BIOCHEMICAL AND BIOPHYSICAL STUDIES ON THE RNA SPECIES OF SINDBIS VIRUS

Thesis by

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To

Phyl, Mom, and Dad

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ABSTRACT

Sindbis virus, a togavirus, contains an infectious, single stranded RNA with a molecular weight of 4.3×10^6 (49S), which in infected cells serves as a messenger RNA for the translation of the virus nonstructural proteins. Also present in infected cells is an RNA with a molecular weight of 1.6×10^6 (26S) which consists of the 3' terminal third of the 49S RNA and functions as a messenger RNA for the translation of the virus structural proteins. In this thesis, three features of these RNA species of Sindbis virus are studied: the 3' terminal poly A tracts on the RNAs and the corresponding poly U tracts on the complementary strand of RNA; the methylation of cytidine residues in the RNA; and the circularization of the 49S RNA molecule.

Both 49S and 26S RNA contain 3' terminal stretches of polyadenylic acid (poly A), which are heterogeneous in size, having a mean length of 70 nucleotides and a size range of from 40 to 200 nucleotides. Poly A isolated from Sindbis virion 49S RNA grown in chicken, hamster and mosquito culture cells have similar size distributions. Although most 49S and 26S RNA molecules contain poly A, a small fraction of intact 49S and 26S RNA molecules contain little poly A. The fact that the fraction of 49S which lacks poly A is only 10% to 20% as infectious as the fraction which contains poly A suggests that poly A is essential for replication of the virus. Sindbis virus double stranded RNA species also contain poly A with a size distribution similar to that of poly A from viral single stranded RNA. The double stranded RNA

species also contain stretches of polyuridylic acid (poly U) which are on the minus strand and have a size distribution identical to Sindbis virus poly A. This indicates that the poly A in Sindbis virus RNA is synthesized by transcription of a poly U template by the virus transcriptase.

Sindbis virus intracellular 26S and 49S RNA contain internal 5-methyl cytidine (m^5C) residues. Sindbis virion 49S RNA contains much less m^5C than intracellular 49S RNA. In the 26S RNA, m^5C residues occur in five oligonucleotides, which are found distributed between two locations, one approximately 4000 nucleotides from the 3' end and the other about 1200 nucleotides from the 3' end (out of a total length of 5000 nucleotides). The distribution of label between these two locations suggests that each contains at least two of the methylated sequences. It thus appears that there are five specific sites for methylation on the 26S RNA. However, only a minority of these sites are modified. Polysomal and nonpolysomal 26S RNA contain equal amounts of m^5C while polysomal 49S RNA contains 60% to 80% more m^5C than nonpolysomal 49S RNA, indicating that m^5C may have a function in translation.

Sindbis virus 49S RNA is capable of assuming a circular configuration through the hydrogen bonding of complementary nucleotide sequences located at the 5' and 3' ends of the 49S RNA molecule. The circular and linear forms of 49S RNA are separable on sucrose gradients containing 0.01 M NaCl. Sindbis virus 49S RNA extracted from virions is completely in the circular form. The melting temperature (T_m) of the

circles is 39.5°C in 0.023 M NaCl and 53.5°C in 0.1 M NaCl. The ΔH for circularization is -160 kcal/mole and the ΔS for circularization is approximately 500 eu. These parameters indicate that the length of the double-stranded region which is formed upon circularization of the molecule is most likely short, on the order of 10 to 20 nucleotides. Our data indicate that extensive mismatching in this double stranded region is unlikely. Intact linear 49S RNA molecules readily renature to form circles under appropriate conditions, the energy of activation for this process being 42.6 kcal/mole. From the measured rate constants for circularization, it is clear that Sindbis virus 49S RNA will circularize readily under physiological conditions of temperature and ionic strength. The virion RNA from Semliki Forest virus also forms circles whose T_m is very similar to that of Sindbis virus RNA circles, suggesting that the sequences involved in circularization have been conserved.

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INTRODUCTION

In this Introduction, the replication of Sindbis virus will be discussed and then compared to the replication of the other RNA-containing animal viruses. Sindbis virus is a member of the togavirus family of animal viruses (for reviews, see Shapiro and Pfefferkorn (1974); Strauss and Strauss (1977)). A togavirus consists of a single piece of RNA contained in an icosahedral nucleocapsid which is in turn, as the name "toga" suggests, enclosed by a membrane "cloak." The togavirus family consists of two genera, the alphaviruses and the flaviviruses, and several ungrouped viruses with the basic togavirus structure. Members of the alphavirus genus include Sindbis virus, Semliki Forest virus, eastern, western, and Venezuelan equine encephalitis viruses, and chickungunya virus. Members of the flavivirus genus include yellow fever virus, Dengue virus, and St. Louis and Japanese encephalitis viruses. The alphavirus and flavivirus genera were originally classified in the arbovirus group. Arboviruses are transmitted between vertebrate hosts by an insect vector, most often a mosquito in the case of the alpha- and flaviviruses. In contrast to the cytopathic replication of arboviruses in vertebrate cells, an arbovirus infection of insect cells is persistent and non-cytopathic. Since the arbovirus group was an epidemiological classification, it contained viruses with

dissimilar structures and modes of replication. The togavirus family is based on structural criteria and includes a few viruses, such as rubella virus of humans and lactate dehydrogenase virus of mice, which are not arboviruses.

There are distinct differences in the structure and the mode of replication of the alphavirus and flavivirus genera. Alphaviruses are conducive to study because of their relatively short growth cycle, their ability to grow to high titers in cultured cells, and their low pathogenicity to man. In contrast, flaviviruses grow relatively slowly, achieve titers of an order of magnitude less than the alphaviruses, and are dangerous human pathogens. Therefore, the replication of the flaviviruses has not been well studied. Since Sindbis virus is an alphavirus, only the replication of the alphaviruses will be discussed here. The replication of the flaviviruses will be discussed when the replication of the alphaviruses is compared to the replication of other RNA-containing animal viruses.

Most of the research on the structure and replication of the alphaviruses has been done in two viruses, Sindbis virus and Semliki Forest virus. Although the structure and replication of these two viruses is very similar, there are some differences which will be pointed out.

