966b proposed that in view of the large difference of about 24% in the G + C content of HSV-I DNA and mammalian DNA and especially because of the shortage in mammalian DNA of the CpG doublet, there may be difficulties in the infected cell for the translation of HSV-I in RNA, and that an altered tRNA population would be required to meet the new demands. Since the G + C content of the DNA of PRV is even higher than that of HSV-I DNA ("Introduction", IV.1.(iii)b) this line of reasoning would suggest that the tRNA population after PRV-infection may be altered; the new tRNAs need not necessarily be virus-coded but the virus may selectively permit or

increase the transcription of some tRNA species and not of others. Since the cellular levels of 4S RNA synthesis are lower in "resting" cells than in exponentially-growing cells, 4S RNA was studied in this system. Three techniques were used in the study of the composition of 4S RNA after PRV-infection (a) methylation labelling studies, (b) nucleotide analysis, and (c) "fingerprinting".

(a) Methylation was studied since in some virus-infected mammalian systems there are changes in the level of tRNA methylase and tRNA methylation e.g. poliovirus-infected HEp-2 cells have a rate of methylation of 4S RNA one-third that of uninfected cells (Grado et al., 1968); foot-and-mouth-disease virus also causes inhibition of 4S RNA methylation, the degree of which varies throughout the infectious cycle (Ascione & Vande.Woude, 1969). Hay & Low, 1970, reported a decrease in methylase levels and in methylation of 4S RNA in Cl3 cells after infection with HSV-I. However, carcinogenic viruses cause tumours with high methylase activity and increased tRNA methylation (e.g. Baguley & Staehelin, 1968).

All these experiments in which decrease in methylation of 4S RNA was observed used techniques where 4S RNA was not separated from the other low molecular weight RNA species, and hence the observed decrease in methylation could have been due to a relative increase in the synthesis of non-methylated species of RNA e.g. 5S RNA.

The methylation studies reported in this thesis (Fig. 44) show no significant change in the methylation level of 4S RNA after infection of "resting" cells, although there may possibly be a very slight drop late in infection; at that stage the low incorporation of counts makes quantitation prone to error. Even if the virus is altering the tRNA population by

selecting transcription of a proportion of the cell tRNA (and is not coding for its own tRNA) then perhaps it should be expected that there will be no gross changes in the level of methylation of total 4S RNA.

(b) Nucleotide analysis of 4S RNA before and after PRV-infection of "resting" cells was performed using ^{32}P -labelled RNA. It is necessary to use a labelling method in a virus-infected system since it is the newly-synthesised RNA of which the nucleotide composition is desired; errors due to uneven equilibration of the triphosphate pools (which should be small during a 3 hour label) have to be assumed to be constant. That equilibration does take place is suggested by the great similarity in the values obtained for uninfected exponentially-growing or "resting" Cl3 cells, and those obtained using the quite separate method of paper chromatography (Table 7). After infection, there were no significant changes in the nucleotide composition of 4S RNA; the analyses were remarkably similar, testifying to the value of this convenient method. The constancy of the nucleotide composition after infection does help to confirm that the 4S RNA is still almost certainly tRNA and is not contaminated by new species of RNA, or mRNA, or mRNA breakdown products (which might be expected to have a G + C content approaching that of the viral DNA, i.e. somewhat in excess of 70%).

(c) "Fingerprint" analysis of T_1 digests of ^{32}P -labelled RNA before and after PRV-infection was performed principally to attempt to detect changes in 5S RNA. Since 4S RNA is so heterogeneous, all spots can be expected to be present. "Fingerprints" of 4S RNA from uninfected and PRV-infected cell sap, and from ribosomes, were all identical and contained all possible spots. A better method of analysis may be to label the methyl groups only of 4S RNA using (^{14}C -methyl)-methionine, which would produce fewer spots; once again, however, the complexity of the 4S RNA population

may mask any differences produced after PRV-infection.

Thus no significant differences in the 4S RNA population produced after PRV-infection could be detected.

VII. 5S RNA in PRV-infected exponentially-growing cells.

The preliminary experiments described in this report in which uninfected or PRV-infected cytoplasmic RNA was labelled for 30 - 45 min periods with radioactive uridine show that in the uninfected cell cytoplasm there is a much more pronounced peak of 5S RNA than in the uninfected cell. For example, a 45 min pulse-label of cytoplasmic RNA indicates that 5S RNA synthesis is only decreased to 54% of the pre-infection level, whereas 4S RNA is present in very small amounts; even if the radioactivity in 4S RNA is added to that in peaks I and II, there is a decrease in synthesis to 33% of pre-infection levels; rRNA synthesis is even more strongly decreased (Table 4). Thus the proportion of low molecular weight RNA synthesis that is 5S RNA is greatly increased after infection, as is the ratio of 5S RNA:4S RNA.

