STUDIES ON THE PROCESSING OF POLYOMA VIRUS RNA

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We accept this thesis as conforming to the
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The University of British Columbia
Vancouver 8, Canada

Date **May 14, 1973**
ABSTRACT

The main objective of these studies was to analyze the processing of polyoma virus RNA transcripts.

An examination was made of the intracellular distribution of polyoma virus RNA molecules synthesized late during productive infection in mouse kidney cells. The virus specific RNA from whole cells, and their nuclear, cytoplasmic and polyribosomal fractions were compared with respect to sedimentation behaviour (on sucrose gradients), electrophoretic mobility (on polyacrylamide gels) and base sequence homology (by competition hybridization tests).

Sedimentation and electrophoretic analysis revealed marked heterogeneity in polyoma RNA from all cell fractions. This heterogeneity and size distribution were essentially the same on dimethylsulfoxide sucrose gradients. The viral RNA resedimented true to its original distribution on a sucrose gradient. The polyoma RNA from different regions of a sucrose gradient contained common sequences as revealed by cross-competition hybridization experiments. However, the polyribosome associated polyoma RNA was devoid of the >28s species found in the other fractions. Thus it appeared that polyoma RNA, including RNA of apparent size in excess of one genome length, was synthesized in the nucleus and cleaved to smaller pieces in association with the polyribosomes.
The accumulation of the polyoma RNA in the nuclear fraction proceeded at a rate similar to that of the cellular RNA, while the corresponding rate of accumulation of viral RNA in the cytoplasmic fraction was similar to the cell RNA for 30 minutes, but did not increase, as did that of the cell RNA, with increased labelling times. The polyoma RNA labelled in the cells during a 15 minute pulse was significantly larger (46% sedimented faster than 28s) than that labelled for 2 hours (25% sedimented faster than 28s). Pulse and chase studies with Actinomycin D and excess uridine revealed that the majority (up to 75%) of the polyoma RNA labelled in a 40 minute pulse was subsequently degraded within the nucleus within one hour of chase, while the remainder disappeared from the nucleus at a slower rate in the next five hours of chase. In addition, the larger (>28s) polyoma RNA in the nuclear fraction was degraded slightly faster than the small (<18s) RNA. In the cytoplasmic fraction, the quantity of labelled viral RNA decreased slowly during the chase period and all size classes were degraded at approximately the same rate.

Polyadenylate sequences were found associated with polyoma RNA. The percentage of the viral RNA molecules containing polyadenylate sequences increased as the RNA was processed from the nuclei through to the polyribosomes, suggesting that only poly (A) containing polyoma RNA molecules can be properly processed.

The methodology utilized in the isolation and detection of poly (A) sequences was examined in more detail. The method of isolation
of poly (A) sequences, by binding to poly (U) fixed to glass fiber filters, was found to be highly dependent on the salt concentration of the binding buffer, through it did select for non-ribosomal heterogeneous cellular RNA which had ribonuclease resistant poly (A) stretches. The method of extraction of the RNA was found to have a considerable effect on the subsequent binding properties of these molecules.
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<tr>
<td>BHK</td>
<td>baby hamster kidney cells</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>$^{14}\text{C}$</td>
<td>carbon - $^{14}$</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>dalton</td>
<td>atomic mass unit</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNAse</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>d-RNA</td>
<td>DNA-like RNA</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FUdR</td>
<td>deoxyribofluorouridine</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>gamma (radiation)</td>
</tr>
<tr>
<td>GF/C</td>
<td>glass fiber filters - type C</td>
</tr>
<tr>
<td>G + C</td>
<td>guanine plus cytosine</td>
</tr>
<tr>
<td>$^3\text{H}$</td>
<td>tritium</td>
</tr>
<tr>
<td>Hm RNA</td>
<td>heteronuclear RNA</td>
</tr>
<tr>
<td>HAU</td>
<td>hemagglutination units</td>
</tr>
<tr>
<td>m-RNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>ml-RNA</td>
<td>messenger-like RNA</td>
</tr>
<tr>
<td>$\mu$l</td>
<td>microliter</td>
</tr>
<tr>
<td>$\mu$Ci</td>
<td>microcurie</td>
</tr>
<tr>
<td>$\mu$gm</td>
<td>micrograms</td>
</tr>
<tr>
<td>$\mu$m</td>
<td>micrometer (micron)</td>
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mCi - millicurie
ME - mouse embryo (cell)
MK - mouse kidney (cell)
MBB - Millipore binding buffer
MEM - minimum essential medium
moi - multiplicity of infection
nm - nanometer
NP-40 - nonidet P-40
PD - petri dish (9 cm)
$^{32}$p - phosphorus-32
PBS - phosphate buffered saline
py - polyoma
py-H - polyoma transformed hamster cells
poly (A) - polyadenylate
poly (C) - polycytidylate
poly (G) - polyguanidylate
poly (T) - polythymidylate
poly (U) - polyuridylate
pfu - plaque forming units
pi - post infection (after infection)
PUBB - polyuridine binding buffer
RNA - ribonucleic acid
r-RNA - ribosomal RNA
RNase - ribonuclease
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<td>RNP</td>
<td>ribonucleoprotein</td>
</tr>
<tr>
<td>RSB</td>
<td>reticulocyte standard buffer</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>S</td>
<td>sedimentation coefficient (seconds)</td>
</tr>
<tr>
<td>s-RNA</td>
<td>soluble RNA (1 M NaCl)</td>
</tr>
<tr>
<td>STE</td>
<td>sodium tris EDTA buffer</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline citrate</td>
</tr>
<tr>
<td>t-RNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>T-antigen</td>
<td>tumor antigen</td>
</tr>
<tr>
<td>tris</td>
<td>tris hydroxymethylaminomethane (buffer)</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet (radiation)</td>
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CHAPTER I: INTRODUCTION

1. History

The pathological manifestations of polyoma (py) virus infection were first observed in 1951 as parotid gland tumors in mice experimentally injected with cell free extracts from leukemic mice (41). This agent was subsequently shown to be present in healthy mice under natural conditions but to lead rarely to tumor development (42). In 1958 the virus was first cultivated in vitro (30) (29) and its properties were subsequently determined (98). In 1960 it was observed that some py infected mouse and hamster cells displayed a change in growth pattern. They lost the property of contact inhibition and hence grew in dense whorls, although in the case of the hamster cells little or no detectable infectious virus was produced (104). On transplantation into newborn hamsters, these cells caused tumors to develop (21). The above observers called these cells virus transformed cells.

2. Nomenclature

The name polyoma was given to the virus by Eddy and Stewart in 1958 (30). This name indicates that the virus is capable of causing tumors at many sites in the infected animal. Because of its
similarity to simian virus 40 (SV40) and rabbit papilloma, it has been included in the group papovavirus (71).

3. Virus Properties

a. Morphology

Electron microscope studies on negatively stained preparations of py virus have shown it to be 40-45 nm in diameter, and lacking a limiting membrane (57). The virus particle has cubic symmetry comprising of 72 capsomers in an icosahedral arrangement (51) (10).

b. Physical and Chemical Properties

i. The py virion

Polyoma virus can be purified by plaque purification (31), by banding in cesium chloride or rubidium chloride, or by sedimentation in sucrose gradients (16) (116). The latter three of these methods have also proved useful in characterizing the virus. The virus has a density in cesium chloride of 1.32 gm per ml (16), and a sedimentation coefficient on sucrose gradients of 250 s (116).

Initial purification of polyoma in CsCl gradients revealed the presence of two types of particles, of which the heavier, with a
buoyant density of 1.32 gm per ml, was the DNA containing particle, while the lighter, with a buoyant density of 1.28 gm per ml, was the empty capsid (16). Later work expanded on this observation and showed that the heavy component consisted of infective virions of a buoyant density of 1.33 gm per ml and non infective pseudovirions of a buoyant density of 1.315 gm per ml which contained partly cellular DNA (70).

ii. Hemagglutinins

Polyoma virus has the ability to agglutinate guinea pig and hamster erythrocytes and human type 0 erythrocytes at 4°C, though not at 37°C (30). A useful assay for the virus titer has been developed using this property of the virus (94).

iii. Resistance to physical and chemical agents.

Viral infectivity is resistant to diethyl ether (43), trypsin (30), RNAse or DNAse (24), repeated freezing and thawing (8), or 3 minutes of sonication (34). Although the viral infectivity is sensitive to ultraviolet radiation, the transforming ability is up to five times less sensitive for an equivalent dosage (63). For storage purposes lyophilization or freezing at -70°C have proven very effective (43) (44).
iv. Polyoma DNA

Early in polyoma research, it was found that DNA, extracted from parotid tumors, was capable, on injection into newborn mice, of causing tumor formation (64) (48). This effect was abrogated on digesting the DNA with DNase prior to injection. It was later shown that py DNA, extracted from purified virions grown in cell cultures, possessed this tumorigenic property and was moreover infectious in mouse embryo cells (24) (107).

Polyoma DNA was found to have a buoyant density on CsCl of 1,709 gm per ml and a base composition of 48% G + C (93) (17). This base composition, along with a unique base sequence and infectious properties, distinguishes py from other papova viruses (117). The viral DNA has a molecular weight of $3 \times 10^6$ (15) (109) and comprises about 10 to 12% of the mass of the virion (116).

Polyoma virus DNA may be separated into three distinct types called type I, II and III. When py DNA is sedimented on a neutral pH sucrose gradient in 0.2 M NaCl, these three types sediment at 20s, 16s and 14s respectively (113). An equivalent separation may be achieved by centrifugation on neutral pH CsCl at a density of 1.50 gm per ml (119). Under alkaline conditions (pH 12.5) py DNA separated in a different manner. In sucrose gradients at pH 12.5 (52a) or CsCl (103) at a density of 1.50 at pH 12.5, py DNA separated into three components of which type I sedimented at 53s, while type II
sedimented at 18s and 16s, and type III at 16s. Type I DNA may be separated from types II and III by isopycnic centrifugation in neutral CsCl at a density of 1.600 gm per ml in the presence of ethidium bromide (83). A similar separation may also be achieved by the use of hydroxyapatite column chromatography (5).

Type I polyoma DNA (py I DNA) has been shown to be a double stranded circular helical molecule supercoiled upon itself. A single strand break led to the conversion of type I into type II molecules, that is, the supercoil to the open circle form (113) (103). It was recently shown that the replication of py DNA involves enzyme mediated strand opening and closing (49). Py DNA III was found to have a molecular weight similar to types I and II but a slightly lower density on CsCl. This is believed to be a result of it being, to a large part, cellular in origin. Virions containing this form of cellular DNA are called pseudovirions and make up varying proportions of viral preparations (70).

v. Proteins

Initial studies on py proteins have revealed that the virus contained one major capsid protein or one major protein and an internal component (101) (33). More recent data have shown that the virus contains six or seven polypeptides having molecular weights between 86,000 and 15,000. Of these proteins, P-2 which is thought to be a capsid
protein, accounts for 50 to 70% of the viral protein content. In addition, there is evidence that proteins P-5 to P-7 are host cell histones (81).

4. The Virus Infected Cell

Polyoma virus has been observed to interact with mammalian cells in three different ways.

In the abortive infection, which was the predominant interaction of py virus with rat embryo cells, the virus was taken up by the cells, and led to the eventual stimulation of cellular DNA synthesis at 10 to 16 hours pi. The virus was eventually lost from most cells and no further effects were observed in the majority of the cells in the population (89). Hamster cells also took up py virus efficiently (4). However, only BHK 21 cells showed a stimulation of DNA synthesis in the absence of virus replication. In addition extensive integration of the viral DNA into the cell DNA was observed in these cells (2) (54). This situation also appeared to represent an abortive infection.

The second type of interaction was transformation. This situation occurred in 0.1 to 12% of the cells in virus infected hamster or rat cultures (89) (104). The affected cells lost their capacity for contact inhibition and their oriented growth pattern, and hence piled up in heavy whorls. These cells also had altered morphology,
an increased growth rate and a higher capacity to be successfully transplanted into animals (104) (22) (99) (67). At least part of the characteristics of the transformed cells is thought to be due to membrane changes. This phenomenon was reflected by the increased ability of the transformed cells to bind concanavalin A and be agglutinated as compared to normal cells (9).

Transformed cells have been shown to carry on the synthesis of viral DNA and RNA and possibly viral protein (115) (3) (36). Only a fraction of the viral genome could transcribe into viral RNA (69), the latter accounting for less than 0.025% of the total RNA synthesized in the cell (3) (53).

A relatively large amount of py virus was found to be needed to cause a transformation event in an infected cell culture. Depending on the cell and virus types, between $10^4$ and $10^5$ plaque forming units (pfu) of virus were needed for such an event (67), although only one virus particle was apparently responsible for the transformation. Since purified py DNA could cause transformation (24), it was considered to be the acting principle responsible for this event.

Polyoma virus has been successfully rescued from transformed rat cells (35). Attempts to rescue the virus from py transformed hamster cells have however met with failure (54). This could be accounted for by the fact that these cells appeared to harbour an incomplete viral genome (53).
Because transformed cells develop into tumors when transplanted into newborn mice and hamsters, they are considered to be an "in vitro" model for tumor formation by py virus in animals.

The third type of virus cell interaction, the lytic or productive infection will be dealt with in more detail below.

5. The Productive or Lytic Infection.

The lytic infection with py virus was found to be restricted almost entirely to mouse cells, particularly mouse kidney and mouse embryo cells. There have been observations that py virus replicates in kidneys of newborn hamsters (46), but hamster cells in general do not support the replication of py virus "in vitro" (54). In consequence, only the mouse kidney and mouse embryo culture systems will be considered.

a. Adsorption and Penetration

In a mouse kidney cell culture infected with py virus at a multiplicity of infection (moi) of 100 pfu per cell, 60% or more of the cells have been observed to produce virus at 30 hours post infection (pi). In a mouse embryo cell culture, infected with py virus, an moi of 1000, 90% to 100% of the cells have been shown to be virus producers (118) (97). These studies showed that in contrast
to the transformation phenomenon, the lytic or productive infection actively involved the majority of the cells of the infected culture, and hence could be studied in a more quantitative fashion.

Studies on adsorption kinetics of py virus indicated that 50% of the virus was taken up by mouse embryo cells in 30 minutes, and nearly 100% of the virus was taken up in 4 hours \((\text{II}_8)\). Through autoradiography, it was shown that the labelled DNA of the infecting virus appeared in the cytoplasm by three hours and in the nucleus by six hours after infection \((\text{61})\).

b. Early RNA Synthesis

After the virus was uncoated in the infected cell, at least part of the viral DNA was transcribed into early py RNA \((\text{3})\). The synthesis of this RNA was essential for the eventual progress of the infection and if interfered with, the infection cycle ceased \((\text{37a})\). This RNA will be considered in more detail below.

c. Enzyme Induction

Following the beginning of early py RNA synthesis, there was an induction of some enzymes involved in DNA and pyrimidine biosynthesis \((\text{27}) (\text{62}) (\text{59})\). It is not known if any of these enzymes are coded for by viral genes.
d. DNA Synthesis

In mouse embryo (ME) cell cultures, infected with py virus at low multiplicity, cell DNA synthesis was stimulated at 12 to 15 hours pi. In this case, the commencement of synthesis of viral DNA lagged about 3 hours behind the stimulation of the cellular DNA synthesis (12). On the other hand, it has been reported that under conditions of high infectious multiplicity, ME cell cultures underwent a depression in their DNA synthesis (7).

In mouse kidney (MK) cells infected with py virus, viral DNA synthesis has been observed as early as 12 hours pi, while cellular DNA synthesis was stimulated at about 14 hours after infection (112) (111) (50). Viral DNA synthesis reached a maximum at about 35 hours pi when it accounted for 11% of the total DNA synthesis as measured by incorporation of $^3$H-thymidine. About 1% of the synthesized DNA was eventually encapsidated (111) (50).

Studies on the replication of the py DNA showed that the replicative form existed as type II DNA which contained at least two single stranded regions and that there was replication in two directions along the molecule (6). In this regard, enzymes have been found in the nuclei of infected cells which could induce single stranded nicks into the superhelical DNA molecule. These enzyme molecules could also have been involved in a swivel mechanism for DNA replication (11).
e. Viral RNA Synthesis

The development of efficient techniques of DNA-RNA hybridization has made it possible to study py RNA synthesis in infected cells. The level of py RNA synthesis was found to be dramatically increased after the replication of py DNA. Hence the py RNA synthesized prior to py DNA synthesis is referred to as "early" py RNA while that synthesized after DNA synthesis is referred to as "late" py RNA (3).

Initial studies on early py RNA were performed in ME cells where it was shown that this species of py RNA made up to 0.01% of the labelled ME cell RNA and was synthesized up to 16 hours pi (3). In later studies done in py infected MK cell cultures early py RNA was shown to be synthesized from 6 to 12 hours pi, when it made up 0.003 to 0.006% of the total pulse labelled cell RNA. Late py RNA synthesis was then observed to begin at 12 hours and to continue up to 30 hours pi. At this time up to 3 to 6% of the pulse labelled cell RNA was py RNA. If py DNA synthesis was blocked with FUdR in the infected cell only early py RNA synthesis occurred (55).