Structure of the Alphavirus Virion

The alphavirus virion is approximately 70 nm in diameter (Acheson and Tamm, 1967) and has the following composition: 57 to 61% protein; 6% RNA; 6% carbohydrate; and 27 to 31% lipid (Pfefferkorn and Hunter, 1963; Laine et al., 1973). The virion RNA has a molecular weight of 4.3×10^6 daltons (49S) which corresponds to approximately 13,000 nucleotides (Arif and Faulkner, 1971; Simmons and Strauss, 1972a). The alphavirus nucleocapsid is composed of the 49S RNA associated with approximately 300 copies of a 30,000 dalton protein known as the capsid protein (Strauss et al., 1969). The nucleocapsid is surrounded by a lipid bilayer, which is derived from the host cell plasma membrane (Pfefferkorn and Hunter, 1963), and in which are embedded two or three virus specific glycoproteins. Sindbis virus contains two glycoproteins, designated E1 and E2, both of which have molecular weights of approximately 50,000 daltons (Strauss et al., 1969; Schlesinger et al., 1972). Along with E1 and E2, Semliki Forest virus also contains a third glycoprotein, E3, with a molecular weight of 10,000 daltons (Garoff, Simons and Renkonen, 1974). The alphavirus structural proteins are all present in the virion in equimolar amounts (Strauss et al., 1969). The bulk of the glycoprotein mass is on the exterior of the virion membrane (Compans, 1971; Harrison et al., 1971). However, part of at least one of the glycoproteins spans

the lipid bilayer, making contact with the capsid protein and providing a source of alignment between the exterior and the interior of the virion particle (Garoff and Simons, 1974). The naked virion 49S RNA is infectious (Wecker, 1959) and thus can serve as the messenger RNA for the translation of virus specific proteins (an RNA molecule capable of serving as a messenger RNA is designated as having plus polarity (Baltimore, 1971)). Indicative of its ability to serve as a messenger RNA, the virion 49S RNA of the alphaviruses is capped on its 5' terminus (Hefti et al., 1976) and polyadenylated in its 3' terminus (Deborde and Leibowitz, 1976; Eaton and Faulkner, 1972; Johnston and Bose, 1972; Frey and Strauss, submitted for publication, and this thesis). A cap is a 7-methyl guanosine residue connected in a 5' to 5' orientation to the adjacent nucleotide by a triphosphate bridge (Shatkin, 1976). Most eukaryotic messenger RNAs are capped and the function of the cap does appear to be in translation (Shatkin, 1976). In in vitro translation systems, the cap is functional in the formation of the messenger RNA-ribosome initiation complex (Roman et al., 1976) and also protects the messenger RNA from nuclease degradation (Furuichi, La Fiandra and Shatkin, 1977). The 5' terminal sequence of Sindbis virus 49S RNA is m^7G (5') ppp (5') Ap Up Gp . . . (Hefti et al., 1976). Many eukaryotic messenger RNAs contain a 3' terminal stretch of polyadenylic acid (poly A) (review:

see Darnell, Jelinek, and Molloy, 1973). The function of poly A in translation is unclear. The poly A stretch of HeLa cell messenger RNA becomes shorter with age of the messenger RNA and therefore may protect the messenger RNA from nuclease degradation (Sheiness and Darnell, 1973). A specific poly A binding protein has been found in messenger ribonucleoproteins of several cell lines (Schwarz and Darnell, 1976). The poly A on alphavirus virion 49S RNA is from 40 to 120 nucleotides long, much shorter than the poly A averaging 200 nucleotides in length found in eukaryotic cell messenger RNA (Darnell, Molloy and Jelinek, 1973; Eaton and Faulkner, 1972; Frey and Strauss, submitted for publication, and this thesis).

Another interesting feature of the alphavirus virion 49S RNA is its ability to circularize by the hydrogen bonding of complementary nucleotide sequences located near the 5' and 3' ends of the 49S RNA molecule (Hsu, Kung and Davidson, 1973). The function of the circular feature is unknown.

Translation of Alphavirus Specific RNA Species in Vertebrate Hosts

Alphaviruses replicate entirely within the cytoplasm of the infected cell. A replicating alphavirus shuts down host cell macromolecular synthesis by an, as yet, undetermined mechanism (Strauss et al., 1969). This shutdown is an aid to research on alphavirus replication, since when

infected cells are given radioactive precursors, primarily virus specific macromolecules are labeled. Usually actinomycin D is added to infected cultures to render the shutdown of host cell macromolecular synthesis complete.

Alphavirus infected cells contain four species of virus specific single stranded RNA (Levin and Friedman, 1971). One species is the 49S RNA which is found primarily in intracellular nucleocapsids but also on polysomes (Mowshowitz, 1973; Simmons and Strauss, 1974b; Söderlund, 1973). Intracellular 49S RNA, both polysomal and non-polysomal, is apparently identical with virion 49S RNA (Wengler and Wengler, 1975). The most abundant virus specific intracellular RNA is an RNA with a molecular weight of 1.6×10^6 daltons (26S) (Simmons and Strauss, 1972a), which is found both in polysomes and in nonpolysomal ribonucleoprotein (Mowshowitz, 1973; Simmons and Strauss, 1974b). The 26S RNA consists of the 3' terminal third of the 49S RNA (Kennedy, 1976; Simmons and Strauss, 1972a; Wengler and Wengler, 1976). Two minor species of alphavirus single stranded RNA with sedimentation coefficients of 33S and 38S have also been detected in infected cells (Levin and Friedman, 1971; Martin and Burke, 1974). The 33S RNA species has been found in polysomes (Simmons and Strauss, 1974b). The 33S and 38S RNAs are apparently conformational variants of the 26S and 49S RNAs, respectively (Kennedy, 1976; Simmons and Strauss, 1974b).

This subdivision of the alphavirus genome represents a control over virus specific translation. The 26S RNA is translated into the virus structural proteins (Cancedda et al., 1974; Clegg and Kennedy, 1974; Simmons and Strauss, 1974a; Wengler, Beato and Hackemack, 1974), which are needed in abundance for virus replication. The 49S RNA, although it contains the genetic information for the virus structural proteins, apparently can only direct the translation of nonstructural virus proteins (Bracha, Leone and Schlesinger, 1976; Simmons and Strauss, 1974b), which are necessary in much smaller quantities during virus replication.