There are various possibilities for the presence of this relatively large amount of 5S RNA production in the infected exponentially-growing cell cytoplasm.

(a) PRV-infection simply has less inhibitory effect on 5S RNA synthesis than it does on 4S RNA or 28S and 18S RNA synthesis. This would imply that 5S RNA synthesis is not co-ordinately repressed with the larger rRNAs.

(b) After PRV-infection a new species of 5S RNA is synthesised which could be associated with ribosomes or cell sap; such a virus-coded cell sap RNA species has been described by Weissman and his co-workers after

adenovirus-infection of KB cells ("Introduction", IV.2.(ii)d).

(c) The 5S RNA peak may be heavily contaminated with precursor RNA species, especially species I~~---~~ and the relatively high level of this species after infection may be artificially elevating the amount of 5S RNA synthesis.

(d) There may be even larger precursors to tRNA than those in the position of species I, which may co-electrophorese with 5S RNA; this need not only be a feature of the PRV-infected cell but could also occur in the uninfected cell. It would be difficult to differentiate between this hypothesis and hypothesis (c).

(e) As mentioned in "Introduction", III.2.(iii)b, there is a nuclear "pool" of 5S RNA, which accounts for 20-25% of the total 5S RNA in the uninfected mammalian cell (Knight and Darnell, 1967; Perry and Kelley, 1968). Since herpesviruses are believed to alter the cell nucleus, making it "leaky", leakage of nuclear 5S RNA might account for the increase in cytoplasmic 5S RNA after PRV-infection.

Further, it is possible that more than one of the above hypotheses contributes to the relative increase in cytoplasmic 5S RNA synthesis after PRV-infection.

5S RNA synthesis in the cytoplasm of exponentially-growing cells. If the relatively high amount of 5S RNA in the infected cell cytoplasm after a 30-45 min pulse-label is due, even if only partly, to the presence of pre-tRNA in the 5S position (possibilities (c) and (d)), then some clarification should come from an examination of uninfected cell cytoplasmic RNA labelled for 5 min or 10 min (because of the retardation in pre-tRNA maturation in the PRV-infected cell). A 5 min pulse-label of uninfected

cell cytoplasmic RNA (Fig. 18a) does indeed prove to have, relative to the amount of 4S RNA present, a high 5S RNA peak, the relative level of which drops after a 10 min label, while the radioactivity in it remains constant. The same phenomenon appears in a report by Bernhardt & Darnell, 1969. Thus it does seem that in the uninfected cell both the proportion of radioactivity in 5S RNA and the 5S:4S ratio is greater at short labelling times than at long labelling times; this is also indicated by the composite graph (Fig. 23a).

In the uninfected cell the only explanations, from the possibilities listed (a) - (e) for an increase in the amount of 5S RNA at short labelling times as compared to longer labels, can be (c) or (d) - i.e., the increase in 5S RNA is due to 5S RNA being contaminated with, or being itself, pre-tRNA. The possibility that some pre-tRNA is as large as 5S RNA has not been reported for mammalian cells, although the results mentioned above from Bernhardt & Darnell, 1969, can be interpreted as such; also Egyházi et al., 1969 have reported that in insects a portion of the very heterogeneous pre-tRNA peak is of the size of 5S RNA. Thus, in the PRV-infected cell, since maturation of pre-tRNA has been shown to be slow, the increased levels of 5S RNA would be maintained after longer labelling times than in the uninfected cell. The composite graph of the time-course of labelling infected cell cytoplasmic RNA (Fig. 23b) does show 5S RNA starting at a high level and taking about 2 hours to reach its steady-state level; the graph drops very steeply at first, then rises and then eventually levels off, as does the graph of the proportion of uninfected cell 5S RNA (Fig. 23a). This rise and fall may well indicate the presence of two species of 5S RNA - one transitory species labelled faster (pre-tRNA?) and one more stable species labelled at a slower rate (stable, ribosome-associated 5S RNA):

The actinomycin D "chase" experiments of infected cell cytoplasmic RNA show disappearance of radioactivity from species I and II in the infected cell and a concomitant increase in the radioactivity in 4S RNA. As discussed in section IV, there was also a loss of radioactivity from 5S RNA, particularly in the 45 min-labelled RNA "chased" for 1 hour (Fig. 22; Table 6) where it seems that the increase in radioactivity in the 4S RNA region can not be wholly accounted for by a loss of radioactivity from species I and II. The counts remaining in 5S RNA presumably represent stable 5S RNA - and mammalian ribosome-associated 5S RNA is a stable RNA species (Watson & Ralph, 1967).