A summary of the chronological order of the metabolic events outlined above may be seen in fig. 1 (110).

A comparison by competition hybridization between late and early py RNA in terms of base sequence, showed that the early py RNA was at least partly homologous to the late py RNA. In addition, most of the early RNA species were also found to be transcribed late in infection (55).
Fig. 1. Tentative scheme for the lytic cycle of polyoma virus in contact-inhibited primary mouse kidney tissue culture cells. The marked asynchrony of the time course of infection (Fig. 2 and ref. 10-16) is due to the varying length, in individual cells, of phase 1. If Py-induced synthesis of viral and cellular DNA is inhibited with 5-fluorodeoxyuridine, little if any capsid protein or chromosomal protein is synthesized; however, the events of phase 1 which lead to the appearance of T-antigen and to the subsequent activation of the cellular DNA-synthesizing apparatus take place just as they do in the absence of the inhibitor.
Preliminary studies on the size of the viral RNA revealed that it sedimented heterogeneously between 4s and 45s in a sucrose gradient (55). The processing of py RNA has been studied to a limited degree (58). Nuclear py RNA was found to be larger than polysomal py RNA. It was also shown that a large portion of the former was degraded within the nucleus without being further processed.

Similar studies have been done on SV40 RNA in terms of size and processing. BSC-1 cells infected with SV40, contained polysome associated, early viral RNA of $8 \times 10^5$ daltons, while the corresponding late RNA appeared to be $7.9 \times 10^5$ and $5.6 \times 10^5$ daltons (102). In SV40 infected VERO cells, early viral RNA from polysomes sedimented at 15 to 17s ($5.6 \times 10^5$ daltons) while the corresponding late RNA sedimented at 28s ($1.7 \times 10^6$) and 17s ($6 \times 10^5$) (68). In these same cells, SV40 specific nuclear RNA sedimented heterogeneously, though there was a predominance of RNA sedimenting around 34s, while species sedimenting as fast as 50s were also observed. Preliminary studies have suggested that SV40 specific RNA which sedimented faster than 28s may be cell-viral linear hybrid molecules (56). In this regard, it is of interest to note that in SV40 transformed cells, virus specific RNA sedimenting faster than 28s had been found (66). This observation along with the evidence that some transformed cell lines harbour less than one full viral genome (53) lends credence to the hypothesis of linear hybrid RNA composed of both cell and viral base sequences.
f. Viral Protein Synthesis

On a theoretical basis, the py genome of $3 \times 10^6$ daltons, of which 50% is transcribed in the virus infected cell (69), could yield an RNA molecule of $1.5 \times 10^6$ daltons. This in turn could code for about $1.5 \times 10^5$ daltons of protein of which not all need be incorporated into the virion.

The proteins coded for by early py RNA are essential for the progress of the infection, as shown by use of protein specific metabolic inhibitors (37a). The only detectable early virus specific protein identified so far is the T antigen (38), while the late viral proteins are believed to be structural viral proteins which have been discussed previously.

More insight into early viral proteins has been obtained by immunoprecipitation assay with SV40. In this case, late viral proteins from infected cells revealed five distinct precipitin bands, while with cells treated with Ara C, a DNA synthesis inhibitor, or transformed cells, only two of these bands were present (25).

6. Biochemistry of RNA Synthesis in Mammalian Cells

a. Processing of RNA

RNA synthesis in mammalian cells has been studied extensively
owing to the development of efficient labelling procedures, specific inhibitors and the occurrence in the cells of large quantities of distinct RNA species, such as ribosomal RNA (r-RNA) and transfer RNA (t-RNA). Ribosomal RNA has been shown to make up about 80% of the cells' RNA content, while t-RNA and other s-RNA made up about 19%. The remainder is mostly messenger RNA (m-RNA) (18).

Actinomycin D, a rapid inhibitor of RNA synthesis, has proven to be a valuable tool in the study of the processing of RNA (39). Although this drug has been criticized with respect to its side effects (75), most of the information about RNA processing obtained through the use of actinomycin D has been confirmed by alternative approaches (88). A strong argument favouring the use of Actinomycin D in our system is that the drug does not affect the replication of RNA viruses (84), or in low concentrations of the drug, of polyoma virus (13). It is, nevertheless, a worthwhile policy to confirm findings on RNA processing obtained with the help of Actinomycin D by an alternate method.

Through the use of Actinomycin D it was shown that r-RNA is transcribed as a molecule sedimenting at 45s which is then sequentially processed into intermediate forms, sedimenting at 41s and 36s. The 36s intermediate breaks down into 18s ribosomal RNA and a 32s intermediate which breaks down into 28s ribosomal RNA. It is the 18s, 28s and 7s RNA molecules which form the ribosomes (18). These studies reveal that the processing of RNA in mammalian cells involves
degradation of a large molecule to smaller units as well as the complete breakdown of a substantial portion of the originally transcribed RNA molecule.

The model for processing of r-RNA proved inadequate to explain the processing of m-RNA (95) (92) (73). Initial studies on m-RNA labelled with $^{32}$P indicated that a large proportion of the labelled molecules sedimented faster than 45s. Though these molecules were not associated with polyribosomes, they did have a DNA like base composition (95), and did not appear to be associated with the molecules as were the r-RNA molecules (105). These two properties differentiate this form of RNA from the r-RNA (73) (18). Because this RNA was found mainly in the nucleus it has been termed heteronuclear RNA (Hn RNA). Since it resembled m-RNA or DNA in base composition; this RNA has also been referred to as messenger-like RNA (ml-RNA) or DNA like RNA (d-RNA) (85) (37).

The Cascade Hypothesis is a model proposed to explain the processing of ml RNA to m-RNA (87) (86). The model states that 3 to 10% of the cell DNA is transcribed into a heterogeneous array of ml RNA molecules varying from 1 to $15 \times 10^6$ daltons. These molecules, which have a number of repeating sequences, become associated with protein immediately after transcription to form ribonucleoprotein complexes (RNP). The less stable regions of these molecules are degraded and the more stable regions of the molecules then sediment between 6 and 35s ($0.05$ to $2 \times 10^6$ daltons).
These RNA molecules associated with protein may then be stored in the nucleus or transported into the cytoplasm. There they may be stored as RNP complexes or processed further into the polysomes for translation.

In duck erythroblast cells, it was found that it takes about 20 minutes for newly labelled RNA to reach the cytoplasm where the half life of the RNA was 3 to 6 hours.

b. Polyadenylate Sequences

Additional significant features of the ml RNA described above are: 1) the biphasic shape of their decay curves (suggesting two possible fates of ml RNA molecules); 2) only 20 to 25% of such RNA is transported into the cytoplasm, and 3) the presence of adenosine rich sequences.

A study of the m-RNA and Hn-RNA (or ml-RNA) molecules revealed that these species have covalently linked with them a 200 nucleotide long polyadenylic acid (poly(A)) region, at the 3' end of the molecule (60) (82) (91). There is evidence that the poly A sequence was added post-transcriptionally (20) (79) and appeared to function in the processing or stabilization of the Hn-RNA and m-RNA (72).

Initial quantitative studies have shown that 10 to 20% of the nucleoplasmic RNA and 46 to 60% of the polyribosome associated RNA contained poly(A) sequences (90). However more recent studies have
shown that in mouse L cells, 20% of the Hn-RNA and 100% of the m-RNA contain poly(A) sequences (40). These data along with the previously mentioned observation that only 20% to 25% of the Hn-RNA entered the cytoplasm from the nucleus, indicate that possibly only Hn-RNA molecules possessing poly A sequences are processed to m-RNA.

In cells infected with nuclear DNA viruses, the association of viral m-RNA with poly A has also been observed. Simian virus 40, the DNA of which has no poly A or poly dT regions corresponding in length to poly A described above, nevertheless has poly(A) associated with its RNA. A quantitative study has shown that 50 to 70% of the SV40 m-RNA from polyribosomes possesses poly(A) sequences (114). A similar study with adenovirus type 2 has revealed that while the viral DNA contained no long poly(dA) or poly(dT) sequences, the adenovirus m-RNA from polyribosomes of infected cells had up to 65 to 80% of its molecules associated with poly(A) (79).

7. Purpose of the Research Project.

The original objective was to use the py RNA as a model system to study the analysis and processing of a defined species of RNA which could be easily and quantitatively detected in the presence of the cellular RNAs.

In this context, it was also of interest to determine if py virus genes are transcribed, processed, and translated in the same manner as the cellular genes.
CHAPTER II: MATERIALS AND METHODS

A. Materials

1. Cells

Primary mouse kidney cells (MK) and secondary mouse embryo cells (ME) were prepared from randomly bred, white Swiss mice, purchased from the Connaught Medical Research Laboratories, Toronto or from the Faculty of Medicine Animal Unit, University of British Columbia. A continuous line of baby hamster kidney cells, BHK-21 (clone 13) and a continuous line of polyoma virus transformed hamster cells, PyH, were purchased from Microbiological Associates, Bethesda, U.S.A.

2. Virus

The wild type large plaque forming strain of polyoma virus used in these experiments was obtained from Dr. R. Weil, Swiss Institute for Experimental Cancer Research.

3. Growth medium and other biological compounds

Dulbecco modified minimal essential medium (MEM) purchased in powder form from Grand Island Biological Company (GIBCO) was used for
growing cells. The medium was sterilized by filtration through Millipore membrane of pore diameter 0.22 μm and made up to 100 units per ml in penicillin, 100 μgm per ml in streptomycin and 0.25 μgm per ml in fungizone. In later portions of the research, the first two of the above antibiotics were replaced by gentamicin (Biocult Laboratories) at 50 μgm per ml. The medium was also supplemented with 10% newborn calf serum purchased from GIBCO or fetal calf serum purchased from Flow Laboratories, Rockville, U.S.A.

Guinea pig blood, for hemagglutination studies was obtained from a guinea pig kept only for this purpose.

<table>
<thead>
<tr>
<th>Name of Compound</th>
<th>Dealer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin D</td>
<td>Merck, Sharp and Dohme, Montreal (Gift)</td>
</tr>
<tr>
<td>Uridine</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>Cordycepin</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>Polyadenylate</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>Polycytidylate</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>Polyuridylate</td>
<td>Sigma Chemical Company: Miles Laboratories</td>
</tr>
<tr>
<td>Ribonuclease A</td>
<td>Sigma Chemical Company</td>
</tr>
<tr>
<td>Deoxyribonuclease</td>
<td>Worthington Biochemical Co.</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Worthington Biochemical Co.</td>
</tr>
<tr>
<td>$^3$H-uridine (42 Ci/m mole)</td>
<td>New England Nuclear Co.</td>
</tr>
<tr>
<td>$^3$H-adenosine (35 Ci/m mole)</td>
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<td>$^3$H-uridine (28 Ci/m mole)</td>
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<td>$^3$H-adenosine (12.1 Ci/m mole)</td>
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<tr>
<td>$^{14}$C-uridine (60 mCi/m mole)</td>
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### Chemicals

<table>
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<td>Cesium chloride</td>
<td>Schwartz Mann Co.</td>
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<tr>
<td>Nonidet-P-40 (NP-40)</td>
<td>Laboratory of Dr. M. Smith (originally purchased from Shell Oil Co.)</td>
</tr>
<tr>
<td>Reagents for polyacrylamide gel electrophoresis</td>
<td>Eastman Organic Chemicals Ltd.</td>
</tr>
<tr>
<td>Spectrafluor</td>
<td>Amersham Searle Co.</td>
</tr>
<tr>
<td>Agarose</td>
<td>Bausch and Lomb Co.</td>
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</tbody>
</table>

All remaining reagents were purchased from Fisher Chemical Co.

All water used was glass distilled.

All glassware used in operations involving nucleic acids was washed with chromic acid.

All tissue culture glass was sterilized by autoclaving at 15 psi of steam for 20 minutes followed by heating in an oven at 170°C for 6 hours.
5. Solutions

a. Phosphate Buffered Saline (PBS) (26)

<table>
<thead>
<tr>
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<th>Concentration (M)</th>
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<tbody>
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<tr>
<td>KCl</td>
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<tr>
<td>(\text{Na}_2\text{HPO}_4)</td>
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<tr>
<td>(\text{KH}_2\text{PO}_4)</td>
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<tr>
<td>MgCl(_2)</td>
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<td>CaCl(_2)</td>
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b. Stock Saline

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<th>Concentration (M)</th>
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<tr>
<td>NaCl</td>
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</tr>
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c. RNA Buffer (55)

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<th></th>
<th>Concentration (M)</th>
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<tr>
<td>NaCl</td>
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</tr>
<tr>
<td>(\text{CH}_3\text{COONa})</td>
<td>0.01</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

adjusted with HCl to pH 5.2

d. Reticulocyte Standard Buffer (RSB\(^0\)) (80)

<table>
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<tr>
<th></th>
<th>Concentration (M)</th>
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<tr>
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<td>0.01</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>0.0015</td>
</tr>
<tr>
<td>Tris</td>
<td>0.01</td>
</tr>
</tbody>
</table>

adjusted with HCl to pH 7.5
e. RSB+

\[
\begin{align*}
\text{NaCl} & \quad 0.1 \text{ M} \\
\text{MgCl}_2 & \quad 0.015 \text{ M} \\
\text{Tris} & \quad 0.01 \text{ M} \\
\end{align*}
\]
adjusted with HCl to pH 7.5

f. Standard Saline Citrate Solution (1 x SSC) (52a)

\[
\begin{align*}
\text{NaCl} & \quad 0.15 \text{ M} \\
\text{sodium citrate} & \quad 0.015 \text{ M} \\
\end{align*}
\]
A stock solution was made up to 2 x SSC and diluted with distilled water to required concentration. Each large batch was treated with a few drops of CHCl₃ as a preservative.

g. STE Buffer (80)

\[
\begin{align*}
\text{NaCl} & \quad 0.1 \text{ M} \\
\text{EDTA} & \quad 0.001 \text{ M} \\
\text{Tris} & \quad 0.01 \text{ M} \\
\end{align*}
\]
adjusted with HCl to pH 7.5

h. Scintillation Fluid

\[
\begin{align*}
\text{Spectrafluor} & \quad 42 \text{ ml} \\
\text{Toluene} & \quad 958 \text{ ml} \\
\end{align*}
\]
i. Polyacrylamide Gel Electrophoresis Solutions

(1) Running buffer

<table>
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<tr>
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<tbody>
<tr>
<td>Tris</td>
<td>0.04 M</td>
</tr>
<tr>
<td>CH$_3$COONa</td>
<td>0.02 M</td>
</tr>
<tr>
<td>EDTA (Na$_2$)</td>
<td>0.001 M</td>
</tr>
<tr>
<td>SDS</td>
<td>0.2% (w/v)</td>
</tr>
</tbody>
</table>

Adjusted with HCl to pH 7.4

(2) Acrylamide gel solutions (106)

Solution A: Acrylamide 90% (w/v)
N,N-bis methylene acrylamide 2.0% (w/v)
Tris 0.48 M

Adjusted with HCl to pH 8.4

Solution B: Ammonium persulphate 0.2% (w/v)
NNN'N'tetramethylethylene diamine 0.1% (w/v)

Solution C: Agarose 0.5% (w/v)
Tris 0.243 M

Tris solution adjusted with HCl to pH 8.4 prior to addition of agarose

J. Millipore Binding Buffer (MBB) (65)

<table>
<thead>
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<th>Component</th>
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<tbody>
<tr>
<td>KCl</td>
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</tr>
<tr>
<td>Tris</td>
<td>0.01 M</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>0.001 M</td>
</tr>
</tbody>
</table>

Adjusted with HCl to pH 7.5
k. Polyuridylylate Binding Buffer (90)

NaCl 0.1 M
Tris 0.01 M
adjusted with HCl to pH 7.5

l. Hank's Balanced Salt Solution (modified) (55)

<table>
<thead>
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<th>Component</th>
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<tbody>
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<tr>
<td>NaCl</td>
<td>0.1470 M</td>
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<tr>
<td>KCl</td>
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<tr>
<td>KH$_2$PO$_4$</td>
<td>0.0044 M</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>0.0150 M</td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.0006 M</td>
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Sterilized by autoclaving at 15 lbs steam for 20 minutes.

m. SDS-EDTA Buffer (50)

<table>
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<tbody>
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<tr>
<td>EDTA</td>
<td>0.01 M</td>
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</table>

adjusted with 1 M NaOH to pH 7.0

n. Alsever's Solution

<table>
<thead>
<tr>
<th>Component</th>
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</tr>
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<tbody>
<tr>
<td>Glucose</td>
<td>0.11 M</td>
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<tr>
<td>Sodium Citrate</td>
<td>0.027 M</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.072 M</td>
</tr>
</tbody>
</table>

Sterilized by autoclaving at 15 lbs steam for 20 minutes.
B. Methods

1. Primary Mouse Kidney Cell Cultures

Baby mice, 7 to 10 days old, were killed and washed in 95% ethanol. Their kidneys were aseptically removed with sterile forceps through a dorsal incision. This operation was carried out in an ultraviolet light sterilized hood.