Since, temperature sensitive mutants of Sindbis virus fall into seven complementation groups (Strauss, Lenches, and Strauss, 1976), seven virus specific proteins must be translated in infected cells.

Consistent with this finding, besides the three virus structural proteins, four nonstructural proteins have been detected in infected cells (Lachmi and Kääriäinen, 1976, 1977).

Alphavirus 26S RNA is capped on its 5' terminus, Sindbis virus 26S RNA having the 5' terminal sequence m^7G (5') ppp (5') Ap Up . . . (Dubin et al., 1977).

Alphavirus 26S RNA also contains 3' terminal poly A of similar size as the 49S RNA poly A (Deborde and Leibowitz, 1976; Eaton, Donaghue, and Faulkner, 1972; Frey and Strauss, submitted for publication, and this thesis). The 26S RNA of Sindbis virus contains internal 5-methyl cytidine residues (Dubin and Stollar, 1975; Dubin et al., 1977; Frey and Strauss, submitted for publication, and this thesis). The intracellular 49S RNA of Sindbis virus also contains internal 5-methyl cytidine residues, although to a lesser extent than the 26S RNA. Since Sindbis virion 49S RNA contains little 5-methyl cytidine, the function of 5-methyl cytidine might possibly be in the translation of viral RNA.

There is only one site for the initiation of translation on the alphavirus 26S RNA (Cancedda et al., 1975; Glanville et al., 1976b). The 26S RNA is translated into a large precursor polyprotein which is then cleaved into its component proteins. Cleavage probably occurs during translation while the polypeptide is still nascent on the ribosome and thus the large precursors are detected in infected cells only under certain circumstances, as will be described (Strauss and Strauss, 1977). The capsid protein is the first protein translated from the 26S RNA and cleaved from the large precursor (Clegg, 1975; Söderlund, 1976). The next protein translated and cleaved is a 62,000 dalton protein (PE2), which is a precursor to the E2 and E3

proteins (Clegg, 1975; Schlesinger and Schlesinger, 1973; Simons, Keranen, and Kääriäinen, 1973). Finally the E1 protein is translated (Clegg, 1975). In cells infected with a Group C temperature sensitive mutant of Sindbis virus (ts-2) at the nonpermissive temperature, cleavage of the structural proteins fails and a 130,000 dalton peptide precursor to the structural proteins accumulates (Strauss et al., 1969; Schlesinger and Schlesinger, 1973). In BHK cells infected with wild type Sindbis virus or Semliki Forest virus, a small amount of 98,000 dalton peptide precursor (B-protein) to proteins E1 and PE2 accumulate (Strauss et al., 1969; Schlesinger and Schlesinger, 1973). These precursors cannot be chased into structural proteins, supporting the idea that cleavage occurs while the precursor is nascent on the ribosome.

When alphavirus 26S RNA is translated in an in vitro system, the following peptides have been observed: (1) capsid protein and unglycosylated E1 and PE 2 (Clegg and Kennedy, 1975); (2) capsid protein and B-protein (Simmons and Strauss, 1974b); and (3) capsid protein with small molecular weight fragments of the envelope proteins (Cancedda and Schlesinger, 1974a,b; Glanville, et al., 1976b; Wengler, Beato and Hackemack, 1974). The difference in results is probably a function of the cell line from which the cell free system is derived and the intactness of the 26S RNA used.

In vivo, newly translated capsid protein is released into the cytoplasm where it rapidly complexes with 49S RNA into nucleocapsids (Söderlund, 1973). E1 and PE2 are inserted into the intracellular membrane system where they are glycosylated by cellular enzymes and eventually emerge at the surface of the cell (Strauss and Strauss, 1977). PE2 is cleaved into E2 and E3 in the cell surface during maturation of the virus (Simons, Keranen, and Kääriäinen, 1973). Since in cells infected with Sindbis virus a precursor to E2 which is cleaved to E2 during virus maturation is found (Schlesinger and Schlesinger, 1973), Sindbis virus must have an E3 component which is somehow lost from the virus surface.

Alphavirus 26S RNA is an interesting messenger RNA because both soluble and membrane bound proteins are translated from it. In eukaryotic cells, soluble proteins and proteins which will be localized in the cytoplasmic face of the cytoplasmic membrane are translated on free polysomes, while integral membrane proteins and proteins for export are translated on membrane bound polysomes and immediately transferred to the intracellular membrane system for processing (Morrison and Lodish, 1974; Palade, 1975). It is theorized that all membrane and exported proteins contain a short hydrophobic "leader" amino acid sequence on their amino terminus which binds the polysome to the membrane while the protein is still nascent (Blobel

and Dobberstein, 1975a,b). Subsequent to this initial interaction, polysomes are thought to be bound to the membrane by both the nascent protein and the 60S ribosomal subunit. The interaction of the nascent protein and the membrane also facilitates the insertion of the protein into the membrane matrix after which the leader sequence is cleaved from the protein.

In cells infected with Sindbis virus, 70% of the 26S RNA is associated with membrane bound polysomes (Martire et al., 1977; Wirth et al., 1977). Membrane bound polysomes isolated from infected cells synthesize both the capsid protein and the two membrane proteins in vitro (Wirth et al., 1977). In this in vitro system, the capsid protein is released free in the supernatant while E1 and PE2 are somehow sequestered in the membrane. As expected, Sindbis virus specific polysomes are bound to membranes by a combination of the ribosome and the nascent peptide.

In contrast to alphavirus 26S RNA, messenger 49S RNA is only found associated with free polysomes (Martire et al., 1977; Wirth et al., 1977). As stated before, translation is initiated by only one site on the 49S RNA, a site different than the 26S RNA initiation site (Glanville et al., 1976b).

The 49S RNA must contain the 26S RNA initiation site, which must somehow be rendered inoperative on the 49S RNA molecule. Possibly this site is made inaccessible to

ribosomes by the secondary structure of the 49S RNA or possibly it must be near a cap group, as it is in the 26S RNA, to be functional.