The in vitro incubations, in which whole cytoplasmic RNA from uninfected cells (10 min labelled) and from PRV-infected cells (30 or 45 min labelled) were incubated with cell extracts, were primarily carried out as an assay for the proposed pre-tRNA inhibitor. These experiments showed that 5S RNA from uninfected or PRV-infected cells, lost only a small amount of radioactivity on incubation. It may be that the precursor element in the 5S RNA peak was very unstable and was lost on storage of the RNA prior to the incubation in vitro; this was clarified by the experiments (discussed later) in which partially-purified 5S RNA was incubated in vitro.

5S RNA in the cell sap of exponentially-growing cells. Since it now seems that at least part of the relatively high level of 5S RNA in the infected cell cytoplasm may be due to pre-tRNA, it was decided to examine RNA prepared from the cell sap, since in the uninfected cell sap there should be no 5S RNA, it being wholly ribosome-associated; pre-tRNA however, should occur in the cell sap. Several difficulties emerged in the course of this work. In several experiments, to avoid possible maturation or nuclease degradation occurring, the final centrifugation used to sediment the

ribosomes was relatively short (60 - 90 min) and, apparently, this gave rise to incomplete precipitation of ribosomes as measured by an absorbancy peak of 5S RNA in the gels of both uninfected and infected cell RNA (Fig. 29). It seemed unlikely that this 5S RNA in the cell sap could have arisen through release of 5S RNA from ribosomes, since mammalian 5S RNA is very strongly attached to ribosomes, as discussed in "Introduction", III.2.(iii)b, and, throughout the preparation of the cell sap, a high concentration of magnesium ions was maintained.

A time-course of labelling cell sap RNA from uninfected or PRV-infected cells (Figs. 30, 31), although difficult to interpret because of the presence of 5S RNA extracted from non-precipitated ribosomes, shows that the proportion of 5S RNA in the infected cell sap is approximately twice that in the uninfected cell sap; it also shows that although the 5S:4S ratio decreases as infection proceeds, even after a 3 hour label when virtually all of species I and II has disappeared, the fraction of low molecular weight RNA in the 5S RNA position is still about 0.2 as compared to 0.1 in the uninfected cell, and the 5S:4S ratio is about 0.40 compared to about 0.13 in the uninfected cell (calculated from composite graph, Fig. 32). These results do suggest that even although part of the high level of 5S RNA synthesised after infection is short-lived, a significant proportion appears to be relatively stable.

However, the fact that this long-lived 5S RNA is probably not a cell sap species was indicated when uninfected and PRV-infected cells were labelled for 4 hours and centrifuged for 16 hours to produce cell sap uncontaminated by ribosomes (Fig. 52); only 4S RNA was present. Thus this postulated long-lived 5S RNA, which occurs in an increased proportion

after infection, is not present in the cell sap and for this reason the ribosome-associated 5S RNA was treated separately and examined in further experiments; these are discussed later in this section.

To examine the stability of the infected cell sap 5S RNA, in vitro incubation studies (Figs. 33-39) were performed on 5S RNA eluted from polyacrylamide gels. The RNA had been prepared from uninfected and PRV-infected cell sap labelled for 1 hour and centrifuged for 90 min so that maturation should not occur; the samples were thus slightly contaminated with ribosomes.

Whereas elution from the polyacrylamide gel had no effect on the other low molecular weight species, 5S RNA from the infected cell produced a main 5S RNA peak and also significant trailing of material towards 4S; this trailing material increased slightly after incubation with buffer. Incubation of this material with cell extract produced a definite peak of 4S RNA and a virtual disappearance of the 5S RNA peak. While calculation of recoveries of radioactivity is difficult to assess owing to the many manipulations involved in working-up this type of experiment, this nevertheless represents an increase in radioactivity in the 4S position which could have come only from 5S RNA. These results indicate that 5S RNA from the infected cell seems to exhibit instability, which results in the formation of 4S RNA; thus in this experiment 5S RNA behaves as a precursor to tRNA.