The kidneys were macerated mechanically by forcing them through a plastic 10 ml syringe whose needle had been replaced by a 40 mesh stainless steel screen. The tissue was then incubated in 0.25% trypsin in Hank’s solution for 20 to 40 minutes at room temperature, on a magnetic stirrer. The cells were collected by centrifugation in an ambient temperature International CS centrifuge for 10 minutes at 2000 rpm in 40 ml centrifuge tubes. The cells were then washed by resuspending them in fresh Hank’s solution and again centrifuging them out of solution. This procedure was repeated two more times.

Following the final wash, the centrifuged cell pellet was re-suspended in MEM prewarmed to 37°C and containing 10% calf serum, at a resuspending ratio of two mouse kidneys per 10 ml of MEM. Ten ml aliquots of the cell suspension were dispensed into 90 mm tissue culture petri dishes (Falcon Plastics) which were then incubated at 37°C in an atmosphere of 5% CO₂ and saturating humidity in a National Appliance Co. CO₂ incubator. The cells formed monolayers within 24 hours.
The overlaying medium and cell debris were removed from the monolayers by aspiration and 10 ml of fresh MEM containing 10% calf serum was added to each culture. Following the medium change, the cell monolayers became confluent within the next 48 hours.

2. Secondary Mouse Embryo Cell Cultures

Mouse embryos aseptically removed from their mothers in their second week of gestation were treated as were the kidneys, above, to produce primary mouse embryo cell cultures. The confluent cell monolayers were dissociated with Hank's solution containing 0.25% trypsin, washed with Hank's solution, resuspended at half of the original concentration in MEM+5% serum and again dispensed into petri dishes. The cells of new monolayer cultures which became confluent within 24 hours, were termed secondary mouse embryo cells.

3. BHK 21 Cells and PyH Cells

These cells were grown as monolayer cultures on 90 mm petri dishes. The cells were subcultured by trypsinizing the confluent monolayer cultures, as was done for the mouse embryo cells, and dispensing the cells in MEM+5% calf serum into new petri dishes at 1/3 of their original concentration. The monolayer cultures became confluent within 24 to 36 hours.
All the py virus used in this study was grown in primary MK cell cultures. These cultures were infected by two methods termed 'in vitro' and 'in vivo'.

a. The 'in vitro' infection: This method was used both in the production of virus stocks and in studies involving virus growth and development.

The overlaying medium of the confluent MK cultures was removed by aspiration and 0.5 ml of py virus suspension containing about $2 \times 10^9$ plaque forming units (pfu) added to each petri dish. During the adsorption period of one hour, the inoculated monolayer cultures were kept in a CO$_2$ incubator. Every 20 to 30 minutes they were withdrawn, and briefly rotated at an angle to redistribute the virus inoculum over the entire monolayer surface.

Following the adsorption period, 10 ml of MEM lacking serum were added to each petri dish culture, which was further incubated for 45 to 48 hours. The overlaying MEM was then aseptically collected and analyzed for virus content by the hemagglutination assay, while the remaining cell monolayer was saved for DNA extraction.

b. The 'in vivo' infection: Newborn mice were each injected subcutaneously with 0.03 ml of py virus containing at least $10^6$ pfu.
At the age of 12 to 14 days the mice were sacrificed and their kidneys used to make MK cultures. After forming a confluent monolayer, these cell cultures were monitored by hemagglutination assay every 12 hours for the appearance of py virus in the medium. By about the fourth day after the confluent monolayer of cells had formed, the level of virus in the medium reached 80,000 hemagglutination units (HAU) per ml. At this point the medium was collected for its virus while the remaining monolayer was used for DNA extraction.

c. Further Treatment and Analysis of the py Virus Preparation.

The medium containing the virus was chilled to 4°C and sonicated with a Bronwill Biosonic III on probe setting of 40, for 2 minutes. The virus was then dispensed in 5 ml amounts into either sterile glass vials or plastic tubes (Falcon Plastics) and frozen at -70°C in a Revco freezer until needed.

d. Hemagglutination Assay

The virus was titrated using guinea pig blood kept in an equal volume of Alsever's solution at 4°C for a period not exceeding 3 weeks. The blood was washed twice with stock saline and the final pellet resuspended up to 1% (v/v) in stock saline. Twelve serial twofold dilutions of 0.025 ml of the virus were made in a microtiter plate.
To each of these were added 0.025 ml of 1% guinea pig blood, and the plates placed at 4°C for 1 to 6 hours. The reciprocal of the highest dilution which produced hemagglutination was used to calculate the virus titer.

Periodically the reproducibility of this assay was tested by also doing a plaque assay on the same virus preparation. The virus titer calculated by the hemagglutination assay and obtained by the plaque assay were found to be in close agreement throughout this study.

5. Preparation of Polyoma Type I DNA (py I DNA) (50) (83)

Infected MK cell monolayers whose medium had been harvested for virus as outlined above, were used as a source of py I DNA. The cells were washed five times with PBS containing 0.001 M EDTA, and lyzed by addition of 0.8 ml of SDS-EDTA buffer to each petri dish. The dishes were gently rotated at an angle until the entire monolayer was exposed to the buffer. The lysates were collected into a 30 ml Corex (Corning) centrifuge tube with the aid of a rubber policeman. The contents of the tube were made up to 1 M in NaCl and mixed by gently inverting the tube 20 times.

After the preparation had been allowed to stand at 4°C for 8 to 16 hours, it was centrifuged at 12,500 rpm for 20 minutes in an SS 34 rotor at 4°C in a Sorvall RC2B centrifuge. While the pellet was discarded, the supernatant was mixed with an equal volume of phenol
saturated with 1 M tris buffer at pH 8.0 at 4°C. The mixture was centrifuged at 10,000 rpm for 10 minutes to separate the phenol from the aqueous phase. The aqueous phase was re-extracted with phenol as above and again re-extracted with chloroform containing 4% isoamyl-alcohol. The aqueous phase from this last extraction was mixed with two volumes of ethanol and kept overnight at 4°C to precipitate the DNA.

The ethanol precipitate, collected by centrifugation at 10,000 rpm at 4°C for 10 minutes, was redissolved in 0.2 x SSC, 0.0001 M EDTA. The equivalent of 10 to 15 ml of the original cell lysate was dissolved in a 4 ml volume. The solution was made up to a density of 1.60 gm per ml with CsCl and to a final concentration of 100 μgm per ml in ethidium bromide. A Bausch and Lomb refractometer was used to determine the refractive index of the solution (a reading of 1.390 corresponded to a CsCl density of 1.60 gm per ml).

Aliquots of about 5 ml of this solution were dispensed into 2½ inch by 5/8 inch nitrocellulose tubes whose inner surfaces had been treated with 1% silane. The tubes were filled to the top with paraffin oil, capped and placed into a Beckman Type 50 rotor and centrifuged for 36 hours at 20°C at 40,000 rpm in a Beckman L2-65 B ultracentrifuge.

Following centrifugation the tubes were observed against a light background for the presence of deep red bands in the gradient. The location of these was marked on the tube surface. The gradients were
collected into fifteen equal fractions by puncturing the bottom of the tubes and dripping the contents through. The fractions containing the lower band, which was located around the middle of the gradient, were pooled and combined with the corresponding fractions from other parallel gradients. After adjustments of the density of these solutions to 1.60 gm per ml, they were again centrifuged and fractionated under the above conditions.

The pooled fractions containing the lower band were then extracted three times with equal volumes of isopropanol (saturated with aqueous CsCl) to remove the ethidium bromide. The CsCl was then removed by dialyzing the preparation against 1000 volumes of 0.01 x SSC and 0.0001 M EDTA for at least 12 hours. The preparation was now considered to consist of pure py I DNA.

6. Labelling and Preparation of RNA from py Infected Cells
   (55)

a. Labelling

Polyoma virus infected cultures were labelled with $^3$H-uridine by replacing the overlaying medium of the monolayers with 2 ml of MEM containing a total of 40 μCi of $^3$H-uridine. The petri dishes were rotated at an angle every 30 minutes to ensure even distribution of the medium over the cell surface.
After 2 hours of incubation the radioactive medium was decanted and the monolayers washed five times at room temperature with PBS and twice with RNA buffer. One ml of RNA buffer containing 1.0% SDS was added to the petri dishes and distributed over the surface by gentle rotation. The cell lysates were collected into a 15 ml or 30 ml Corex tube with the help of a rubber policeman.

b. Phenol Extraction

The cell lysate was mixed with an equal volume of phenol (saturated in RNA buffer) by vigorous agitation. The mixture was incubated in a water bath at 60°C for 3 to 4 minutes, again agitated and plunged into an ice water bath, for an additional 5 minutes. The preparation was then centrifuged at 10,000 rpm at 4°C for 10 minutes in a Sorvall centrifuge. The phenol phase was discarded while the interphase and the aqueous phase were re-extracted two more times with RNA buffer saturated phenol at 4°C. In these two extractions the aqueous phase alone was collected. This aqueous phase from the last extraction was made up to 0.2 M in NaCl and mixed with 2 volumes of ethanol and kept at -20°C for at least 4 hours to precipitate the RNA.

The precipitate was collected by centrifugation at 10,000 rpm for 10 minutes at 4°C, made up to 2 ml in RNA buffer at 4°C and reprecipitated with ethanol under above conditions. This ethanol
precipitate was resuspended in 2 ml of RNA buffer at pH 7.2, and
treated with RNase free DNAse at 20 μg/ml for 20 minutes at 37°C. The reaction was stopped by adding SDS to a concentration of 0.5%
(w/v) and the preparation was extracted with cold phenol saturated
in RNA buffer. The aqueous extract was precipitated two more times
with ethanol, and finally made up to 0.1 to 0.3 ml 0.001 M EDTA.

c. Analysis and Storage of RNA

Samples of this RNA (10 μl) were analyzed for absorbance spectrum
in the ultraviolet range on a Beckman DBG spectrophotometer and for
the radioactivity on a Nuclear Chicago Mark II scintillation counter
which had a background of 15 cpm. A sample was also hybridized with
denatured py I DNA to measure the amount of py RNA present.

The remainder of the RNA was frozen at -70°C. It was generally
used between 1 to 4 weeks after freezing.

7. Modifications of the RNA Extraction Method

a. Use of Pronase

Pronase (fungal protease (Sigma)) was used in conjunction with
1% SDS in RNA buffer to lyse the infected cell monolayer. The lysis
was carried out for 15 minutes followed by phenol extraction as out-
lined above.
b. Use of Phenol-Chloroform (77)

A preparation of equal parts phenol and chloroform saturated in RNA buffer was used in place of phenol. The extraction was done at 4°C.

c. Use of Phenol at pH 9.0 (65)

A preparation of phenol saturated with 0.05 M KCl, 0.05 M tris pH 9.0 and 0.001 M MgCl₂ was used in place of phenol. The extraction was also carried out at 4°C.

8. Cell Fractionation

a. Cytoplasmic and Nuclear Extracts (76) (14)

Virus infected ³H-uridine labelled MK cell monolayer cultures were washed 5 times with RSB⁺, and treated with 0.4 ml of RSB⁺ containing 1% NP₄O which was spread over each monolayer by gentle rocking. The cultures were kept at 4°C for 10 minutes and periodically rocked. The monolayers, which were already showing signs of disintegration, were scraped with a rubber policeman into a 6 ml Dounce homogenizer (Kontes) and further disrupted by two or three strokes. The broken cell preparation was collected into a 15 ml
conical centrifuge tube and centrifuged for 10 minutes at 2000 rpm in a swinging bucket rotor in an International Clinical Centrifuge. The supernatant which contained the cytoplasmic extract was removed with a Pasteur pipette while the pellet was resuspended in RSB° for 5 to 10 minutes, and again homogenized with five or six strokes of the Dounce homogenizer. The preparation was again centrifuged as outlined above, the supernatant discarded while the pellet which contained the nuclei was collected and resuspended in RNA buffer. Both the cytoplasmic extract and the nuclei, which were made up to 0.5% in SDS (w/v), were extracted with phenol saturated with RNA buffer at pH 5.2.

In initial work in this study no detergent was used. Instead, the washed cells were swollen in RSB° for 15 minutes, scraped off the petri dish with a rubber policeman and disrupted with 5 or 6 strokes of a Dounce homogenizer. The remaining procedure was as outlined above.

b. Polyribosomes (80)

For a polyribosome preparation, the cytoplasmic extract was layered on a preformed 15% to 30% (w/v), RSB° sucrose gradient in a 3\(\frac{1}{2}\) by 1 inch nitrocellulose tube. The gradient was centrifuged at 4°C for 2 hours at 25,000 rpm in an SW27 rotor in a Beckman L2-65 B ultracentrifuge. The gradients were fractionated into 1 ml
fractions on an Isco (Instrumentation Specialties Inc.) fraction collector and ultraviolet monitor by downward displacement of the gradient. If the cytoplasmic extract was from an $^3$H-uridine labelled preparation, 25 µl samples of each fraction were analyzed for radioactivity.

Appropriate fractions of the above gradient were pooled, diluted 1:1 with ice cold RSB$^+$ and again sedimented on an SW27.1 rotor at 4°C for 6 hours at 27,000 rpm to pellet the polyribosomes. These were resuspended in RNA buffer, made up to 0.5% (w/v) with SDS and the RNA extracted with hot phenol as outlined above. Alternatively pools of fractions were made up to 0.5% (w/v) in SDS and mixed with 2 volumes of ethanol. The preparation was kept at -20°C overnight to precipitate the RNA. The precipitate was resuspended in RNA buffer and extracted once with hot phenol.

9. Preparation of Ribosomal RNA (80)

Monolayers of BHK or PyH cells were labelled with $^{14}$C-uridine at 0.2 µCi/ml in 5 ml of MEM for 16 hours. The labelling medium was removed and the cells incubated an additional 8 hours with non-labelled MEM. The cytoplasmic extract of these cells was made up to 0.5% (w/v) in SDS and extracted twice with hot phenol saturated in RNA buffer. RNA in 25 µl amounts was placed into vials and frozen at -70°C.
10. Fractionation of RNA

a. Sucrose Gradients (80)

Five ml sucrose gradients of 5 to 20% sucrose in STE-SDS buffer were formed in a Buchler two chamber gradient maker. On these were layered 25 to 200 μgm of RNA in a volume not exceeding 200 μl. The gradients were centrifuged at 42,000 rpm in a Beckman L2-65 B ultracentrifuge for $\frac{3}{2}$ hours at 15°C, then fractionated by downward displacement into 20 equal fractions. Samples of 10 μl were withdrawn from each fraction and assayed for radioactivity. Periodically the fractions of the gradient were analyzed for $A_{260}$ on a Beckman DBG spectrophotometer.

b. Polyacrylamide Gel Electrophoresis (106)

Polyacrylamide gels were made up by mixing one part solution A, one part solution B and two parts of molten solution C at room temperature, and then pouring the mixture into 150 mm by 16 mm glass tubes sealed at the bottom by parafilm stoppers. The solution was allowed 15 minutes to harden, whereupon the tubes were blocked at the other end with a dialysis membrane, and turned upside down in a Buchler electrophoresis stand. The gels were allowed to polymerize for two hours, the parafilm plugs removed and chambers filled with running buffer.
The RNA was made up to 50 μl in 10^{-3} M EDTA and 30% (w/v) in sucrose and 0.0001% bromophenol blue. The mixture was applied to the surface of the gel with a syringe. The anode was connected to the bottom of the gel and a current of 5 v per cm of gel applied. The electrophoresis was continued for 2\frac{1}{2} hours in which time the dye marker had migrated to within 1 cm of the bottom of the gel.

The gels were removed from the tubes under tap water pressure and sliced with a razor blade into 75 equal pieces. These were deposited directly into scintillation vials, which contained 2 ml of methanol to dehydrate the slices. Eight ml of scintillation fluid were added to each vial, and these were counted for radioactivity.

11. Hybridization (52)(52a)

a. Denaturation and Fixation of DNA

A known mass of Py I DNA in 0.01 X SSC was denatured by heating in a boiling waterbath for 20 to 25 minutes, then by rapidly chilling in an icewater bath to prevent renaturation. This DNA was made up to 50 ml in 6 x SSC and passed by gravity flow through a 4.5 cm Millipore filter presoaked in 6 x SSC. The filter was then washed with two 20 ml volumes of 6 x SSC under slight vacuum assistance. The filter was partially dried and cut into at least 20 equal size
discs 0.65 cm in diameter. Care was taken that only the surface in contact with the DNA was used. By this procedure, only about two thirds of the filter area exposed to the DNA was recovered. These filters contained approximately equal precalculated amounts of denatured DNA.