The virus nonstructural proteins which are translated from the 49S RNA are produced in such small quantities that they are difficult to detect in infected cells. However, four virus specific nonstructural proteins, with molecular weights of 86,000, 78,000, 70,000, and 60,000 daltons (designated ns86, ns78, ns70 and ns60, respectively) have been found in cells infected with Semliki Forest virus (Lachmi and Kääriäinen, 1976, 1977). Two of the three proteins, with molecular weights of 90,000 and 63,000 daltons (which probably correspond to ns86 and ns60) have been shown to be components of the Semliki Forest virus RNA replicase (Clewley and Kennedy, 1976). In Sindbis virus infected cells, three, and possibly four, nonstructural virus proteins have been detected (Brzeski and Kennedy, 1977). Similarly to the structural proteins, the alphavirus nonstructural proteins are processed from large polyprotein precursors (Lachmi and Kääriäinen, 1976, 1977; Clegg, Brzeski, and Kennedy, 1976). In Semliki Forest virus infected cells, two precursor proteins can be detected: a 155,000 dalton protein which has the peptide content of ns86 and ns70; and a 135,000 dalton protein which has the peptide content of ns78 and ns60 (Lachmi and Kääriäinen, 1976, 1977). The order of translation of the

nonstructural proteins from the 49S RNA is (5') ns70, ns86, ns78, ns60 (3').

Alphavirus 49S RNA has been translated *in vitro* with mixed results. In some studies, the polypeptides translated from the 49S RNA include capsid protein, fragments of the membrane proteins, and unidentified polypeptides (Cancedda *et al.*, 1974b, 1975; Glanville *et al.*, 1976a; Smith *et al.*, 1974). In other seemingly more careful studies, the only proteins translated are of large molecular weight and bear no resemblance to the virus structural proteins (Glanville *et al.*, 1976b; Simmons and Strauss, 1974b). The intactness of the 49S RNA preparations used could explain the differences in the results. Since *in vivo*, translation of the virus structural proteins is dependent on the continued synthesis of 26S RNA (Bracha, Leone, and Schlesinger, 1976), it seems likely that the 49S RNA cannot serve as a messenger for the translation of the virus structural proteins.

Transcription of Alphavirus RNAs

Alphavirus specific RNA is transcribed in infected cells by a virus specific RNA-dependent-RNA-polymerase or transcriptase. Alphavirus transcriptase activity is tightly associated with the host cell membranes (Grimley *et al.*, 1972; Friedman *et al.*, 1972). Solubilized extracts which show transcriptase activity have been derived from Semliki Forest virus infected cells (Clewley and Kennedy,

1976; Michel and Gomatos, 1973; Sreevalsen and Yin, 1969). However, these *in vitro* transcription systems have the drawback of being template independent and extremely labile. As mentioned before, the transcriptase of Semliki Forest virus has been isolated and has been shown to contain two virus specific proteins (Clewley and Kennedy, 1976).

When alphavirus infected cells are given radioactive RNA precursors, the first virus specific RNA to be labelled is a partially double stranded species called the replicative intermediate (RI) (Friedman, 1968; Simmons and Strauss, 1972b). The structure of the RI is presumably a double stranded RNA backbone with single stranded RNA tails. It is not clear whether transcription occurs on a double stranded template *in vivo* or whether the replicating virus RNA is mostly single stranded, but collapses together and hydrogen bonds during deproteinization, thus forming the RI (Weissmann *et al.*, 1968; Thach and Thach, 1973; Thach *et al.*, 1974). The RI has been shown to contain a minus strand equal in size to the 49S plus strand RNA (Bruton and Kennedy, 1975). The minus strand RNA must be transcribed off plus strand RNA, and vice versa but the factors which discriminate between plus and minus strand transcription and heavily favor plus strand transcription are not understood.

When treated with ribonuclease to cut off single stranded RNA tails, alphavirus RIs give rise to three species of double stranded RNA known as RFs (Simmons and

Strauss, 1972b). RFI has a molecular weight of 8.8 x 10^6 and contains the plus stranded nucleotide complement of the 49S RNA. RFII and RFIII have molecular weights of 5.6 x 10^6 and 3.2 x 10^6 and contain the plus stranded nucleotide complement of the non26S and 26S regions of the 49S RNA respectively. Thus, it is theorized that RFII and RFIII are derived from an RI engaged in the synthesis of 26S RNA.

Since the alphavirus minus strand RNA is equal in size to 49S RNA (Bruton and Kennedy, 1974) and 26S RNA represents the 3' end of 49S RNA (Kennedy, 1976), initiation of transcription of 26S RNA must occur at an internal position on the minus strand RNA molecule. Since RI molecules are interconvertible between the RFI and RFII:RFIII configurations (Sawicki, Kääriäinen, and Gomatos, 1977), the factor which discriminates between 26S transcription and 49S transcription must be reversible. Conceivably it could be a distinctive secondary structure feature in the minus strand RNA near the 26S RNA initiation site which is recognized either by a 26S RNA specific polymerase or a protein which facilitates transcription of 26S RNA by the transcriptase which synthesizes 49S RNA (Strauss and Strauss, 1977). Members of one of the RNA$^-$ complementation groups of Sindbis virus are deficient in the transcription of 26S RNA at the nonpermissive temperature (Scheele and Pfefferkorn, 1969; Waite, 1973). A curious feature of alphavirus RFs is that the plus strand of RFII is slowly

labelled, yet no single stranded virus RNA with the plus strand nucleotide complement of RFII has ever been detected in infected cells (Simmons and Strauss, 1972b).

Since the minus strand of both Semliki Forest virus and Sindbis virus contain poly U similar in size to the plus stranded poly A (Sawicki and Gomatos, 1976; Frey and Strauss, submitted for publication, and this thesis), poly A is most likely added to alphavirus RNA by transcription of the poly U template by the virus transcriptase. It is unknown if the alphavirus RNA is capped and methylated by a virus or host cell enzyme.