The control experiments - in vitro incubation studies on 5S RNA from the uninfected cell - are difficult to interpret since eluted 5S RNA produced two peaks, one of 4S and the other of 5S RNA; this is probably because the main low molecular weight RNA species in uninfected cells is 4S RNA, which may well contaminate the 5S RNA sample. Incubation with

buffer had little effect but incubation with cell extract caused most of the 5S RNA material to disappear leaving little material besides the 4S RNA peak; this 4S RNA did not seem to be increased. Thus in the uninfected cell most of the 5S RNA was unstable to the incubation conditions used; this may be because the low level of substrate RNA allowed non-specific nuclease degradation to occur.

These experiments do suggest that, in the PRV-infected cell at least, some 5S RNA becomes 4S RNA, and also, that 5S RNA, from uninfected or infected cells, exhibits some degree of instability to the incubation conditions used. We cannot rule out the possibility that in the uninfected cell, too, some 5S RNA becomes 4S RNA (i.e. is pre-tRNA) but, because of its faster maturation, such 5S RNA was not present in the sample used.

It has been shown that part of the increased level of 5S RNA after infection is due to the presence of some pre-tRNA in the 5S RNA position. However, after longer labels in which even infected cell pre-tRNA has mostly matured, the ratio of 5S:4S in the infected cell still remains higher than in the uninfected cell, and reasons for this continuing high 5S RNA level must be considered. One which has already been suggested - possibility (a) - is that ribosomal 5S RNA is inhibited less strongly after PRV-infection than 4S RNA. This was examined by following the synthesis of ribosome-associated 5S RNA.

5S RNA synthesis in relation to that of the other ribosomal RNAs in exponentially-growing cells. The length of time of processing of Cl3 cell ribosomal RNA from nucleus to cytoplasm was first examined and shown to be of the same order as for HeLa cell RNA (Fig. 48). A four hour labelling period was used to study ribosomal RNA synthesis (Fig. 51); it was found that

ribosome-associated 5S RNA synthesis decreased to 54% after infection, whereas ribosome-associated 4S RNA decreased much more, to 36% (Table 4); the level of decrease of ribosome-associated 4S RNA synthesis is of the same order as the decrease in cell sap 4S RNA synthesis. Thus this leads to an increased 5S:4S ratio after infection and would account in part for the relative increase in levels of 5S RNA after long labels in the PRV-infected cell.

This lack of decrease in 5S RNA synthesis is striking especially since the larger rRNAs are strongly repressed to <20% of normal levels (Table 8). Such separate rates of decrease are possible since 5S RNA is synthesised separately from 45S RNA, the precursor to the other rRNAs. However, in the uninfected cell there is normally some degree of control in the production of these two types of rRNA e.g. anucleolate mutants of Xenopus laevis which lack the genes for 28S and 18S RNA do not accumulate 5S RNA even although the DNA homologous to 5S RNA is present (Brown & Weber, 1968); another example of such co-ordination of synthesis occurs in the oocytes of Xenopus laevis which, as they synthesise 28S and 18S RNA at very high rates using repeated genes, also synthesise 5S RNA coordinately, even although its genes are not amplified (Brown & Dawid, 1968). Lack of co-ordination is, however, theoretically possible because of the separate origin of 5S from 28S and 18S and has indeed been shown, in that low levels of actinomycin D inhibit the synthesis of 28S and 18S but leave 5S RNA synthesis unaffected (Perry & Kelley, 1968). A very recent report by Ford, 1971 also claims that in the early stages of oogenesis of Xenopus laevis there is non-coordinated synthesis and accumulation of 5S RNA. Thus there are precedents for non-coordinated synthesis of 5S RNA with 28S and 18S RNA, as seems to occur in the PRV-infected cell.

VIII. 5S RNA synthesis in PRV-infected "resting" cells.

5S RNA in the cell sap and ribosomes of "resting" cells. "Resting" cells were used as a means to study 5S RNA synthesis for the reasons previously discussed, viz., the lower background level of RNA synthesis. The preparation of cell sap free of ribosomes was subject to the difficulties outlined earlier. In the series of experiments in which "resting" cells were infected and labelled for 30 min periods at different stages throughout the infective cycle (Fig. 42) there is little 5S RNA synthesis in the uninfected controls (uninfected "resting" cells, exponentially-growing "high serum" cells, and cells to which serum had been replaced). On infection, there is little change until 3 - 3.5 hours P.I. when a small amount of species I and II and a slightly increased level of 5S RNA synthesis appears. The amount of these species builds up until, by 7 - 7.5 hours P.I., there is a very prominent 5S RNA peak in addition to RNA species I and II. Since this 5S RNA has no counterpart in the controls and appears at the same time as species I and II, it seems reasonable to suggest that this supports the findings from work with exponentially-growing cells that at least part of 5S RNA is pre-tRNA.