The filters were air dried, heated in an oven at 78°C for 2 to 4 hours. They were then stored at room temperature for periods up to 6 months. Blank filters were prepared in an identical fashion, but without DNA.

b. DNA-RNA Hybridization

The RNA preparation was made up in a scintillation vial to a total volume of 1 ml in 6 X SSC, 0.0001 M EDTA and 0.1% (w/v) SDS. To this were added duplicate filters containing DNA and duplicate blank filters. The vials were tightly sealed and incubated at 65°C for 20 to 24 hours in a water bath. The filters were marked with a pencil for identification.

The filters were removed, washed in batch with 50 ml of 2 x SSC, and incubated for 30 minutes at 37°C in 50 ml of 2 x SSC containing 20 μgm per ml of RNase A. They were again washed with 50 ml of 2 x SSC and counted for radioactivity. The radioactivity of the blank filters was subtracted from radioactivity of the DNA containing filters in all calculations.
12. Poly (A) Studies

a. Filtration through Millipore Filters (65)

A sample of labelled RNA was made up to 1.0 ml in Millipore binding buffer (MBB) and chilled to 4°C. The solution was passed by gravity flow through a Millipore filter, presoaked in MBB. The filter was washed in MBB and the filtrate and washings collected, and their radioactive content determined.

The amount of py specific RNA on the Millipore filter was determined by incubating the filter, cut into four equal sections, in a scintillation vial containing 1 ml of 0.5% SDS (w/v), 6 x SSC, 0.001 M EDTA and two Millipore filters each bearing 0.25 μg of py I DNA. The original cut up Millipore filters served to determine the background.

b. Filtration through Poly (U)-GF/C Filters (90)

A solution of polyuridylate (poly U) at 1.0 mg per ml was made up in distilled water. Of this 0.150 ml were dispensed onto glass fibre filters (Whatman GF/C). The filters were allowed 2 hours at 37°C to dry. The dry filters were irradiated with a 15 watt germicidal ultraviolet lamp at 22 cm from the filter for 10 minutes each side. About 70% of the poly(U) was retained by the filters after they were washed with 20 ml of distilled water.
A volume of 0.5 ml of RNA made up at room temperature in poly-
uridylic acid binding buffer (PUBB) was placed on one of the above filters
(also presoaked in PUBB) and passed through under gravity flow.
The filter was then washed with 4.5 ml of PUBB and dried under a
low vacuum. The filtrate and washings were collected and their
radioactivity determined by precipitation with 6% trichloroacetic
acid (TCA).
The RNA bound to the filter was removed by eluting the filter
with 1.5 ml of PUBB which was 0.001 M in NaCl, 0.001 M in tris pH
7.5. The eluted RNA was made up to 0.2 M in NaCl and precipitated
with 2 volumes of ethanol.
CHAPTER III: RESULTS-I

Characterization of Py RNA in Infected Cells

1. Polyoma Virus RNA Synthesis During the Course of Virus Infection

A preliminary step in the study of py virus RNA was to determine the pattern of RNA synthesis during the course of infection in our particular system. As outlined in fig. 2, infected MK cell cultures were labelled with $^3$H-uridine, at various times after infection, and their RNA extracted and purified. Samples of each RNA preparation were hybridized with py I DNA to determine the relative amount of py RNA synthesized. The appearance of this RNA in the infected culture with respect to time after infection, is represented by the solid line in fig. 2 which revealed that there was a very low level of virus RNA in the cell culture up to 16 hours pi, whereupon it increased linearly up to 40 hours pi, after which it remained constant.

The overlaying medium of each culture was saved, and was analyzed for py virus titer by hemagglutination. The pattern of virus release is shown by the broken line in fig. 2. Virus was first detected at 32 to 40 hours after infection and was then released in increasing amounts with time.

These results not only confirmed previously reported information of viral RNA synthesis (55), but they were also important from a
Twenty-one confluent primary MK monolayer cultures were infected with py virus at an moi of 50 pfu/cell. At specified times pi, the overlaying medium was removed from a group of 3 dishes, pooled and frozen at -70°C. The cell cultures were then incubated with 3H-uridine at 40 μCi/2 ml of MEM/dish for 2 hours. The RNA was extracted from the cells and fractions comprising one eighth of each preparation were analyzed for py RNA content. A total of about 300 μgm of RNA with a specific activity of about 3,500 cpm/μgm were obtained from each of the above samples.

The virus titer of the overlaying medium was determined by hemagglutination.
Fig. 2:

![Graph showing the relationship between c.p.m. hyb. x 10^-3 and H.A.U. x 10^-4 over hours post-infection (P.I.). The graph plots two datasets: one represented by solid circles and the other by triangles.](image-url)
technical point of view in that they demonstrated that in the system used in this laboratory viral RNA may be labelled from 24 to 36 hours pi. Other relevant information to come out of this experiment was that at 40 hours pi about 1.5% of the labelled RNA was virus specific and at 16 hours pi only 0.02% was virus specific while no net decrease in the total cell RNA synthesis was observed throughout the infection (data not shown). These values are also in agreement with previous reports (55).

2. Comparison of Py RNA Synthesis in MK, ME and BHK-21 Cells

Although py virus replicates mainly in mouse cells, there have been reports that it replicates in hamster tissue (46). An attempt was therefore made to compare viral RNA synthesis in py infected MK, ME and BHK-21 cells at 8 hours and 40 hours after they were infected with py virus. As shown in table 1, BHK-21 cells exhibited no significant synthesis of py RNA at any time after infection, while ME cells and MK cells exhibited an increase of py RNA to 0.005% (twice the background level) at 8 hours and 0.2% at 40 hours after infection.

It is of interest to note that while py virus was found to infect BHK-21 cells and integrate its DNA into the cellular DNA (2), there was no detectable transcription of the viral RNA.
Table I: Py RNA Synthesis in MK, ME and BHK-21 Cells.

Six petri dish cultures of secondary ME cells and of BHK-21 cells were treated as follows: Two cultures of each cell type were labelled with $^3$H-uridine for 2 hours at 200 μCi/2 ml of MEM/dish, and their RNA extracted and purified. The remaining cultures were infected with py virus at an moi of 50 pfu/cell. At 6 hours pi and 38 hours pi, two petri dishes of each cell type were labelled with $^3$H-uridine as outlined above, and their RNA extracted and purified. Samples (60 μgm) of each RNA preparation were analyzed for radioactivity and hybridized with 1 μgm of py I DNA. Specific activity of ME RNA was about 50,000 cpm/μgm and that of BHK-21 RNA was about 100,000 cpm/μgm.

The above experiment was repeated with BHK-21 and primary MK cell cultures. Specific activity of MK cell RNA was 3,000 cpm/μgm. Values of RNA hybridized at 40 hours were 850, 60 and 7,000 for MK, BHK-21 and ME RNAs.
Table I: Py RNA synthesis in MK, ME and BHK-21 Cells

<table>
<thead>
<tr>
<th>Time pi.</th>
<th>% cpm hybridized with Py DNA</th>
<th>Mouse Kidney</th>
<th>Mouse Embryo</th>
<th>BHK-21</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hours</td>
<td>0</td>
<td>0.003%</td>
<td>0.0007%</td>
<td></td>
</tr>
<tr>
<td>8 hours</td>
<td>0.008%</td>
<td>0.005%</td>
<td>0.0004%</td>
<td></td>
</tr>
<tr>
<td>40 hours</td>
<td>0.23%</td>
<td>0.20%</td>
<td>0.0003%</td>
<td></td>
</tr>
</tbody>
</table>
3. a. Size of Py RNA in Infected Cells

Preliminary studies on py RNA have shown that it sedimented heterogeneously in sucrose gradients and did not appear to contain any population of discrete size molecules (55). These results were confirmed and extended in the present study. Thus as shown in fig. 3(a) the py RNA was found to be a heterogeneous population of molecules, sedimenting from 4s to beyond 45s.

b. Sedimentation on DMSO Sucrose Gradients

In order to rule out the possibility that the heterogeneous behaviour of py RNA during sedimentation was due to configurational changes between molecules of similar size, RNA from the same preparation as used in fig. 3(a) was centrifuged on a sucrose gradient made up in dimethyl sulfoxide (DMSO). Sedimentation in DMSO tends to minimize the secondary structure in RNA molecules (100). As seen in fig. 3(b), the viral RNA still sedimented heterogeneously throughout the gradient as in fig. 3(a).

c. Polyacrylamide gel Electrophoresis

Polyacrylamide gel electrophoresis was also used as a method for analysis of py RNA size distribution, since the method resolves
The RNA was obtained from py infected MK cell cultures infected at an input multiplicity of 50 pfu/cell, incubated with $^3$H-uridine between 29 and 31 hours pi at 40 μCi/2 ml/dish, and harvested for RNA extraction at 31 hours pi. Specific activity of the RNA was 3,500 cpm/μgm. About 50 μgm of this RNA were sedimented through a sucrose gradient or DMSO sucrose gradient. After removal of 10 μl of each fraction for total radioactivity measurement on glass fibre filters, the fractions were pooled in pairs and used for the hybridization reaction with filters bearing 0.5 μgm of py DNA. Prior to hybridization, the DMSO fractions were dialyzed against 1,000 volumes of 0.001 M EDTA to remove the DMSO.

Direction of sedimentation was to the left.
Table 2: Elution of Py RNA from Polyacrylamide Gels.

Four 50 μgm quantities of nuclear RNA (7,000 cpm/μgm) from py infected MK cells were respectively placed into 13 x 150 mm test tubes. To each of 2 test tubes were added 0.5 ml of unpolymerized agarose-polyacrylamide solution; to one test tube only polyacrylamide solution, and to one test tube distilled water. The tubes were kept at room temperature for 2 hours and the contents of the tubes placed in a hybridization mixture with 1.0 μgm of py 1 DNA. One agarose-polyacrylamide gel was macerated with a pasteur pipette prior to hybridization.
Table 2: Elution of Py RNA from Polyacrylamide Gels

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CPM hybridized</th>
<th>% Py CPM recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA in polyacrylamide agarose</td>
<td>1324</td>
<td>74</td>
</tr>
<tr>
<td>RNA in polyacrylamide agarose (macerated)</td>
<td>1430</td>
<td>80</td>
</tr>
<tr>
<td>RNA in polyacrylamide</td>
<td>1560</td>
<td>88</td>
</tr>
<tr>
<td>RNA in solution</td>
<td>1791</td>
<td>100</td>
</tr>
</tbody>
</table>
Polyacrylamide gels, prepared as outlined in the Methods chapter, were overlayed with 100 μg of the total cell RNA preparation used in fig. 2. In addition, about 1,000 cpm of C-ribosomal RNA prepared from py H cells were also layered on the gel. The RNA was electrophoresed at 7 v/cm of gel for 3 hours, and sliced manually. The slices were individually dehydrated in scintillation vials with 2 ml of absolute methanol, and counted by addition of 8 ml of scintillation fluid to the vials. After counting for radioactivity, the slices were removed from the toluene, washed twice with 95% ethanol, and twice with 6 x SSC. They were then sequentially pooled in groups of 5 slices, and hybridized with 0.5 μg of py DNA.

Direction of migration is to the right.
Fig. 4:

![Graph showing fraction number vs. counts per minute (CPM) values for a sample experiment. The graph includes markers for 28s and 18s RNA, and the y-axis represents counts per minute (CPM) multiplied by 10^-3.]
Fig. 5: Resedimentation of py RNA in a Sucrose Gradient.

Purified, radioactive (10,000 cpm/μg) RNA was prepared from two MK cell cultures which had been infected with py virus at moi of 100 and labelled with $^3$H-uridine at 40 μCi/2 ml of MEM/dish. The RNA (100 μg) was layered on each of 2 sucrose gradients, centrifuged and fractionated. The fractions of one gradient, pooled in threes, were hybridized with py I DNA, while the fractions of the second gradient were monitored for radioactivity, pooled as indicated in fig. 5(a) and the pools were precipitated with ethanol. The ethanol precipitates were resuspended in 0.001 M EDTA and again sedimented on individual sucrose gradients. The fractions of the gradients were monitored for radioactivity and fractions, pooled in groups of 3, hybridized with py I DNA.
Fig. 5:
RNA species according to their molecular weights (106). A preliminary study on the elution of py RNA from the gel slices was done as outlined in table 2. This study indicated that incubation of the RNA trapped in the polyacrylamide agarose gel in a hybridization mixture led to the elution of at least 75% of the py RNA. This method was therefore considered adequate for monitoring py RNA distribution on gels after electrophoresis of infected cell RNA.

The results of such a study are shown in fig. 4. The viral RNA appeared to migrate heterogeneously with a size range similar to that found in sucrose gradients.

4. Resedimentation of Py RNA on Sucrose Gradients

To further test that the sedimentation characteristics of py RNA were not due to an artefact, the RNA which sedimented to different regions of a sucrose gradient, was recentrifuged on another sucrose gradient. As shown in fig. 5, virus infected cell RNA was fractionated on a sucrose gradient (5a), and RNAs in fractions corresponding to regions of 28s + (5b), 28s to 18s (5c) and 18s to 4s (5d) were recentrifuged. The viral RNA resedimented true to its original sedimentation value, with a fraction sedimenting as slightly smaller units. These may have been due to breakdown during handling or incomplete resolution on the original gradient.
5. Polyoma RNA from Nuclear and Cytoplasmic Fractions of Virus Infected Cells.

The preparation of nuclear and cytoplasmic extracts from MK cells proved difficult, using conventional techniques (76), since the cells would not readily detach from the monolayer. Accordingly, the procedure was modified so that washed cell monolayers were either swollen with RSB or treated with RSB$^+ \text{ containing } 1% \text{ NP}_40, \text{ for 15 minutes. The swollen or detergent treated cells were then removed from the culture dish with a rubber policeman. Although about half of the cells were broken by this method, as seen by phase contrast microscopy, the resuspended cells had to be further treated in a Dounce homogenizer to give near complete breakage of the cells.}

The isolated nuclear fraction of the above preparation was then resuspended in RSB and further disrupted with a Dounce homogenizer. No whole cells could be detected by phase contrast microscopy in the nuclear fraction so treated. When the supernatant of the re-homogenized nuclear fraction was further examined, it was found to contain about 10% of the radioactivity and py RNA content of the cytoplasmic extract. This RNA also sedimented on a sucrose gradient in a manner indistinguishable from that of the cytoplasmic extract.

Cell breakage was monitored by phase microscopy which revealed whole, cytoplasm free nuclei. Infected cells were also labelled with $^3\text{H-thymidine for 16 hours, further incubated in non-radioactive}$
Fig. 6: Sucrose Gradient Sedimentation of Py RNA from (A) Nuclei and (B) Cytoplasm, of Py Infected MK Cells.

Conditions of infection, labelling, sedimentation and hybridization were the same as in legend to fig. 3(a). Seventy-five μgm of nuclear RNA and 150 μgm of cytoplasmic RNA with respective specific activities of 7,200 cpm/μgm, and 1,700 cpm/μgm, were used.
medium for 8 hours, and nuclear and cytoplasmic fractions separated as above. Less than 3% of the TCA precipitable radioactivity was found in the cytoplasmic fraction, indicating that the nuclei were not significantly disrupted (14).

A quantitative study on a number of preparations of nuclear and cytoplasmic fractions showed that about 75% of the mass of RNA is extracted from these preparations as compared to direct extraction from the whole cell cultures.

In general, the nuclear RNA mass was about 20% that of the cytoplasmic RNA, while the total uridine radioactivity of the RNA from cultures labelled for 2 hours, was about equal in the nuclear and cytoplasmic extracts.

When RNA preparations, purified from nuclear and cytoplasmic fractions of virus infected cells, were centrifuged on sucrose gradients, as shown in fig. 6, the py RNA was found to sediment as did the py RNA from whole cells.

6. Leakage of RNA from Nuclei During Fractionation

The above results were further analyzed for cross contamination between nuclear and cytoplasmic RNAs. The distribution of total radioactivity revealed that in the nuclear RNA there was a barely detectable peak at 18s though no peak at 4s, which indicated a negligible amount of contamination with cytoplasmic RNA. The cyto-
Conditions of infection and labelling were described in the legend to fig. 2. Specific activity of the NP4O cytoplasmic RNA was 860 cpm/μgm. Seventy-five μgm of this RNA were analyzed by the usual sucrose gradient method. In this case, fractions were pooled in groups of three for the hybridization test with filters bearing 0.5 μgm of py DNA.
plasmic RNA gradient did contain radioactivity sedimenting beyond 28s, though there was no distinct 45s peak present, thus demonstrating that the cytoplasmic RNA was relatively free of nuclear RNA (76).

In order to further substantiate this conclusion, a different method of RNA extraction was used which involved the use of NP40 and no mechanical breakage of the cells. The cytoplasmic extract obtained under these conditions was extracted for RNA. This RNA sedimented on a sucrose gradient as shown in fig. 7. The py RNA was detected throughout the gradient as in fig. 6(b), though no significant amount of cellular RNA sedimenting beyond 28s was detected.