Upon completion of transcription, most 49S RNA molecules are complexed into nucleocapsids, but some become associated with polysomes (Simmons and Strauss, 1974a). Though the mechanism of this assortment is unclear, it is possible that 49S RNA preferentially associates with capsid protein. Early in replication, when the virus nonstructural proteins are needed and capsid protein is present only in small amounts, is when a significant fraction of the newly transcribed 49S RNA is free to associate with polysomes (Lachini and Kääriäinen, 1977).

Comparison of the Replication of Alphaviruses to other Animal Viruses

The strategies of replication followed by the RNA containing animal viruses are incredibly varied. In this section, the important features of the replication of the

other groups of RNA containing animal viruses will be briefly discussed and compared to the important features of alphavirus replication. The virus groups will be discussed more or less in increasing order of complexity of their replication.

The picornaviruses are the family of RNA containing animal viruses most closely related to the alphaviruses and flaviviruses (for review, see Rekosh, 1977). The most studied members of the picornavirus family are poliovirus, encephalomyocarditis virus, Mengovirus, and the rhinoviruses. The genomic RNA of picornaviruses is a single piece of plus polarity RNA with a molecular weight of 2.5 to 2.8 x 10^6 daltons. The genomic RNA is complexed with 4 virus specific proteins to form the icosahedral virion. Picornaviruses replicate entirely within the cytoplasm of the host cell and their replication completely inhibits host macromolecular synthesis. There is no subdivision of the genomic RNA during picornavirus replication. The picornavirus virus messenger RNA has the same size and nucleotide composition as the virion RNA. Both picornavirus virion and messenger RNA are polyadenylated on their 3' terminus, but neither RNA species is capped; the 5' terminus of both RNA species of polivirus begins with pU . . . . Besides picornavirus messenger RNA, the only other species of eukaryotic messenger RNA which has been found not to be capped in its 5' terminus is the messenger RNA of

the plant virus satellite tobacco necrosis virus (Wimmer, et al., 1968). The picornavirus virion and messenger RNAs are not identical in one respect: the virion RNA of poliovirus has been recently shown to contain a small virus specific protein covalently bound to its 5' terminus (Flanagan et al., 1977; Lee, 1977). At this date, poliovirus virion RNA is the only RNA known to be covalently attached to a protein, although the DNA of adenovirus contains proteins covalently attached to its ends (Robinson et al., 1973). Translation of picornavirus RNA begins at a single initiation site and the individual proteins are produced by cleavage of a large precursor. There is thus no control over picornavirus translation, and all picornavirus proteins, both structural and nonstructural, are synthesized in equimolar amounts. Picornavirus RNA is transcribed by an intracellular transcriptase composed of nonstructural virus proteins. Similar to the alphaviruses, poly A is added to the picornavirus RNA by transcription of a poly U template in the minus strand by the virus transcriptase. The picornavirus transcriptase activity is tightly associated with smooth cellular membranes.

Thus the replication of the picornavirus and the alphaviruses is similar in that the genomic RNA of both virus groups is plus stranded and serves as a messenger RNA in infected cells. The messenger RNAs of both virus groups are multicistronic and contain one translation initiation site,

the individual proteins being processed by the cleavage of a large precursor. The subdividion of the alphavirus genome provides a simple control over the abundance of structural and nonstructural virus proteins in infected cells, a control not present in picornavirus replication.

The structure of the flavivirion differs from an alphavirion in that it is slightly smaller and contains two nucleocapsid proteins and one membrane glycoprotein (for reviews: see Pfefferkorn and Shapiro, 1974; Strauss and Strauss, 1977). The flavivirus virion RNA is plus polarity, has a molecular weight of 4.2×10^6, and is polyadenlyated on its 3' terminus. The structure of the 5' terminus of flavivius RNA is unknown. Flavivirus replication is entirely cytoplasmic and is slightly toxic to host cell macromolecular synthesis. The only species of flavivirus messenger RNA is identical in size to the virion RNA. Flavivirus messenger RNA is unique among eukaryotic messenger RNAs in that it contains internal initiation site for the translation of the virus specific proteins (Westaway, 1977). Since flavivirus proteins are not present in infected cells in equimolar amounts (Westaway, 1973), the abundance of these proteins could be controlled at the initiation of translation. Flavivirus RNA is transcribed by an intracellular, membrane bound, virus specific transcriptase.

The only other group of animal viruses whose virion contain plus polarity single stranded RNA are the

retroviruses (for review: see Gilden, 1977). Members of the retrovirus group contain reverse transcriptase and replicate through integration of a DNA copy of the virus RNA into the host cell's genome. Many of the retroviruses are capable of transforming the host cell. Thus, the replication cycle of the retroviruses is radically different from the replication cycles of the other RNA containing animal viruses. For this reason, the replication of the retroviruses will not be discussed except to note two similarities to the replication of the alphaviruses. One similarity between alphavirus and retrovirus replication is that retrovirus RNA isolated from infected cell polysomes consists of both molecules of similar size to the virion RNA and smaller molecules which are subsets of the virion RNA. The second similarity is that retrovirus proteins are processed by cleavage from large polyprotein precursors.

The virion RNAs of the remaining RNA containing animal virus groups is either single stranded and of negative polarity or double stranded. The two animal virus groups whose virions contain a single piece of single stranded RNA of negative polarity, the rhabdoviruses and the paramyxoviruses, will be discussed first. Though the best known member of the rhabdovirus group is rabies virus, by far the most studied is vesicular stomatitis virus (VSV) of cattle (for review: see Bishop and Smith, 1977). VSV virion RNA (molecular weight, 3.8×10^6) is complexed with three virus