Experiments were performed in which infected or uninfected "resting" cells were labelled for 3 hour periods prior to the preparation of cell sap RNA (Figs. 43 - 45). In none of the uninfected control samples was there more than a trace amount of 5S RNA, but in all the infected samples there was considerably more 5S RNA, albeit still a small portion; the amounts of 5S RNA found varied in different experiments but, e.g., in Figs. 44 and 45, a 4 - 7 hour P.I. pulse-label produced a significant 5S RNA peak. Any radioactive 5S RNA found after a 3 hour label is unlikely to be pre-tRNA ~~---~~

and little evidence for the presence of the other pre-tRNA species I and II was found. As has been discussed in relation to the experiment, it would be surprising if the ribosomes which precipitated in the controls did not precipitate after infection, and indeed little ribosomal RNA can be found on top of the gel. However, in view of the larger decrease in rRNA synthesis after infection compared to 5S RNA synthesis, it is possible that the 5S RNA seen could have come from a very small fraction of unprecipitated ribosomes; very recent work by Ben-Porat, Rakusanova & Kaplan, 1971 has shown that there are more single ribosomes in the infected cell than in the uninfected cell and hence it is possible that precipitation of ribosomes may become less complete as infection proceeds. Thus the 5S RNA which occasionally occurs in the cell sap of infected cells after a long label may be partly ribosome-associated. However, its appearance when there is so little ribosomal RNA on top of the gel must mean that, as in the exponential system, rRNA synthesis in the "resting" cell is depressed to a greater extent than ribosomal 5S RNA synthesis, and this indeed can be calculated from Figs. 53 and 54 (see P. 197): 5S RNA synthesis seems to be depressed co-ordinately with that of the larger rRNAs on going to "rest", but whereas the synthesis of 28S and 18S RNAs is depressed on subsequent PRV-infection, that of 5S RNA seems to be unaffected.

It should be noted, in passing, that the heterodisperse material which appears around 18S in 2.5% polyacrylamide gel fractionation of ribosomal RNA from these PRV-infected "resting" cells (Fig. 54) is of a similar size to viral mRNA in cells infected with HSV-I ("Introduction", IV.2.(ii)b); in a recent report PRV mRNA is also approximately this size (Rakusanova et al., 1971).

Studies on the composition of 5S RNA in the PRV-infected cell. Studies on the long-lived 5S RNA in the infected cell sap showed that it is unmethylated; this is expected for 5S RNA and also for pre-tRNA. Nucleotide analysis revealed small differences between it and uninfected 5S RNA in that its composition was found to be more like that of 4S RNA. This could support the hypothesis that this 5S RNA is pre-tRNA, or it could be the result of contamination with 4S RNA.

A more sophisticated way to study 5S RNA is by "fingerprinting", since the homogeneity of normal ribosomal 5S RNA means that there should be relatively few oligonucleotide spots. It was found impossible to get enough ^{32}P -radioactivity into cell sap 5S RNA (because the 3 hour pulse-label necessary for equilibration was too long for the short-lived 5S RNA), and so ribosome-associated 5S RNA was "fingerprinted". Within the limitations of the results, discussed at the time (Fig. 47) no differences emerged between ribosome-associated 5S RNA from uninfected and PRV-infected cells.

Thus, taking together the evidence from both exponentially-growing and "resting" cells, it appears that the high levels of 5S RNA after infection are due to (i) the slower processing of pre-tRNA after PRV-infection, since some pre-tRNA electrophoreses as 5S RNA, and (ii) the relative lack of depression of ribosome-associated 5S RNA synthesis after PRV-infection.

Two of the possibilities, (a) - (e), mentioned at the beginning of section VII have not yet been considered. One, (a), is that a new species of 5S RNA is coded for by the virus, and this could only be shown by molecular hybridisation experiments. However, the fact that the infected

cell sap 5S RNA is normally unstable, and that the ribosome-associated 5S RNA is probably unchanged after infection, would argue against this possibility.