7. Polyribosomal RNA

In order to characterize the functional py specific m-RNA, it was necessary to examine the polysomes of the infected cells. As shown in fig. 8(a) and 8(b), the level of polyribosomes in the infected cell culture was twice the level in the contact inhibited uninfected cell cultures which indicated an increase in the synthesis of functional m-RNA in the infected cells. The size distribution of py RNA from the polyribosomes was considerably different from that of the nuclear and cytoplasmic fractions. No virus specific RNA sedimenting faster than 28s was detected, implying that the large py RNA in the cytoplasm was cleaved upon entering the polyribosomes or simply did not incorporate into the polyribosomes. The isolated
Fig. 8: Sucrose Gradient Sedimentation of (A) Cytoplasmic Extract of Mock Infected Cells; (B) Cytoplasmic Extract of Py Infected Cells and (C) Polyribosome Associated RNA of Py Infected Cells.

Cultures of mock-infected and py infected (50 pfu/cell) MK cells were incubated with $^3$H-uridine at 40 μCi/2 ml/dish between 30 to 32 hours pi and harvested at 32 hours pi for cell fractionation. The cytoplasmic fractions obtained by Dounce homogenization were analyzed on 15 to 30% RSB+ sucrose gradients (A, B). Infected polyribosome fractions sedimenting faster than 80s were pooled, and extracted for RNA, 150 μgm of which were analyzed on a 5 to 20% sucrose gradient (C) as outlined in legend to fig. 2. Specific activity of the polyribosomal RNA was 600 cpm/μgm.
polyribosomes were predominantly hexamers which indicated that no significant breakdown of the m-RNA had occurred during their isolation. It should be pointed out however, that no species of py RNA sedimenting faster than 28s could be found in the subribosomal region of the gradient shown in fig. 8(b), although cellular RNA exceeding 28s in sedimentation was found here.

8. Comparison of Nuclear and Cytoplasmic RNA by Competition Hybridization

A study of the base sequence homology between the nuclear and cytoplasmic py RNAs was performed in order to further compare these molecules. Milligram quantities of non radioactive and radioactive RNA were prepared both from the nuclear and cytoplasmic fractions of infected cells. As depicted in fig. 9, increasing amounts of non labelled RNA were hybridized with a set amount of py I DNA bound to filters. The filters were then washed and incubated with the radioactive RNA. The fraction of the labelled py RNA that hybridized with the filter as a function of the concentration of the non labelled py RNA is shown.

Both the nuclear and cytoplasmic py RNA competed efficiently with themselves and each other, which indicated that in terms of base sequence homology, the two py RNA preparations resembled each other closely.
Fig. 9: Competition Hybridization Between Nuclear and Cytoplasmic RNA.

Nuclear and cytoplasmic RNA were obtained as outlined in the Materials and Methods, using the Dounce homogenization procedure. The specific radioactivity of nuclear RNA was 11,200 cpm/µgm and of the cytoplasmic RNA was 4,525 cpm/µgm. The hybridization mixture of the first hybridization step contained the indicated amounts of unlabelled RNA, and 0.1 µgm of py I DNA. In the second step, the hybrid containing filters were incubated with 20 µgm of the labelled RNA. Both hybridizations were carried on for 24 hours. The 100% value of the graph denotes 300 cpm for nuclear RNA and 200 cpm for cytoplasmic RNA. The upper line denotes a control hybridization with non-labelled yeast RNA and labelled nuclear RNA. The lower curve the following:

- ▲ labelled nuclear RNA, unlabelled nuclear RNA.
- • labelled nuclear RNA, unlabelled cytoplasmic RNA.
- ▲ labelled cytoplasmic RNA, unlabelled cytoplasmic RNA.
- • labelled cytoplasmic RNA, unlabelled nuclear RNA.
cpm HYBRIDIZED % of control

μg of COMPETING RNA

100
200
300
400

50
100
9. Comparison of Nuclear and Polyribosomal py RNA by Competition Hybridization.

A similar study was done, comparing polyribosomal py RNA with nuclear py RNA from infected cell cultures. As shown in fig. 10, the nuclear py RNA competed efficiently with polyribosomal py RNA indicating that all sequences in polyribosomal py RNA were present in the nuclear py RNA. However the polyribosomal py RNA did not compete efficiently with nuclear py RNA, nor with itself. In this regard, it was difficult to say with certainty that the polyoma sequences present in the nuclear RNA were conserved as the py RNA was processed to the polysomes. It is believed that the low amounts of polysomal py RNA available accounted for this low efficiency of hybridization.

10. Comparison of Large and Small py RNA by Competition Hybridization.

Polyoma specific cytoplasmic RNA populations sedimenting beyond 20s and below 18s on a sucrose gradient were compared by competition hybridization, as shown in fig. 11. Both RNA species competed efficiently with themselves and with each other, indicating that the large py RNA had a very similar base sequence to the small py RNA.
Fig. 10: Competition Hybridization Between Nuclear and Polyribosomal Py RNA.

Nuclear and polyribosomal py RNA were obtained as outlined in the Methods. Since a number of batches of infected cells were necessary, care was taken that both the nuclei and polyribosomes from each batch were used in equal amounts in each pool. Polyribosomal RNA was considered to be all RNA from ribosomes sedimenting in excess of 80s. In addition, one third of each batch preparation was always labelled with $^3$H-uridine at 60 µCi/2 ml/dish in order to obtain the radioactive RNA. The specific radioactivity of the nuclear RNA was 13,000 cpm/µgm and of the polyribosomal RNA was about 2,000 cpm/µgm.

The hybridization mixture of the first step involved indicated amounts of unlabelled RNA with 0.1 µgm of py I DNA. In the second step 20 µgm of nuclear RNA or 60 µgm of polysomal radioactive RNA were used. Both hybridizations were carried out for 24 hours. The 100% value of the graph denotes 1,200 cpm for the nuclear RNA and 200 cpm for the polysomal RNA. The upper line denotes a control hybridization with non-labelled yeast RNA and labelled nuclear RNA. The lower curve the following:

- labelled polyribosomal RNA, non-labelled nuclear RNA
- labelled polyribosomal RNA, non-labelled polyribosomal RNA
- labelled nuclear RNA, non-labelled polyribosomal RNA
- labelled nuclear RNA, non-labelled nuclear RNA
Fig. 11: Competition Hybridization Between Large and Small Py RNA.

Approximately 1 mg of labelled (specific activity 4,525 cpm/μg) and 2 mg of non-labelled cytoplasmic RNA were centrifuged through 5 to 20% STE SDS sucrose gradients in an SW40 rotor for 4.5 hours at 15°C. The gradients were fractionated, monitored for A260 and radioactivity. The >18s and <18s fractions were separately pooled for each gradient. The adjacent fractions of the pools were omitted. The RNA was recovered by ethanol precipitation. The indicated amounts of unlabelled RNA were used in each hybridization mixture along with 0.1 μg of py I DNA. In the second step, 50 μg of small RNA or 25 μg of large RNA were used in each appropriate mixture. The values of the hybrids were 150 cpm in the case of the large RNA and 75 cpm in the case of the small RNA. The upper line denotes a control hybridization with non-labelled yeast RNA and labelled large RNA. The lower curve the following:

△ labelled large RNA, unlabelled large RNA
○ labelled large RNA, unlabelled small RNA
△ labelled small RNA, unlabelled small RNA
○ labelled small RNA, unlabelled large RNA
Fig. 11:
CHAPTER IV: RESULTS II

Processing of Py RNA in Virus Infected Cell Cultures

1. Kinetics of Labelling of Nuclear and Cytoplasmic Py RNA

In the previous chapter, the py RNA from different compartments of the infected cell was characterized with respect to base sequence homology and size distribution. It therefore became possible to study the actual processing of py RNA from its transcription time onward. A first step in this direction was to study the kinetics of labelling of py RNA in the nuclear and cytoplasmic fractions of the infected cells.

In the experiment, the results of which are depicted in fig. 12, py infected cells were exposed to \(^{3}\)H-uridine for periods of 15 minutes to 4 hours. The RNA was extracted from these cells, purified and analyzed for absorbancy, radioactivity and also hybridized with py I DNA to determine the virus specific content.

The incorporation of radioactive label into the total nuclear RNA started immediately, and increased at a constant rate. On the other hand, the incorporation of label into the total cytoplasmic RNA was very slow for about one hour, whereupon it began to increase at a rate parallel to that of the nuclear RNA. This lag in the incorporation of the label was considered to reflect the time
Fig. 12: Variable Pulse Labelling of Infected Cell RNA

Fifteen MK cell cultures, infected with py virus at an input multiplicity of 100 pfu/cell, were labelled at 30 hours pi with $^3$H-uridine at 100 $\mu$Ci/2 ml/dish. At 15, 30, 60, 120 and 240 minutes after labelling, RNA was extracted from 3 of the above cultures, purified and monitored for $A_{260}$ and radioactivity. One eighth of each RNA sample (50 $\mu$gm of cytoplasmic RNA and 15 $\mu$gm of nuclear RNA) was incubated with 0.5 $\mu$gm of py I DNA for hybridization. All values were normalized with respect to the $A_{260}$ of the nuclear and cytoplasmic RNA preparations.

nuclear RNA - dark symbols

cytoplasmic RNA - light symbols
Fig. 12:
taken for the labelled RNA to be transported from the nucleus to the cytoplasm in the infected MK cells.

The incorporation of label into the py RNA in the nuclear fraction increased at a constant rate, similar to that of the cellular RNA, up to two hours, after which it slowed down. On the other hand, the cytoplasmic py RNA was observed to become labelled at a constant rate, which was parallel to the cytoplasmic cell RNA for the first hour, but did not undergo a sharp increase as did the latter over a longer labelling period.

A comparison of the viral and cellular RNA in the above study showed that the nuclei contained about 10 times as much of the labelled py RNA as did the cytoplasm. Of the total labelled RNA in the nuclear fraction, only about 1% was virus specific, regardless of the labelling period. This implied that during transcription a constant portion of the transcribed RNA was viral RNA which was then processed in the nuclei at a rate similar to the cell RNA. On the other hand, the percentage of the label present as py RNA in the cytoplasmic RNA decreased after 30 minutes of labelling. This most likely reflected the accumulation of long lived r-RNA in the cytoplasm.

The py RNA was synthesized and processed in the nuclear fraction at a rate comparable to the total cell RNA. Its appearance in the cytoplasmic fraction was similar to that of the total cell RNA for the first 30 minutes, after which it did not appear to accumulate at
the rate of the cell RNA, but increased at a rate which was about 10% of its rate of increase in the nuclear fraction. In similar experiments the cytoplasmic RNA made up to 20% of the nuclear py RNA, but this fraction also remained constant regardless of the labelling period. The implication of these data was that regardless of the time of pulse, only 10 to 20% of the viral RNA transcribed in the nuclear fraction was processed on to the cytoplasmic fraction in py infected MK cell cultures.

2. Py RNA in Pulse Labelled Cells.

The size distribution of py RNA pulse labelled for 15 minutes was examined on a sucrose gradient as shown in fig. 13. Based on the results of the above experiment (fig. 12) the labelled viral RNA was still mainly in the nuclear fraction and hence would be subject to a smaller degree of processing than py RNA labelled for a 2 hour period (shown in fig. 3).

As shown in fig. 13, the 15 minute pulse labelled RNA sedimented in a heterogeneous fashion, predominantly faster than 18s with up to 46% sedimenting faster than 28s. Since no distinct labelled r-RNA peaks were evident in the gradient, the RNA was considered to have been subject to a minimal amount of processing. Although shorter pulse labelling studies were not performed (since an insufficient amount of viral RNA would be labelled), this study alone suggested
Py infected (moi 100 pfu/cell) MK cells were labelled at 31 hours pi with $^3$H-uridine at 200 $\mu$Ci/2 ml/dish for 15 minutes. RNA was extracted and purified from these cells, and 75 $\mu$gm of this RNA (specific activity 1,160 cpm/$\mu$gm) were analyzed on a sucrose gradient as outlined in the Methods. The gradient fractions were monitored for $A_{260}$, 10 $\mu$l sampled for radioactivity, and the remainder pooled sequentially in pairs and hybridized with 0.5 $\mu$gm of py I DNA.

Direction of sedimentation was to the left.
Fig. 13:

![Graph showing the fraction number and corresponding values for cpm Py RNA $\times 10^{-2}$ and $3^H$ cpm $\times 10^{-3}$.

Key:
- $28s$ and $18s$ markers indicate specific fractions.

FRACTION NUMBER:
- 5 10  15  20

$3^H$ cpm $\times 10^{-3}$:
- Values range from 0 to 3.

cpm Py RNA $\times 10^{-2}$:
- Values range from 0 to 3.

Graph indicates a decreasing trend in both values as the fraction number increases.
that the py RNA species were transcribed as a population of large molecules, predominantly greater than 28s. A similar size distribution of py RNA labelled for 20 minutes was recently observed by other workers (1).

3. Pulse and Chase Studies.

Pulse and chase studies, which involved pulse labelling of cells for a brief period of time, followed by the addition of Actinomycin D to stop incorporation of the label, have proven useful in studying the synthesis and processing of r-RNA. A similar type of approach to study the processing of py RNA in infected cells was attempted by the use of not only Actinomycin D, but also excess non labelled uridine, and cordycepin. The behaviour of the cellular RNA and viral RNA in the nuclear and cytoplasmic fractions, under the above conditions of pulse and chase, was examined. The result of such an experiment is shown in fig. 14(a) and (b).

In Actinomycin D treated cells, the radioactivity of the total nuclear RNA showed a rapid rate of decay in the first hour, followed by a slower rate for the remaining 5 hours of chase. A total decrease of 82% was observed throughout the entire chase period. The radioactivity of the py nuclear RNA behaved in a similar fashion, with the radioactivity decreasing by 90% during the six hour chase period.

In the same cell cultures, the total cytoplasmic RNA radioactivity
Fig. 14: Pulse and Chase Studies on Infected Cell RNA.

Twenty-four py infected (moi 100 pfu/cell) MK cell cultures were pulse labelled with $^3$H-uridine, at 100 μCi/2 ml/dish for 40 minutes. The RNA was immediately extracted from two cultures, while the remainder were washed with MEM and one half incubated with MEM containing 5 μgm/ml of Actinomycin D, and the other half with MEM containing $2 \times 10^{-4}$ M uridine. RNA was extracted from these cultures at 1, 2 and 6 hours after the chase began. The purified RNA preparations were monitored for $A_{260}$, radioactivity, and one eighth of each sample (12 μgm of nuclear RNA, and 40 μgm of cytoplasmic RNA) hybridized with 0.5 μgm of py I DNA to determine viral RNA content. The data expressed in this figure were all normalized to a constant mass of respective nuclear or cytoplasmic RNA.

A. Total cell RNA
B. Viral RNA
C. % cpm hybridized

- Actinomycin D chase, nuclear RNA
- uridine chase, nuclear RNA
- Actinomycin D chase, cytoplasmic RNA
- uridine chase, cytoplasmic RNA.
Fig. 14:

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)
increased about threefold, while the corresponding py specific radioactivity decreased about threefold during the chase with Actinomycin D.

A comparison of the above values (fig. 14(c)) showed that in the nucleus the py RNA makes up a constant fraction of about 3% of the cell RNA. On the other hand in the cytoplasm, the py RNA fraction of the cell RNA decreased during the chase period from 2% to 0.1%. This was believed to be due to the different rates of processing of the py RNA and the longer lived species of cellular RNA such as r-RNA.

The shapes of the decay curves of the py RNA in the nuclear and cytoplasmic fractions throughout the chase with Actinomycin D, indicated that the processing of py RNA was biphasic in nature. In the first hour over 75% of the viral RNA was degraded in the nucleus, without being transported to the cytoplasm. In the next five hours, the rates of loss of py RNA from the nucleus and cytoplasm were nearly parallel, which was consistent with processing from the nucleus to the cytoplasm and degradation in the cytoplasm.

In a parallel chase experiment, $10^{-4}$ M uridine was used in place of Actinomycin D. As shown in fig. 14, the cellular nuclear radioactivity appeared to remain constant, reflecting the fact that incorporation of $^{3}H$-uridine continued, to some extent, in the presence of excess unlabelled uridine. This was thought to be due to the large intracellular pool of uridine compounds in mammalian cells (18). The nuclear py RNA radioactivity also showed a more gradual decrease compared with the Actinomycin D treated cells.
The uridine chased cell RNA radioactivity from the cytoplasmic fraction appeared to be relatively unaffected by the addition of uridine up to six hours of chase. The labelled RNA continued to accumulate in this fraction, in contrast to the situation in the Actinomycin D chase. The virus specific radioactivity remained at a constant level showing no decrease. These results were in agreement with those of the Actinomycin D chase, on the assumption that the excess uridine was an inefficient chasing system.