specific proteins to form a helical nucleocapsid. The nucleocapsid is surrounded by a lipid bilayer which contains an external virus glycoprotein and an internal virus membrane matrix protein. As necessitated by the negative polarity of the virion RNA, the rhabdovirion contains an RNA transcriptase activity which is remarkably complicated. In vitro, partially solubilized rhabdovirions are capable of synthesizing and releasing the five rhabodvirus messenger RNAs which are all capped, methylated, and polyadenylated. Poly A is added to rhabdovirus messenger RNA post-transcriptionally as the virion RNA contains no significant stretches of polyuridylic acid. Rhabdoviruses replicate entirely within the host cell cytoplasm. As in vitro, in vivo, the rhabdovirus virion RNA is transcribed into five monocistronic messenger RNAs, each of which is translated into one of the five virus specific proteins. One of the rhabdovirus specific proteins is found in very small amounts relative to the amounts of other virus specific proteins in infected cells. Since the messenger RNA which codes for this protein is also found in small amounts relative to the other virus messenger RNAs, some control of translation of rhabdovirus proteins may occur at the level of transcription. There is one site for the initiation of transcription of the rhabdovirus messenger at the 3' end of the virion RNA molecule. The messenger RNAs are transcribed sequentially and released from the template

as transcription of their section of the virion RNA is complete and while transcription of the next section of the virion is still ongoing. In infected cells, some rhabdovirus transcriptase complexes transcribe full-length plus stranded RNA from which progeny rhabdovirion RNA is synthesized, a process termed replication. The factors which discriminate between transcription and replication are unknown. In both transcription and replication, both the transcriptase activity and the template RNA are associated with nucleocapsid particles.

Noteworthy members of the paramyxovirus family are measles and mumps viruses of humans, Newcastle disease virus of chickens, and Sendai virus of mice (for review: see Kingsbury, 1977). Paramyxoviruses are distinguished from rhabdoviruses in the shape of their virion and the presence of hemaglutinin, neuraminidase, and a cell fusion factor in the virion membrane surface. The strategies of replication of these two virus groups are very similar and therefore the replication of the paramyxoviruses will not be discussed here.

The primary viruses of the orthomyxovirus family of animal viruses are the influenza viruses (for review: see Nayak, 1977). Myxoviruses have a segmented genome consisting of several pieces of single stranded RNA of negative polarity (total molecular weight is approximately 5×10^6). Each piece of RNA is complexed into a separate helical

nucleocapsid all of which are surrounded by a membrane which contains a virus membrane matrix protein and two virus glycoproteins which are arranged as spikes in the exterior surface of the virion. Myxoviruses and isolated nucleocapsids exhibit a transcriptase activity *in vitro*. Myxoviruses replicate in the cytoplasm of the host cell but require an unknown host nuclear function for their replication. *In vivo*, each piece of myxovirion RNA serves as the template for the transcription of a single monocistronic messenger RNA. Influenza virus messenger RNA is capped and polyadenylated, the poly A being added postranscriptionally. Myxovirus proteins are synthesized in unequal amounts during infection, but the level at which control of translation is exercised is not understood. As with the rhabdo- and paramyxorviruses, myxovirus transcriptase activity always resides with nucleocapsid structures. The mechanism by which myxovirus nucleocapsids assort so that each virion receives the correct RNA components is unknown. Not surprisingly, myxoviruses show a high degree of genetic reassortment and virion strains with defective virions lacking pieces of virion RNA are readily found.

Another family of animal viruses with a segmented, negative polarity, single stranded RNA genome are the bunyavirus family (Petterson and Kääriäinen, 1975). Members of the bunyavirus family are tick born arboviruses. The replication of the bunyaviruses has not been well studied,

but the virion contains three circular helical nucleocapsids which exhibit transcriptase activity. The genomic RNA's are also circular, held in the circular configuration by the presence of complementary nucleotide sequences located near the ends of the RNA molecules (Hewlett, Petterson and Baltimore, 1977). The circular structure of the bunyavirion RNAs is comparable to the circular structure of the alphavirus 49S RNA.

The last group of RNA containing animal viruses to be discussed are the reoviruses. Reoviruses multiply asymptomatically in the respiratory tract of the host organism. The reovirus genome consists of 10 pieces of double stranded RNA (total molecular weight of 1.5×10^6), which is complexed in an icosohedral nucleocapsid which in turn is surrounded by an outer icosohedral protein shell. Trypsinized reovirions demonstrate transcriptase activity. Reoviruses replicate entirely within the cytoplasm of the host cell. As with the negative strand viruses, there are transcription and replication phases of reovirus RNA synthesis. During transcription, 10 species of reovirus monocistromic messenger RNA, each corresponding to a double stranded RNA segment of the genome, are transcribed. Reovirus messenger RNAs are capped but are not polyadenylated. Reovirus proteins are synthesized in unequal amounts, but the control over translation is not understood. During both transcription and replication, reovirus

transcriptase activity is associated with nucleocapsid cores.

Besides the obvious difference in the polarity of the virion RNAs, there are other distinct differences in the replication of the alphavirus and negative strand RNA viruses. The alphavirus transcriptase is composed of non-structural virus proteins and is associated with host cell membranes. The nucleocapsid of the negative strand RNA viruses is the transcriptional complex, carrying out transcription *in vitro* and transcription and replication *in vivo*. The helical arrangement of the negative strand virus RNA in the nucleocapsid may facilitate transcription. It will be interesting when the structure of the alphavirus replicative complexes is elucidated to compare it to the structure of the negative strand RNA virus transcription complex. There are interesting differences in the transcriptase activities of the alphaviruses and the negative strand RNA viruses. Transcription of alphavirus RNA can begin internally while transcription of the negative strand virus RNA begins at the 3' terminus of the template RNA strand. This may be due to the greater array of functions which the negative strand RNA virus transcriptase seemingly possesses. The negative stranded RNA virus transcriptase can terminate at internal locations, can add capping and methyl groups, and can polyadenylate messenger RNA without a template. Alphavirus transcriptase may be able to cap

progeny RNA, but polyadenylation occurs by transcription of a poly U template after which transcription terminates. By being able to initiate internally, the alphavirus transcriptase can use the same poly U stretch and termination site for the two species of virus RNA which it transcribes.

At the level of translation, alphavirus messenger RNAs are polycistronic, but contain only one initiation site so individual proteins are cleaved from large polyprotein precursors, The messenger RNAs of the negative strand RNA viruses also contain one translation initiation site, but being monocistronic no protein processing by cleavage of polyprotein precursors needs to occur. In effect, the negative strand RNA viruses carry the simple subdivision of the alphavirus genome to its extreme case where every protein has its own messenger RNA. Thus, in the replication of the negative strand RNA viruses, the translation of every virus protein can be controlled separately, and not in clusters as is the case with the alphaviruses. In the case of the rhabdoviruses and paramyxoviruses, there is some evidence that control of the abundance of each virus protein is exercised at the level of transcription in that messenger RNAs are transcribed in the same relative abundance in which the proteins they code for occur. This is really not much different from the control of translation exercised by the alphaviruses.