The other possibility, (e), is that infection causes leakage of the nuclear 5S RNA "pool"; however, the nuclear "pool" comprises only 20 - 25% of the cell 5S RNA and, even if it all leaked, this would not account for the labelling increase after short pulse-labels.

Thus, the reason for the relative increase in the amount of 5S RNA after PRV-infection of exponentially-growing or "resting" cells seems to be:

- (i) the slower processing of pre-tRNA which affects that portion of 5S RNA in the infected cell - and probably also in the uninfected cell - which is pre-tRNA,
- and (ii) the non-coordinated depression of 5S RNA with the other ribosomal RNAs such that the synthesis of 5S RNA is less strongly repressed by the virus than any of the other cytoplasmic RNAs (4S, 28S, 18S).

SUMMARY

In this thesis, some effects of pseudorabies virus infection on RNA metabolism in BHK-21/Cl3 cells were examined; in particular, the production of 4S RNA and 5S RNA was studied.

1. The concentration of uridine in the medium that allows maximum incorporation of isotopically-labelled uridine into RNA in Cl3 cells was determined.
2. A 10% polyacrylamide gel electrophoresis system was calibrated and used to fractionate low molecular weight cytoplasmic RNA, excellent separation between 5S RNA and 4S RNA being obtained.
3. Infection of exponentially-growing cells with pseudorabies virus caused a steady decrease (to 15% of the uninfected level) in incorporation of ^3H -uridine into RNA by 5 hours after infection. The production of cytoplasmic 28S and 18S RNAs was found to be most strongly inhibited while the production of 4S and 5S RNAs was much less affected; the decrease in synthesis of 4S RNA from either total cytoplasm, cell sap or ribosomes was found to be virtually identical.
4. In some experiments, serum-depleted "resting" cells were used; in these cells DNA synthesis was shown to be <1% of the exponential level, and the rates of synthesis of 28S, 18S, 4S and 5S RNAs were all found to be decreased.
5. In the uninfected, exponentially-growing Cl3 cell, a pulse-label of ^3H -uridine for a period of about 30 - 60 min produces cytoplasmic low molecular weight RNA in which almost all of the radioactivity is in 5S RNA

and 4S RNA. It was found that in pseudorabies virus-infected cells, such a pulse-label produced radioactive low molecular weight RNA mainly of a size between 5S and 4S. This material eluted as a single heterodisperse peak from a Sephadex G100 column, but resolved on 10% polyacrylamide gel electrophoresis into two peaks; the peak nearer 5S RNA was designated species I and the other species II.

6. Several lines of evidence suggested that species I and II in the pseudorabies virus-infected cell are precursors to tRNA, and that they remain so clearly labelled because their maturation to 4S RNA is retarded as a result of virus-infection. Such evidence included experiments involving time-courses of labelling, in vivo "chase" experiments using actinomycin D, and in vitro incubation experiments. It was also shown that pre-tRNA from uninfected Cl3 cells (pulse-labelled for 5 - 10 min) electrophoresed on 10% polyacrylamide gels as two peaks, analogous to peaks I and II from the virus-infected cell.

7. In vitro incubation experiments failed to demonstrate any difference in the ability of cell extracts from virus-infected or uninfected cells to convert precursor RNA to 4S RNA; both extracts successfully matured unpurified or partially-purified species I and II to 4S RNA. These experiments supported evidence from the kinetic experiments that species I may become species II en route to 4S RNA.

8. After infection of "resting" cells with pseudorabies virus, 4S RNA synthesis was found not to be decreased and possibly even increased, until late in infection; this may be correlated with appearance of species I and II indicating retardation of the maturing process. Alterations in level of methylation and nucleotide composition of 4S RNA after infection of "resting" cells were shown to be negligible.

9. The increase in 5S RNA synthesis, relative to other cytoplasmic RNA species, which occurs after pseudorabies virus-infection of exponentially-growing cells, was found to be due to two factors : (a) the synthesis of ribosome-associated 5S RNA is not strongly decreased after infection (much less than that of the larger rRNAs) and (b) some 5S RNA appears to be a precursor to tRNA; a similar situation may occur in the uninfected cell.

10. After infection of "resting" cells, it was found that ribosome-associated 5S RNA synthesis did not seem to be further decreased. Significant levels of cell sap 5S RNA, probably pre-tRNA, appeared late in infection. After infection of "resting" cells, there were no significant alterations in nucleotide composition of 5S RNA or in the sequence as determined by electrophoretic separation of oligonucleotides ("fingerprinting").

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