In a similar experiment, cordycepin at 50 μg/ml was used to produce a chase. The effect on the total and viral nuclear and cytoplasmic RNA was intermediate between that of Actinomycin D and uridine. Although this drug inhibits the processing of m-RNA (76a), it did not affect the entry of labelled py RNA into the cytoplasm. Since the process was not investigated further, it is possible that insufficient quantities of cordycepin were used to completely block the processing of viral RNA.

4. Size Distribution of Py RNA in Pulse and Chase Studies

Pulse labelled and chased RNA from nuclear and cytoplasmic fractions of infected cells were analyzed on sucrose gradients. As shown in fig. 15(a) and (b), all size classes of nuclear RNA decreased in the chase with Actinomycin D. The greatest decrease was observed by the large py RNA, and the smallest decrease with the small py RNA.
Fig. 15: Sucrose Gradient Analysis of Py RNA from the Pulse-Chase Study.

RNA preparations from pulse and chase studies such as outlined in fig. 14 were analyzed on sucrose gradients. About 200 μgm of cytoplasmic RNA (specific activities from 49 to 730 cpm/μgm) were analyzed on the gradients which are shown in sections B and F. Similar analyses of about 60 μgm of nuclear RNA (specific activities from 8,000 to 1,540 cpm/μgm) are shown in sections A and E. The fractionated sucrose gradients were analyzed for virus specific radioactivity. Fractions 1-8, 9-14 and 15-20 from each preparation were respectively summed for large, medium and small py RNA, and these sums appear opposite to their corresponding gradients. Sections A to D correspond to Actinomycin D chased RNA, while sections E to H are for uridine chased RNA. All values were normalized to a constant mass of nuclear or cytoplasmic RNA.

For Sections A, B, E, F:

control

2 hours chase

6 hours chase

For Sections C, D, G, H:

'large RNA' (region of fractions 1-8)  - o -  o -

'medium RNA' (region of fractions 9-14)  - o -  o -

'small RNA' (region of fractions 15-20)  - △ -  △ -
Fig. 15:

3H cpm HYBRIDIZED $\times 10^{-2}$

FRACTION NUMBER

HRS OF CHASE

Cpm HYB./region $\times 10^{-2}$
Fig. 15:

- E: Graph showing 3H cpm HYB. × 10^{-2} with fraction numbers 2, 4, 6 on Y-axis and fraction number range 10 to 20 on X-axis. Key markers indicate 28s and 18s.

- G: Graph showing cpm HYB. region × 10^{-2} with hours of chase range 2 to 6 on X-axis.

- H: Similar graph to G with hours of chase range 2 to 6 on X-axis.
These data indicate that there was a preferential breakdown of the large nuclear py RNA to smaller molecules during the chase period.

A similar analysis of the cytoplasmic py RNA from a pulse and chase study (fig. 15(b), (d)), revealed that all size classes were degraded at a very similar rate. Though here again the small py RNA appeared most stable.

The rates of decay of the various size classes of py RNA pulse labelled and chased with excess uridine, were not as pronounced as with Actinomycin D. These results, shown in fig. 15(e), (f), (g), (h), did none the less, agree with those obtained with the Actinomycin D chase in that the decay curves followed a similar pattern.

5. Involvement of Polyadenylate Sequences with Py RNA.

Polyadenylate (poly (A)) sequences, attached to the 3' terminus of the m-RNA, have been shown to be involved in the processing of these molecules (65). The role of poly (A) in regard to the processing of py RNA was therefore examined in the py infected MK cell system.

Purified RNA from py infected cells as well as from their nuclear cytoplasmic and polyribosomal components was analyzed for the presence of poly (A) sequences, by its ability to bind to Millipore filters in the presence of 0.5 M MBB. As shown in Table 3, the degree of binding to Millipore increases as the py RNA is
Table 3: Poly (A) Content of Py Infected Cell RNA

These are average values derived from three separate experiments measuring poly (A) content from various components of the virus infected cell. Less than 30 μgm of each RNA was used. One sixth of the surface area of each filter was assayed for radioactivity while the rest was used for hybridization.
Table 3: Poly (A) Content of Py Infected Cell RNA.

<table>
<thead>
<tr>
<th>RNA</th>
<th>% of total RNA containing poly (A)</th>
<th>% of Py RNA containing poly (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell</td>
<td>8.3 ± 0.2</td>
<td>16.3 ± 4</td>
</tr>
<tr>
<td>Nuclear</td>
<td>10.9 ± 2.3</td>
<td>6.1 ± 1.3</td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>14.6 ± 2.8</td>
<td>30.2 ± 1.1</td>
</tr>
<tr>
<td>Polysomal</td>
<td>31%</td>
<td>58%</td>
</tr>
</tbody>
</table>
processed from the nuclei through to the polyribosomes. Hence the percentage of poly (A) containing molecules is lowest in the nuclear fraction and highest in the polyribosomes. This suggests that only polyadenylate containing py RNA molecules can be properly processed and be incorporated into polyribosomes for translation.
Studying the Isolation and Enumeration of Poly (A) Containing RNA Molecules

1. Parameters Involved in Binding Poly (A) Containing RNA to Millipore Filters (72)

In the process of measuring the relationship between poly (A) sequences of py RNA and the degree of processing, a number of parameters of poly (A) binding to Millipore were examined.

The first such parameter examined was the efficiency of poly (A) binding to Millipore in the presence of MBB containing sodium (Na\textsuperscript+MBB) compared with MBB containing potassium (K\textsuperscript+MBB). In each case approximately 5% of the total cell RNA radioactivity bound to the Millipore filter, the values being 5.3% for Na\textsuperscript+MBB and 5.7% for K\textsuperscript+MBB. The potassium salt was therefore used in the ensuing studies to accord with other workers.

The second parameter examined was the elution of bound RNA from the Millipore filter. One of the RNA containing filters was placed in a 1 ml solution of 0.1 M Tris (pH 9.0) and 0.5% SDS and incubated at 4\degree C for 30 minutes. This solution and filter were sampled for radioactivity and after the former was brought to pH 7.0 with HCl, were assayed for py RNA by hybridization to py I DNA. Another Millipore
filter containing an equal amount of RNA was placed directly into a hybridization mixture (0.5% SDS, 6 x SSC and 0.001 M EDTA) containing 0.5 μgm of py I DNA. In the former case, 0.45%, and in the latter case, 0.85% of the cpm bound to the filter subsequently hybridized. Thus py RNA eluted from Millipore more efficiently if the filter was placed directly into the hybridization mixture; rather than with the eluting buffer, followed by hybridization. In addition, it was observed that the original Millipore filters, which were eluted by placing them directly into the hybridization mixture, retained virtually no radioactivity. This process of elution was, therefore, used in all subsequent experiments.

2. Ribonuclease Resistance of RNA Bound to Millipore.

Polyoma infected cell RNA preparations, labelled with ³H-uridine or ³H-adenosine, were passed through Millipore filters in MBB, sampled for total radioactivity and stored frozen in 0.5 x SSC. The RNA, precipitated out of the filtrates by the addition of 2 volumes of ethanol, was redissolved in 0.5 x SSC, and sampled for radioactivity. Both the filters and the filtrates were incubated with pancreatic RNAse, at 37°C, rapidly chilled to 0°C, and made up to 6% in TCA. The TCA insoluble radioactivity on both filters and filtrates was determined. As shown in table 4, the ³H-adenosine labelled RNA bound to Millipore filters was more RNAse resistant
Table 4: Resistance of Millipore Bound and Non-Bound RNA to Pancreatic Ribonuclease.

Thirty-one µgm of $^3$H-adenosine labelled RNA (specific activity 1,600 cpm/µgm) and 43 µgm of $^3$H-uridine labelled RNA (specific activity 3,100 cpm/µgm) were separately made up to 0.5 ml in MBB, passed through Millipore filters at 4°C and washed with 4.5 ml of ice cold MBB. The filters, of which one sixth of the exposed area was sampled for radioactivity, were frozen in 1.0 ml of 0.5 x SSC at -70°C while the RNA was recovered from the pooled filtrates and washes by precipitation with 2 volumes of ethanol. The RNA from the ethanol precipitates were also made up to 1 ml of 0.5 x SSC and along with the filters were individually mixed with 10 µgm of pancreatic RNase and incubated at 37°C for 40 minutes. The preparations were then precipitated with 6% TCA at 0°C and the precipitates, collected on glass fiber filters, were counted for radioactivity.
Table 4: Resistance of Millipore Bound and Non-Bound RNA to Pancreatic Ribonuclease.

<table>
<thead>
<tr>
<th>Isotope</th>
<th>No RNAse</th>
<th>RNAse</th>
<th>% RNAse resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm on filter (bound)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^3$H-adenosine</td>
<td>3,091 cpm</td>
<td>217 cpm</td>
<td>7.05</td>
</tr>
<tr>
<td>$^3$H-uridine</td>
<td>4,960 cpm</td>
<td>114 cpm</td>
<td>2.27</td>
</tr>
<tr>
<td>cpm in filtrate (non-bound)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^3$H-adenosine</td>
<td>54,981 cpm</td>
<td>587 cpm</td>
<td>1.03</td>
</tr>
<tr>
<td>$^3$H-uridine</td>
<td>143,348 cpm</td>
<td>1,400 cpm</td>
<td>1.01</td>
</tr>
</tbody>
</table>
than the corresponding $^3$H-uridine labelled RNA, while this difference was not evident with RNA from the filtrates from these two species. These data provided evidence that the RNA bound to the Millipore filters in MBB had RNAse resistant $^3$H-adenosine labelled regions which could well have been poly (A) sequences, while the RNA from the same preparation, which did not bind to Millipore, lacked this property.

3. Capacity, Washing and Sampling of Millipore Filters with Bound RNA.

(a) Washing

An analysis was made of the degree of washing needed to remove non specifically bound RNA from a Millipore filter. As depicted in fig. 16, two similar quantities of $^3$H-adenosine labelled RNA and $^3$H-uridine labelled RNA, were separately passed through Millipore filters. The filters were washed with successive volumes of MBB, and the RNA was collected from the washes and filtration by ethanol precipitation.

Figure 16 shows that if the filters, through which RNA has been filtered, were washed with 0.5 ml of MBB, about 75% of the now bound RNA was removed. If they were washed with an additional 1.0 ml, up to 94% of the non bound RNA was removed. Washing with
Fig. 16: Effects of Washing on Millipore Bound RNA.

Two 50 µgm quantities of $^3$H-adenosine labelled RNA (1,600 cpm/µgm) and $^3$H-uridine labelled RNA (3,000 cpm/µgm) from virus infected cells, were filtered individually through Millipore. The filters were washed with increasing volumes of MBB and the washes collected separately. The radioactivity of the filters was determined directly and that of the washes was obtained from the TCA precipitate of each wash. In a parallel experiment, only one third of each wash was TCA precipitated while the remainder was mixed with 2 volumes of ethanol. The RNA precipitated by the ethanol was resuspended in 0.5 x SSC, incubated for 30 minutes with pancreatic RNase at 10 µgm/ml, and precipitated with ice cold 6% TCA. The TCA insoluble material was counted.

$^3$H-adenosine labelled RNA - (○-)

$^3$H-uridine labelled RNA - (-○-)
Fig. 16:

![Graph showing total $^3H$ cpm in wash vs volume of MBB in ml]

- **Total $^3H$ cpm in wash** $\times 10^3$
- **Volume of MBB** (in ml)

The graph illustrates the relationship between the volume of MBB and the total $^3H$ cpm in the wash. As the volume of MBB increases, the total $^3H$ cpm in the wash also increases, reaching a plateau at higher volumes.
greater volumes (2.5 ml to 8.5 ml) removed all of the non bound RNA. From these data, the protocol of applying the RNA to the filter in 0.5 ml of MBB and eluting with 5.4 ml of MBB was developed.

An examination of the RNAse resistance of \(^3\)H-adenosine and \(^3\)H-uridine labelled RNA from such washes revealed that the filtrate and 0.5 ml wash of the former species had 2.88\% RNAse resistant TCA insoluble radioactivity, while the corresponding value for the latter RNA was 1.64\%. These values did not change if the filters were washed with a total of 2.5 ml of MBB. Further studies showed that if the above protocol, of washing with 4.5 ml of MBB, was employed, the \(^3\)H-adenosine labelled RNA in the pooled filtrates and washes was about 1.0\% RNAse resistant.

These data indicate that washing the RNA bound to the Millipore filter with MBB removes only the non Poly (A) containing species of RNA.

(b) Capacity of Millipore Filters for Binding RNA Associated with Poly (A) Sequences.

Four aliquots containing 5, 10, 15, and 30 \(\mu\)gm of \(^3\)H-adenosine labelled py infected cell RNA (specific activity 1,600 cpm/\(\mu\)gm) were applied to Millipore filters, which were washed with 4.5 ml of MBB. For each respective RNA sample, 7.5\%, 8.1\%, 5.7\% and 5.8\% of the counts were found to bind to the Millipore filter. Thus in
this and other similar experiments, the extent of binding was proportional to the amount of RNA filtered.

(c) Sampling of Millipore Filters.

Since incubating the Millipore filter, containing the bound RNA in a hybridization mixture, was found to be the best way of eluting the RNA, a sample of the uneluted filter was necessary to determine the total radioactivity bound. The method chosen was to punch out a circle which was one sixth of the filter area exposed to the RNA. In order to investigate the reliability of this technique, three Millipore filters, through which were filtered equal amounts of labelled infected cell RNA, were sampled by this technique from three different sites on the exposed filter areas.

The total radioactivity per filter, obtained from the sum of the three samples of the filter, was in close agreement for all three filters. However, the radioactivities of single samples from the same filter differed by 40 to 60%. It was concluded from these data that the RNA bound to Millipore filters to a reproducible extent, although it did not bind homogeneously across the filter surface. Hence this method of sampling Millipore filters was not accurate, though the use of multiple filters for each determination would be adequate.
4. Binding of RNA to Millipore, Nitrocellulose and to Polyuridylate-Glass Fiber Filters.

In order to gain some insight into the nature of the binding process, a comparison was made between Millipore filters (composed of mixed cellulose esters), and Scheicher and Schuell nitrocellulose filters in terms of their ability to bind RNA from infected cells. As shown in table 5(a), the nitrocellulose filter bound much more RNA than did the Millipore filter. In addition a smaller fraction of the RNA which bound to the nitrocellulose filter was RNAse resistant, compared with RNA which bound to Millipore filters.

These data, though limited in scope, did nevertheless show a profound difference in the binding properties of the nitrocellulose and Millipore filters. Taken by themselves, these observations implied that the interaction between poly (A) and Millipore was more complex than anticipated.

On the other hand, a comparison of the RNA binding between Millipore and polyuridylate-GF/C (poly (U)-GF/C) filters, as shown in table 5(b), revealed that the latter system was relatively inefficient in binding cell RNA or RNAse resistant $^3$H-adenosine labelled RNA. Since the system was considered to work on a principle different from Millipore binding, the fact that the degree of poly (U)-GF/C binding of RNA was not similar to the Millipore binding makes an unambiguous interpretation of the binding results
Table 5(a):

Two 20 μgm preparations of \(^3\)H-adenosine labelled py infected cell RNA (specific activity 2,160 cpm/μgm) were made up to 0.5 ml in MBB and filtered through Millipore and Nitrocellulose (Schleicher and Schuell) filters. One sixth of each filter was sampled for radioactivity and the filtrates and washes were mixed with ethanol to precipitate the RNA. The RNA precipitate was resuspended in 0.5 x SSC of which one quarter was sampled for radioactivity and the rest, together with the filters, incubated with pancreatic RNAse at 10 μgm/ml for 40 minutes at 37°C. The digested RNA was precipitated with 6% TCA for radioactive determination.

Table 5(b):

The operations performed above were repeated with Millipore filters and GF/C filters on which was bound poly (U). The binding of poly (U) and GF/C was described in the Methods. The binding buffer for application and washing of the RNA on the filter was 0.12 M NaCl, 0.01 M Tris pH 7.0.
Table 5(a) and 5(b): Comparison of RNA binding to Millipore, Nitrocellulose and Poly (U)-GF/C Filters.

### 5(a)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Millipore</th>
<th>Nitrocellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm bound to filter</td>
<td>1,130</td>
<td>10,400</td>
</tr>
<tr>
<td>cpm in filtrate</td>
<td>34,000</td>
<td>24,300</td>
</tr>
<tr>
<td>% cpm bound to filter</td>
<td>3.2</td>
<td>29.1</td>
</tr>
<tr>
<td>cpm of bound RNA which was RNAse resistant</td>
<td>80</td>
<td>332</td>
</tr>
</tbody>
</table>

### 5(b)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Millipore</th>
<th>Poly (U)-GF/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm bound to filter</td>
<td>3,993</td>
<td>851</td>
</tr>
<tr>
<td>cpm in filtrate</td>
<td>72,288</td>
<td>60,920</td>
</tr>
<tr>
<td>% cpm bound to filter</td>
<td>5.25</td>
<td>1.38</td>
</tr>
<tr>
<td>cpm of bound RNA that was RNAse resistant</td>
<td>228</td>
<td>124</td>
</tr>
</tbody>
</table>
difficult. It thus became important to examine the above RNA binding systems further, in order to explain the discrepancies.