The replication of all of the RNA containing animal

viruses (with the exception of the retroviruses) is similar in that there is no gross temporal control over transcription and translation. Most virus-specific RNAs and proteins are synthesized in the same relative abundances at all states of infection. This is in contrast to many animal DNA viruses in whose replication cycle there are definite classes of RNAs and proteins which are synthesized at different stages of infection. There are thus more levels of control over the replication of a DNA containing animal virus. This is probably a result of two tendencies. First, many DNA containing animal viruses are much more complex than the RNA containing animal viruses and thus have more genetic potential to control their own replication. Secondly, most DNA containing viruses replicate in the cell nucleus where they can utilize cellular control systems.

Scope of this Thesis

The primary emphasis of my research on Sindbis virus has been on studying the chemical structure of the virus RNA species. By doing so, I have been able to both characterize the virus RNA and determine the effect which some features of the RNA have in the replication of the virus. In the first chapter, I will describe my research in the poly A of Sindbis RNA. In the second chapter, I will present research I have done on the internal 5-methyl cytidine residues present in Sindbis virus RNA. In the

final chapter, I will describe a method of separating the circular and linear forms of Sindbis virus 49S RNA which I have used to characterize some of the biophysical and biochemical properties of the circularization of this RNA.

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Chapter 1

Replication of Sindbis Virus: Poly A and Poly U in Virus Specific RNA Species

by

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SUMMARY

Polyadenylic acid (poly A) isolated from all species of Sindbis virus RNA is heterogeneous in size with a mean length of 70 nucleotides and a size range of from 40-200 nucleotides. Poly A stretches isolated from Sindbis virion RNA grown in chicken, hamster and mosquito culture cells have similar size distributions. Base composition analysis of Sindbis virus poly A indicates that it is preceded on its 5' end by a short stretch of pyrimidine residues. Oligo dT cellulose chromatography of whole viral RNA reveals that most Sindbis virus 49 S and 26 S molecules contain poly A. However, a reproducible fraction of these molecules will not bind to oligo dT cellulose and contains little poly A. The oligo dT cellulose unbound fraction from virion RNA contains a discrete species of RNA which does not appear to be due to random degradation. This RNA is only 10-20% as infectious as oligo dT cellulose bound or whole virion RNA, suggesting that the poly A is essential for replication of the virus. Poly A is found in Sindbis virus double stranded RNA species. This poly A has a size distribution similar to poly A isolated from viral single stranded RNA. RF I and RF III contain poly A while RF II does not. Stretches of polyuridylic acid (poly U) are also found in Sindbis virus double stranded RNA. This poly U is on the minus strand, has a size distribution identical to Sindbis virus poly A, and occurs once per double stranded molecule. Poly U is found in RF I and RF III but not on RF II. This evidence indicates that the poly A in Sindbis virus

RNA is synthesized by transcription of a poly U template on the viral minus strand.

INTRODUCTION

The genomic and messenger RNAs of both the picornaviruses and the togaviruses (alphaviruses and flaviviruses) contain stretches of polyadenylic acid (poly A) (Armstrong et al., 1972; Brawner et al., 1973; Johnston and Bose, 1972b; Nair and Owens, 1974; Plagemann and Miller, 1972; Yogo and Wimmer, 1972). These viruses have in common that they contain infectious (plus polarity) single stranded RNA and replicate entirely within the cytoplasm. Although the function of this poly A is unclear, it could be needed for replication, for translation, or for protection of the RNA from nuclease activity. Spector and Baltimore (1974) succeeded in removing the poly A from poliovirus RNA by hybridization to poly dT followed by digestion with RNase H. This deadenylated RNA lost substantial infectivity without losing its ability to direct translation of viral proteins (Spector and Baltimore, 1974; Spector, Villa-Komaroff, and Baltimore, 1975). Since cells infected with this deadenylated polio virus RNA show no evidence of virus specific RNA synthesis, the authors conclude that the poly A must serve a function early in the course of viral RNA replication (Spector, Villa-Komaroff, and Baltimore, 1975). Hruby and Roberts (1976) also obtained evidence that poly A is essential for picornavirus replication by isolating fractions of encephalomyocarditis virus RNA with different poly A lengths by differential chromatography on oligo dT cellulose and measuring the infectivities of these fractions. Encephalomyocarditis

virus RNA containing normal size poly A was much more infectious than virus RNA containing very short poly A or no poly A.

Alphavirus infected cells contain several species of virus specific RNAs (Levin and Friedman, 1971). These include the 4.5×10^6 dalton genomic RNA (49 S) (Arif and Faulkner, 1972; Simmons and Strauss, 1972a), which is infectious (Wecker, 1959) and also serves as an intracellular messenger for the translation of nonstructural virus proteins (Bracha et al., 1976; Glanville et al., 1976; Lachmi and Kääriäinen, 1977; Mowshowitz, 1973; Simmons and Strauss, 1974a,b). The predominant intracellular virus messenger RNA species is an RNA of 1.5×10^6 daltons (26 S) (Kennedy, 1972; Mowshowitz, 1973; Rosemond and Sreevalsen, 1973; Simmons and Strauss, 1974b), which contains the 3' terminal third of the 49 S nucleotide sequences (Kennedy, 1976; Wengler and Wengler, 1976) and codes for the three structural viral polypeptides (Cancedda and Schlesinger, 1974; Clegg and Kennedy, 1974; Simmons and Strauss, 1974a; Wengler, Beato, and Hackemack, 1974). Two minor viral intracellular species of RNA, the 33 S and 38 S, are apparently conformational variants of the 26 S and 49 S RNA respectively (Kennedy, 1976; Simmons and Strauss, 1974b). Alphavirus RNA is replicated on double stranded replicative complexes which contain a full length minus strand RNA and pieces of plus stranded RNA ranging in size up to full length (Bruton and Kennedy, 1975; Friedman, 1968). When treated with ribonuclease, these complexes give rise to three species of double stranded RNA, RF I which contains full length stretches of both plus and minus strands, and RF II and RF III, whose molecular weights add to that of RF I (Simmons and Strauss, 1972b).