5. Effects of Salt Concentration on the Interaction Between Cellular RNA and Poly (U)-GF/C Filters.

The effects of the salt concentration of the poly (U) binding buffer (PUBB) on the RNA binding by the poly (U)-GF/C filters is depicted in fig. 17. Evidently, the extent of the RNA binding was strongly influenced by the salt concentration.

Although the RNA binding to the poly (U)-GF/C filters did not appear to have reached a plateau, it did show a distinct reproducible biphasic effect above 0.5 M NaCl. On the other hand, increasing the salt concentration had no detectable effect on the binding of this same RNA onto irradiated GF/C filters lacking poly (U).

6. Binding of $^3$H-Adenosine Labelled RNA to Other Polynucleotides.

In an effect to distinguish between the specific binding of the long poly (A) tract of RNA to poly (U) on the filter and any less specific binding that might occur due to "A rich" regions of these molecules, the RNA preparations used for fig. 17 were exposed to GF/C filters containing fixed polyadenylate or polycytidylate. In this case it was assumed that the poly (A) and poly (C) would
Fig. 17: The Effect of Salt Concentration on RNA Binding to Poly (U)-GF/C Filters.

Virus infected cell RNA labelled with $^3$H-uridine (specific activity 3,370 cpm/μgm) was made up in quantities of 9.0 μgm in the PUBB concentrations indicated on the graph, passed through poly (U)-GF/C filters and washed with 8.0 ml of the same PUBB concentration. The RNA was precipitated out of the filtrates with 6% TCA and the filters and TCA insoluble RNA in the filtrates were counted for radioactivity. Blank filters were treated identically except that they did not contain poly (U).

(●) Blank GF/C filter
(○) Poly (U)-GF/C filter
Fig. 17:

NaCl CONCENTRATION IN PUBB

% cpm BOUND

0.5 1.0 1.5

NaCl CONCENTRATION IN PUBB
Table 6: Binding of $^3$H-adenosine Labelled RNA to GF/C Filters Containing Poly (A) and Poly (C).

The RNA preparation used in fig. 17 was made up in equal quantities of 0.5 $\mu$gm in the PUBB concentrations indicated and passed through the GF/C filters containing either poly (A) or poly (C) as outlined in the legend to fig. 17. The poly (A) and poly (C) were attached to the filter by UV irradiation as was outlined in the Methods.
Table 6: Binding of $^3$H-Adenosine Labelled RNA to GF/C Filters Containing Poly (A) and Poly (C).

<table>
<thead>
<tr>
<th>NaCl concentration in PuBB</th>
<th>% cpm bound to Poly (A)-GF/C Filter</th>
<th>% cpm bound to Poly (C)-GF/C Filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 M</td>
<td>1.15</td>
<td>0.82</td>
</tr>
<tr>
<td>0.1 M</td>
<td>1.73</td>
<td>2.44</td>
</tr>
<tr>
<td>0.3 M</td>
<td>4.90</td>
<td>N.A.</td>
</tr>
<tr>
<td>0.5 M</td>
<td>8.00</td>
<td>19.26</td>
</tr>
<tr>
<td>0.9 M</td>
<td>45.4</td>
<td>N.A.</td>
</tr>
<tr>
<td>1.5 M</td>
<td>56.3</td>
<td>23.6</td>
</tr>
</tbody>
</table>
fix to GF/C filters by U.V. light to the same degree as poly (U) did.

The results displayed in table 6 indicated that there existed "U rich" and "G rich" regions in the RNA which caused it to bind to such a degree to poly (A) and poly (C) filters. Alternatively some non specific binding to poly (A) and poly (C) may occur at the higher salt concentration. Since a high degree of binding to the above polynucleotides occurred at moderate salt concentrations (1.3 M - 0.5 M) these data could not be used to distinguish between the salt concentrations which would lead to specific binding of poly (A) tracts and that which promoted the binding of "A rich" regions.

7. Elution of RNA from Poly (U)-GF/C Filters.

Another approach was attempted to resolve which salt concentration would produce specific binding of poly (A) to the poly (U), and which would lead to non specific binding. This involved examining the dissociation of the poly (A) containing RNA from the poly (U). As shown in fig. 18, poly (U)-GF/C filters onto which RNA had been bound in 1.5 M PUBB were eluted with lower concentrations of salt. It was thought that authentic poly (A)-poly (U) hybrids would dissociate at a different salt concentration from the non specifically bound RNA. The salt dependent elution
Fig. 18: Elution of RNA from Poly (U)-GF/C Filters.

Equal quantities (18 µg) of $^3$H-adenosine labelled RNA (specific activity 3,370 cpm/µg) were applied to each of seven poly (U)-GF/C filters. Through each filter was then passed 10 ml of PUBB whole salt concentration varied from 1.5 M to 0.01 M as shown in the graph. The RNA eluted by this treatment was precipitated with 6% TCA and counted for radioactivity along with the poly (U)-GF/C filters. The percentage of cpm eluted was the cpm eluted as a fraction of the total cpm which was bound to the filter prior to elution.
Fig. 18:

- **% cpm ELUTED** vs. **NaCl CONCENTRATION IN PUBB**
  - Ranges from 0.0 M to 1.5 M on the x-axis.
  - Values on the y-axis range from 0 to 100.
  - The graph shows a curve indicating a relationship between NaCl concentration and % cpm eluted.
observed was similar to the reverse of the binding curve. The point of inflection of the curve occurred between 0.3 and 0.5 M PUBB, though this was not a sharp change. Although these results did not distinguish between poly (A)-poly (U) hybrids, and RNA non specifically bound to poly (U), they did prove that it was feasible to elute RNA from the poly (U) GF/C filters by using 0.001 M NaCl.

8. Binding of Ribosomal RNA to Poly (U)-GF/C Filters

A possible elucidation of the problem of non-specific binding of RNA to poly (U)-GF/C filters could be obtained by studying the binding of labelled r-RNA to these filters, especially as a function of the salt concentration of the PUBB. To do this, $^{14}$C-uridine labelled r-RNA was passed through poly (U)-GF/C filters at different PUBB concentrations. As illustrated in fig. 19, there was a low level of binding of RNA to the filters which in this case, though not in others, increased with the PUBB concentration. However, the maximum of 2.5% achieved with the highest PUBB concentration was in marked contrast with the 12% of the label which bound when infected cell RNA was so assayed.

These data give strong evidence that the non ribosomal RNA was responsible for the binding to the poly (U)-GF/C filters.
Fig. 19: Binding of Ribosomal RNA to Poly (U)-GF/C Filters.

Six equal 7.5 μg samples of $^{14}$C labelled ribosomal RNA (specific activity 2,175 cpm/μg) from BHK-21 cells were made up to 1.0 ml with the various PUEB concentrations shown in the graph. These were filtered through poly (U)-GF/C filters and the RNA in the filtrates precipitated with TCA. The filters and the TCA insoluble RNA in the filtrates were counted for radioactivity.
Fig. 19:

NaCl CONCENTRATION IN PUBB

% cpm BOUND

0.5M 1.0M 1.5M
9. Sucrose Gradient Sedimentation of RNA which was Bound and Eluted from Poly (U)-GF/C Filters.

A more detailed analysis of the $^3$H-adenosine labelled RNA that was bound to, and eluted from, poly (U)-GF/C filters was made by centrifuging this RNA, and its RNAse digest, on sucrose gradients as shown in fig. 20. This RNA was very heterogeneous in size sedimenting from the 4s to greater than 45s regions of the sucrose gradient. In addition, it was found to possess no detectable labelled ribosomal RNA species such as were seen in a similar but unfiltered RNA preparation (fig. 3). This information along with the observation that r-RNA did not bind to poly (U)-GF/C were strongly indicative that the RNA which binds to poly (U)-GF/C filters was neither r-RNA nor t-RNA, but could have been Hn-RNA and/or m-RNA. It was also observed that the RNA which bound to the poly (U)-GF/C filters in 0.3 M NaCl and subsequently eluted sedimented in a similar fashion to that shown in fig. 20.

The RNAse digested RNA did not appear to sediment faster than 5s. This was interpreted to mean that either the RNA examined did not contain poly (A) sequences sedimenting from 5 to 7s as observed in other systems (19), or that the RNAse digestion was too severe and resulted in a very limited breakdown of some of the poly (A) sequences. These RNAse resistant TCA insoluble $^3$H-adenosine labelled counts comprised up to 4% of the undigested
Fig. 20: Sucrose Gradient Sedimentation of RNA Which Was Bound and Eluted from Poly (U)-GF/C Filters.

Twenty-five μgm of 3H-adenosine labelled infected cell RNA (specific activity 4,500 cpm/μgm) were filtered through each of two poly (U)-GF/C filters, eluted from them with 0.001 M PUBB and precipitated with ethanol. The precipitate, resuspended in 0.1 x SSC, was divided into three equal parts, of which one part was layered on a sucrose gradient, whilst the other two parts were together incubated with pancreatic RNAse at 10 μgm/ml. The reaction was stopped by the addition of SDS to a final concentration of 0.5%, and preparation layered on a sucrose gradient. Centrifugation and fractionation were done, as outlined in Fig. 3, except that centrifugation was at 42,000 rpm for 3 hours and only the TCA precipitable RNA of each fraction was counted.

(○) labelled RNA

(●) RNAse treated labelled RNA

arrows indicate 28s, 18s and 4s markers.
Fig. 20:

![Graph showing fraction number vs. $3H_{cpm}$](image)

- Fraction numbers: 5, 10, 15, 20
- $3H_{cpm}$ values: 0-300
- Marked points for 28s, 18s, and 4s
RNA. In contrast, RNase digested $^3$H-uridine labelled RNA gave no detectable TCA precipitable radioactivity on the top of the gradient or elsewhere.

10. Extraction of RNA from Infected Mouse Kidney Cells by Three Different Techniques.

The results in the above section show that very little 5 to 7s RNase resistant $^3$H-adenosine labelled RNA may be detected in RNase digests of RNA eluted from poly (U)-GF/C filters. This prompted an investigation into the nature of the RNA, and particularly its poly (A) region that is bound to the poly (U)-GF/C filter. A method of investigating this would be to prepare the cell RNA by different methods, one of which has been shown to yield complete recovery of poly (A) sequences (77).

Accordingly, RNA was prepared from three pairs of infected cell monolayer cultures labelled with $^3$H-adenosine, by the standard pH 5.2 phenol extraction at 60°C; by the phenol-chloroform extraction; and by phenol extraction at pH 9.0. The latter two techniques yielded some DNA in the extracts but this was removed by the DNAse treatment. A fraction of the RNA isolated and purified by each of the three techniques was passed through a poly (U)-GF/C filter in 0.3 M NaCl and 0.1 M NaCl. As shown in table 7, the binding of the RNA extracted by the standard pH 5.2 phenol
Table 7: Binding of RNA Extracted by Three Different Methods to Poly (U)-GF/C Filters.

Three preparations of $^3$H-adenosine labelled RNA were obtained by extracting infected MK cell cultures with phenol pH 5.2 at 60°C; with phenol-chloroform at room temperature, and with phenol pH 9.0. They had respective specific activities of 6,250, 7,500 and 8,000 cpm/µg. One pair of 14 µg samples from each of these preparations was passed through individual poly (U)-GF/C filters in 0.1 or 0.3 M PUBB. The filters were counted as was the TCA insoluble RNA in the filtrates.
Table 7: Binding of RNA Extracted by Three Different Methods to Poly (U)-GF/C Filters.

<table>
<thead>
<tr>
<th>RNA</th>
<th>Parameter</th>
<th>cpm on filter</th>
<th>cpm in filtrate</th>
<th>% cpm bound to filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenol 60°C</td>
<td>pH 5.2</td>
<td>1,121</td>
<td>84,670</td>
<td>1.30</td>
</tr>
<tr>
<td></td>
<td>0.1 M PUBB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>phenol CHCl₃</td>
<td>pH 5.2</td>
<td>7,235</td>
<td>98,026</td>
<td>6.86</td>
</tr>
<tr>
<td></td>
<td>0.1 M PUBB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>phenol pH 9.0</td>
<td></td>
<td>2,413</td>
<td>70,467</td>
<td>3.30</td>
</tr>
<tr>
<td></td>
<td>0.1 M PUBB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>phenol 60°C</td>
<td>pH 5.2</td>
<td>3,598</td>
<td>90,987</td>
<td>3.82</td>
</tr>
<tr>
<td></td>
<td>0.3 M PUBB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>phenol CHCl₃</td>
<td>pH 5.2</td>
<td>12,032</td>
<td>98,942</td>
<td>10.80</td>
</tr>
<tr>
<td></td>
<td>0.3 M PUBB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>phenol pH 9.0</td>
<td></td>
<td>4,539</td>
<td>68,767</td>
<td>6.20</td>
</tr>
<tr>
<td></td>
<td>0.3 M PUBB</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
at $60^\circ$C method bound the least efficiently, while the phenol-chloroform extracted RNA bound most efficiently. The phenol (pH 9.0) extracted RNA was intermediate in binding to poly (U)-GF/C filters.

Further tests were done on these three RNA preparations. Samples of RNA were sedimented on sucrose gradients to check if there was some unique component present which would account for increased binding. None was detected, though the phenol-chloroform extracted RNA did not contain as much rapidly sedimenting material. The overall yield of RNA, the radioactivity, specific activity and py RNA content were similar for all three preparations.

Samples of these RNA preparations were then bound to poly (U)-GF/C filters, eluted, part of the sample digested with RNAse, and the digested and undigested RNA sedimented on individual sucrose gradients. A considerably greater amount of the phenol chloroform extracted RNA was RNAse resistant as compared to the phenol pH 5.2 extracted RNA. The non digested RNA sedimented heterogeneously in a sucrose gradient, not unlike that of fig. 19, though it appeared to be composed of slightly smaller size classes. The RNAse resistant RNA did not however have a prominent 5 to 7s peak.

This last experiment raises a number of questions about the RNA preparation techniques and the basis for binding of RNA to poly (U)-GF/C filters, as well as providing a possible explanation for the results so far obtained in this chapter.

These issues will be discussed in the next chapter.
CHAPTER VI: DISCUSSION

The purpose of this study as stated in the Introduction, was to examine the processing of py RNA in virus infected cells. The last three chapters have directly or indirectly contributed to this study.

1. Viral RNA Synthesis in Infected Cells.

In order to correlate this work with other studies on polyoma virus, the time course of viral RNA synthesis was measured. This time course, under the conditions used in the experiments performed in this thesis, was found to be comparable to that of other workers (3)(112). It was also of interest to note, although results were not shown, that a relatively constant amount of cell RNA was labelled throughout the infection. This confirmed previous observations (112) that the initial stages of viral replication did not have much effect on the cellular RNA metabolism.

The observation that py infected BHK-21 cells did not produce detectable amounts of viral RNA, while under identical conditions MK and ME cells produced viral RNA, and viral DNA was shown to integrate into the BHK-21 cells (2), was of interest. Hamster tissue has moreover been shown to be capable of supporting the replication of py virus (46), and the presence of viral RNA has
also been demonstrated in transformed hamster cells (53). The above observation denotes that either a very small percentage of virus infected BHK-21 cells produce viral RNA, or that the entire cell population produces py RNA but in less than one tenth the quantity produced in productively infected ME cells early in infection. It is possible that only the infected BHK-21 cells, which are destined to be transformed, synthesize viral RNA at levels similar to ME cells early in infection. Under such circumstances, the detection of this viral RNA would have been beyond the scope of the technique used.

2. Size Distribution of Py RNA in Infected Cells.

The observation that py RNA from infected MK cells had a heterogeneous size distribution from 4s to \( > 45s \) when analyzed in a sucrose gradient, was in agreement with other workers (55) (1) (58). This phenomenon has been more rigorously examined by sedimentation in DMSO sucrose gradients, by polyacrylamide gel electrophoresis, and by resedimentation of RNA from different regions of the sucrose gradient. All of these studies showed that py RNA consisted of a heterogeneous population of molecules. Since py DNA is a small molecule (\( mw \ 3 \times 10^6 (10^9) \)) it would not be expected to code for a large variety of monocistronic m-RNA molecules, nor for a polycistrionic m-RNA representing a complete DNA transcript which was greater than \( 1.5 \times 10^6 (28s) \). Although the hydrogen bond mediated form-
ation of aggregates, to account for py RNA sedimenting faster than 28s, has been ruled out by use of DMSO sucrose gradients, it was still possible that the smaller py RNA molecules could have been due to RNase mediated breakdown. This possibility was unlikely, since in cases where $A_{260}$ measurements of the gradient fractions were performed, the 28s r-RNA fraction was a distinct peak containing about twice the absorbance of the 18s r-RNA, as would be expected for undegraded mammalian cell RNA. Likewise the RNA preparations were also always scanned for absorbance at 260 nm and 280 nm and the $\frac{A_{260}}{A_{280}}$ ratio was always 2.0, which indicated that the RNA was free of sufficient protein contamination to influence its sedimentation properties.

A number of hypotheses could explain the heterogeneous distribution of py RNA. It is possible that the py RNA undergoes a very rapid turnover, as was observed for $\phi$ x 174 DNA (47), hence a number of different sizes of partially synthesized and broken down RNA could account for the heterogeneity. In this case the 28s py RNA would have been transcribed from the continuation of transcription of a single circular genome. The fact that py DNA is a circular molecule supports this hypothesis. Alternatively, py RNA may be transcribed from the py DNA which was integrated into the cell DNA (2). In this case, the large number of different size molecules could be due to this integrated viral DNA that had undergone further recombinations with the cell DNA. As well several
copies of viral DNA may integrate in tandem and thus transcribed would lead to the formation of py RNA 28s. Finally, it is possible that viral DNA may be transcribed from integrated py DNA in such a way that it is a linear hybrid of viral and cell RNA. If the viral DNA has many integration points, a number of different size hybrid molecules could arise which would be greater than 28s. This last hypothesis is supported by the observation that in py transformed cells, the viral RNA has a similar size distribution (66). In these cells the viral DNA was not only integrated into the cell DNA but was likely smaller than one complete genome in size. Similar observations have been made with SV40 transformed cells (68).

3. Py RNA in Nuclear, Cytoplasmic and Polyribosomal Fractions.

The py RNA in the nuclear and cytoplasmic fractions of infected cells was similar in both size and base composition. In contrast to these observations, most of the viral RNA in the nuclei of SV40 infected cells was larger than in the cytoplasmic extracts (68) (102). In order to rule out the possibility that anomalies in cell fractionation techniques were responsible for the similarity between nuclear and cytoplasmic py RNA, two methods of fractionating the infected cells were used, namely, Dounce homogenization (76) and NP40 treatment (14).

In all cases, phase contrast microscopy revealed almost
quantitative breakage of cells and virtual absence of cytoplasmic "tabs" on the nuclei. According to the criteria of Penman (76) these cell fractions were relatively "clean" judging from the small amounts of heterogeneous-nuclear RNA in the cytoplasm and of 18s RNA in the nuclei. Although it is probable that some cross contamination occurred (i.e. leakage of nuclear RNA into the cytoplasm and binding of ribosomes to nuclear membranes) such levels could not account for the amount of >28s viral RNA in the cytoplasm.

If it is assumed that the presence of >28s RNA in the cytoplasm was due to "leakage" from the nuclei during cell fractionation, then a comparison of the amount of radioactive RNA in this region of a sucrose gradient between nuclear and cytoplasmic fractions would indicate the maximum extent of this "leakage". These values were therefore calculated for three separate experiments which comprised a complete analysis of nuclear and cytoplasmic viral RNA (using the Dounce homogenization technique) on sucrose gradients. The values for total >28s radioactive RNA ranged from 8.5% to 21.0% while the corresponding percentages for the virus specific >28s RNA (cytoplasm as percent of nucleus) ranged from 27% to 43%. Therefore unless there was a preferential leakage of viral specific >28s RNA, as opposed to cellular >28s RNA, it had to be concluded that virus specific RNA of greater than single genome size could be transported across the nuclear membrane. This conclusion was supported by the more limited studies using the NP40 technique.
and by examining the distribution of radioactivity in the cell fractions of $^3\text{H}$-thymidine labelled cells. In the latter case, less than 3% of the label was found in the cytoplasmic extract, which indicated a minimum of nuclear fragmentation (14).

These studies have shown that py RNA is transcribed in the infected cell nucleus as a heterogeneous collection of RNA molecules of different sizes, including RNA larger than one genome in length. A similar size distribution of viral RNA was recovered from the cytoplasm, and competition hybridization tests revealed that all nuclear py RNA sequences were represented in the cytoplasmic py RNA.

Virus specific RNA extracted from the polyribosomes was devoid of the $>28s$ species, implying that py RNA in excess of one genome in length was cleaved as part of its processing. This cleavage did not appear to be an artifact of purifying and characterizing the RNA, since the sedimentation behaviour of the polyribosomes indicated that no breakdown had occurred up to this point in the procedure. Since the polyribosomes were then immediately disrupted with SDS and the RNA extracted with hot phenol, it is unlikely that any enzymatic breakdown of the viral RNA occurred after this point. In addition the ribosomal RNA markers did not appear to have been degraded by this extraction process. Studies with SV$_{40}$ infected cells also showed that viral RNA in polyribosomes was smaller than in the nuclei (68).

The py RNA found in the polyribosomes sedimented at or below
18s, corresponding to a maximum molecular weight of about $0.5 \times 10^6$. This could well represent a monocistronic m-RNA coding for the capsid protein which has a molecular weight of $4.5 \times 10^4$ \((81)\). Since this protein comprises up to 70% of the virion, it is possible that the bulk of the py RNA codes for this protein.

It is not definite if all py RNA species eventually become associated with the polyribosomes. Because of technical limitations unequivocal results from competition hybridization experiments with py RNA from polyribosomes could not be obtained. It was significant however, that in py RNA from the cytoplasmic extract the small species (<18s) could efficiently compete with larger py RNA in a competition hybridization study. If the small species represented predominantly those present in the polyribosomes, then this observation would imply that all species of py RNA become eventually associated with the polyribosomes.

A comparison of these results with those of other workers, showed that there was agreement with the work of Cheevers \((13)\), who noted that the level of polyribosomes was stimulated in infected cells, as was also shown above. On the other hand, a study of the py RNA from the polyribosomes \((58)\) revealed that in a sucrose gradient, the majority of the py RNA sedimented below 18s, but there also appeared a distinct peak of viral RNA at 28s. No evidence for this peak was ever observed in the studies described in this thesis.
4. Processing of py RNA in Infected Cells.

The study of the kinetics of labelling of nuclear and cytoplasmic RNA of infected cells late in infection revealed three important facts: (a) the level of labelled py RNA in the nucleus was about 10 times as high as the level in the cytoplasm; (b) in the nucleus and labelling of py RNA progressed at the same rate as the labelling of the cell RNA; (c) labelled py RNA accumulated in the cytoplasm at the same rate as cell RNA for 30 minutes of labelling, after which the latter accumulated faster. These facts will now be discussed further.

The consistently higher level of py RNA in the nuclear fraction over the cytoplasmic fraction implied that most of the RNA in the nucleus may not be processed to the cytoplasm. This implication was confirmed by the pulse and chase studies which will be discussed later. That this observation was made at this stage is very significant since the possibility of it being a potential artefact caused by the use of Actinomycin D is eliminated.

The observation that in the nucleus, the viral RNA forms a constant fraction of the cellular RNA (~1.0%) regardless of the labelling time, was also important since it implies that the same mechanism is involved in the transcription of both RNA species. If this were indeed the case, the viral RNA could then be considered as a typical m-RNA and a study of its processing would thus be a more meaningful model for the cellular RNA.
In this regard the accumulation of labelled viral and cellular RNA in the cytoplasm appeared to be compatible with the concept of viral RNA being a typical m-RNA molecule. For the first 30 minutes, the viral RNA formed a constant fraction of the cellular RNA, after which the rate of accumulation for the cell RNA in the cytoplasm increased, while the rate for the viral RNA was constant. This observation may be explained by postulating that the RNA processed into the cytoplasm for the first 30 minutes of labelling was mostly m-RNA, after which the r-RNA and t-RNA, which have longer half lives, began to appear. The constant rate of accumulation of py RNA may thus be taken as an indication of the accumulation of m-RNA in the cytoplasm.

A more detailed extension of the kinetics of labelling was done in a pulse and chase study which examined the subsequent fate of py RNA labelled for 40 minutes, by stopping further labelling of RNA with Actinomycin D or excess uridine. The most significant observation was that about 75% of the labelled viral RNA in the nuclear fraction decayed within the first hour of chase without being transported to the cytoplasm. After the first hour of chase the rates of decay of the nuclear py RNA and cytoplasmic py RNA were roughly parallel. These observations, which were also reported by Kajioka (58), imply that there are two types of viral RNA labelled in the nuclear fraction. The more abundant type is very similar to the Hn-RNA in terms of its rapid labelling and
equally rapid decay properties, while the second type is a much more stable form which may have been derived from the first type. This observation could also be explained by postulating that the two types of RNA are transcribed from integrated and non-integrated DNA respectively.

A more detailed examination of the above RNA on sucrose gradients revealed that the rapidly decaying nuclear RNA was very similar in size distribution to the more stable form. Further examination of the sucrose gradients also indicated that during the chase, the large (>28s) py RNA was broken down at a slightly faster rate than the small (<18s) py RNA. Since such a trend was also observed in the cytoplasm, this implies that the large py RNA in the course of processing is broken down to 'intermediate' and finally into 'small size' species prior to translation. This argument is supported by the observation that in py RNA pulse labelled for 15 minutes, 46% sedimented faster than 28s, but in py RNA labelled for 2 hours only 25% sedimented in this region. Further support comes from the work of Acheson et al (1).

This similarity in processing of viral and cellular RNA of the nuclear fraction was also observed in the pulse and chase study with Actinomycin D. Both species appeared to decay with similar rates with py RNA comprising about 3% of the cell RNA.

It should be pointed out that the above conclusions are based on pulse labelling studies as well as pulse and chase studies using
an inhibitor of RNA synthesis. It is significant that the results obtained by all these studies are at least qualitatively in agreement.

5. Polyadenylate Sequences.

The fraction of py RNA molecules that were associated with poly (A) sequences increased as the py RNA was processed from the nuclear through to the polyribosomal fraction of the infected cell. In this regard, py RNA behaved as a typical cellular species of m-RNA studied in other cell lines (65) (19). The increased association of the py RNA from the cytoplasmic and polyribosomal fractions with poly (A) may indicate that only py RNA which was so modified was capable of being properly processed, while the rest was degraded either in the nuclear or cytoplasmic fractions. This hypothesis would account for the rapid degradation of py RNA in the nuclear fraction and its rather inefficient transfer to the cytoplasmic and polyribosomal fractions. In addition, when the relationship of poly (A) sequences to py RNA in a pulse and chase experiment was examined (data not shown), the fraction of py RNA in the nuclear fraction associated with poly (A) was seen to diminish throughout the chase period. This result was also in agreement with the above hypothesis.

The method of assaying for poly (A) sequences was taken, with very few modifications, directly from Brawerman (65). A considerable variation was observed in the values of the degree of poly (A)
association with the RNA, hence these results were considered only from a qualitative point of view.

6. Poly (A) Isolation Techniques.

In the initial studies, the results obtained by Brawerman (65) were confirmed and expanded. Millipore filters were shown to specifically bind RNAse resistant $^3$H-adenosine labelled RNA. The filters were not saturated by the amounts of RNA used, nor did yeast RNA compete with the binding of labelled cell or viral RNA. A protocol was also established in which RNA was applied to the filter in 0.5 ml and the filter washed with 4.5 ml of buffer. None of the poly (A) containing RNA was removed by the washing. These studies were essential from a technical point of view.

Further studies showed that there were uncertainties associated with this technique. In contrast to the homogeneous distribution of single stranded DNA on Millipore filters (52), the RNA was not distributed homogeneously across the filter surface, hence methods of sampling the filter were unsatisfactory. A considerable discrepancy was also seen between the amount of RNA bound to Millipore, nitrocellulose and poly (U)-GF/C filters. These observations placed the actual mechanism of the binding of the RNA to Millipore in question.

Some studies on the mechanism of binding of poly (A) sequences
to different cellulose derivatives have implied that it was the lignin impurities in the cellulose which were responsible for the binding properties (23). Further analysis of the Millipore and nitrocellulose filters could possibly clarify this problem.

The failure of the poly (U)-GF/C filters, which represent a better defined system, to bind significant quantities of viral RNA in 0.12 M NaCl led to further investigation of this system. The uncertainties inherent in this method were: (a) the optimal salt concentration to be used, and (b) the amount of 'non-poly (A)' binding that would occur at different salt concentrations. The degree of binding as a function of salt concentration followed a biphasic pattern from which 0.9 M NaCl was taken to be the optimal salt concentration. This observation was in marked contrast with studies performed by Kates and co-workers (90) who showed that the optimal salt concentration for binding poly (A) to the poly (U)-GF/C filters was 0.12 M NaCl. However, this study was performed with pure labelled poly (A), hence this result may not readily apply to an RNA molecule of which only a small terminal portion is poly (A).

A number of studies were performed to investigate the degree of non-specific binding of 'A rich' regions to the poly (U) on the filter as a function of the salt concentration. Ribosomal RNA, which comprises the bulk of the cell RNA, was shown to bind to a very small degree and this was only marginally increased by
increasing the salt concentration. A study of the elution pattern of the RNA, that had been bound to the filter in 1.5 M NaCl, as a function of the salt concentration, revealed that the RNA eluted in a biphasic manner which reflected a reverse of the above binding pattern. In this study, as well as in a similar study by other workers (90), only 80% of the RNA bound to the filters was eluted. The nature of the non-eluted RNA was not examined. Neither of these studies led to a differentiation between the poly (A) binding and the non-specific binding by 'A rich' regions.

A comparison of the effects of increasing salt concentration on the binding of RNA to poly (A), poly (U) and poly (C)-containing glass fibre filters was made in a further effort to resolve the question of non-specific binding. The high degree of binding to poly (A) and poly (C) filters at even relatively low salt concentration indicated that there must be regions in mammalian cell RNA rich in U and G, an observation also made recently by other workers (74). Moreover, the high degree of binding (60%) to poly (A) filters at high salt concentration indicated that this U rich region may well be associated with the ribosomal RNA, which is the only species present in such large amounts.

The question of non-specific binding was partially resolved by sucrose gradient sedimentation of the RNA bound to and eluted from poly (U)-GF/C filters at various salt concentrations (0.1, 0.3 and 0.9 M). The sedimentation pattern of all these preparations
revealed that the RNA was heterogeneously distributed throughout the gradient and lacking any distinct r-RNA or t-RNA markers. This observation indicated that only messenger-like RNA or m-RNA would bind to poly (U)-GF/C filters. No change in this pattern was observed for the various salt concentrations in which the RNA was bound to the filters, indicating that at least in size, the RNA which binds to the filters is the same regardless of the salt concentration in which it is bound.

The RNA which bound to and was eluted from the poly (U)-GF/C filters did not appear to have been degraded since, if it represented messenger-like RNA, it sedimented in a similar manner to pulse labelled cell RNA, which was predominantly messenger-like RNA (fig. 13). When the above $^3$H-adenosine labelled RNA, eluted from poly (U)-GF/C filters, was first treated with RNAse and then analyzed on a sucrose gradient, its acid precipitable radioactivity sedimented at 4s, while that of similarly treated $^3$H-uridine labelled RNA was hardly detectable even on the top of the gradient. This was taken as firm evidence that there are poly (A) sequences associated with RNA trapped by the poly (U)-GF/C filters, although these sequences were shorter than those reported by other workers (19) (32).

In view of this last finding, an attempt was made to determine if the length of the poly (A) sequences is responsible for the lack of binding to poly (U) in the presence of the lower salt concentrations. RNA preparations were extracted by three different methods,
of which at least one (phenol-CHCl₃) was shown to give undamaged poly (A) sequences (77). From this it was found that the method of extraction of the RNA has a profound effect upon its ability to bind to poly (U).

An analysis of the size distribution of the (CHCl₃-phenol extracted) RNA binding to poly (U) before and after RNAse treatment revealed that although the ³H-adenosine labelled, RNAse resistant sequences still sediment around 4s, they appear somewhat larger in size and greater in concentration. (figure not shown).

These facts point to an explanation for the poly (A) binding characteristics observed above. If one assumes that extraction with hot phenol, followed by cold phenol as done for the most part in this study, is relatively inefficient in extracting whole un-fragmented poly (A) sequences (a fact also shown recently by Perry) (74), then the poly (A) sequences attached to this RNA could be shorter. These short sequences, while still binding to poly (U), may not form as stable a hybrid in low salt as in higher salt concentrations. When the extraction was done with phenol chloroform, shown by the above authors to be efficient in extracting whole poly (A) sequences, then the longer sequences form stable hybrids under lower salt concentrations. It was however puzzling that the poly (A) sequences from these RNA preparations still sedimented at 4s. This observation has also been observed by Faulkner for an arbovirus RNA (28). It is possible that mouse
cells may have shorter poly (A) sequences, or alternatively the RNAse concentration used (10 to 20 μg/ml) could have been too high, and resulted in some breaks in the poly (A) sequences.
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