Together, RF II and RF III contain the plus strand complement of the complete Sindbis virus genome; RF III contains the 26 S nucleotide sequences and RF II contains the non-26 S sequences (Simmons and Strauss, 1972b). Simmons and Strauss (1972b) have proposed a model in which RF I and the RF II - RF III complex are the sites for the transcription of 49 S and 26 S RNA respectively.

Poly A in alphavirus RNA has been studied in both Sindbis virus and Semliki Forest virus. With Sindbis virus, both 49 S and 26 S species of Sindbis virus contain poly A (Eaton and Faulkner, 1972; Eaton, Donaghue, and Faulkner, 1972; Johnston and Bose, 1972a,b). This poly A is 3' terminal (Deborde and Leibowitz, 1976) and has a heterogeneous size distribution, with mean lengths of 30-40 nucleotides (Deborde and Leibowitz, 1976) and 50-70 nucleotides (Eaton and Faulkner, 1972) having been reported. A small fraction of the Sindbis virus poly A is much larger, reaching lengths of up to 200 nucleotides (Eaton and Faulkner, 1972); viral RNA with these long stretches of poly A has a similar specific infectivity to viral RNA containing average length poly A stretches. Similarly, poly A has been demonstrated in all species of Semliki Forest virus intracellular RNA (Clegg and Kennedy, 1974). The poly A is again 3' terminal (Sawicki and Gomatos, 1976; Wengler and Wengler, 1976) and possesses a heterogeneous size distribution, mean lengths of 40-50 nucleotides (Wengler and Wengler, 1976) and 80-90 nucleotides (Sawicki and Gomatos, 1976) having been reported.

The double stranded species of Semliki Forest virus RNA have been shown to contain 3' terminal plus stranded poly A with a size

distribution similar to the poly A species of single stranded RNA (Bruton and Kennedy, 1975; Sawicki and Gomatos, 1976). Poly A could be added to the viral RNA by direct transcription of a minus strand polyuridylate (poly U) template by the viral replicase or by a post-transcriptional adenylating enzyme. With poliovirus, the minus strand contains a 5' terminal stretch of poly U equal to or longer in size than the plus strand poly A (Spector and Baltimore, 1975; Yogo and Wimmer, 1973; Yogo, Teng, and Wimmer, 1974). Poliovirus replicase purified free of terminal adenylate transferase produces viral RNA containing poly A, which must therefore be transcribed from the minus strand (Dorsch-Häsler, Yogo, and Wimmer, 1975). None of the minus strand RNA viruses (rhabdoviruses, myxoviruses, or paramyxoviruses) contain poly U, but their virion associated transcriptases synthesize plus strand RNA containing poly A, which in this case must be added post-transcriptionally (Banerjee and Rhodes, 1973; Plotch and Krug, 1977; Villarreal and Holland, 1973; Weiss and Bratt, 1974). Bruton and Kennedy (1975) were unable to demonstrate the presence of poly U in the minus strand of Semliki Forest virus RNA replicative complexes. Recently, however, Sawicki and Gomatos (1976) were able to isolate a stretch of poly U from the viral minus strand RNA. This poly U was similar in size to the plus stranded poly A and was shown to be 5' terminal.

In this paper we ask the question of whether all molecules of Sindbis virus RNA contain poly A and present evidence that poly A is essential for viral replication. We also demonstrate that Sindbis virus double stranded RNA replicative complexes contain stretches of

both poly A and poly U, in agreement with the results of Sawicki and Gomatos (1976). A preliminary report of some of the findings in this paper was presented elsewhere (Frey and Strauss, 1976).

MATERIALS AND METHODS

Materials. [2-^{3}H]adenosine (24 Ci/mmole), [5,6-^{3}H]uridine (58 Ci/mmole) and [2-^{14}C]uridine (50 mCi/mmole) were purchased from Amersham-Searle. [^{32}P]phosphoric acid (carrier free) was obtained from ICN. Unlabeled polyadenylic acid and polyuridylic acid as well as pancreatic ribonuclease (Type X-A) were supplied by Sigma. Ribonuclease T-1 and T-2 were obtained from Calbiochem. Oligo dT cellulose (Type T-3) and oligo dA cellulose were purchased from Collaborative Research, Inc. Materials for gel electrophoresis were obtained and handled as described previously (Simmons and Strauss, 1972a) except for agarose, which was supplied by Seakem. Liquefied phenol from Mallinckrodt was redistilled and stored at -20°.

Cell cultures and virus strains. Except where mentioned, all work described in this paper was done with viral material grown in primary cultures of chicken embryo fibroblasts (Pierce, Strauss and Strauss, 1974). When used, BHK-21 cells were maintained in Eagle's minimal essential medium (Eagle, 1959) containing 10% fetal calf serum. Aedes albopictus cells (ATCC cell line) were grown at 30° in Mitsuhashi and Maramorosch medium (Mitsuhashi and Maramorosch, 1964) containing 15% inactivated fetal calf serum (56 C, 60 min); labeling was in D-20 (Echalier and Ohanessian, 1970) medium containing 5% inactivated

dialyzed fetal calf serum and 0.5 μg/ml actinomycin D. Both the wild type strain (furnished by Dr. B. Burge) and the HR strain (Burge and Pfefferkorn, 1966) of Sindbis virus were used in these experiments; the two strains gave identical results.

Preparation of RNA. At least 6 hr after adding fresh medium confluent cell monolayers were infected with Sindbis virus at a multiplicity of 35 plaque forming units per cell. Infection and labeling of chick and BHK cells were done at 37° in Eagle's medium containing 3% dialyzed fetal calf serum and 1 μg/ml actinomycin D. One ml of inoculum was used if the cells were in a 75 cm^2 T flask and 10 ml of inoculum if the cells were in a 800 cm^2 roller bottle. One hr after infection, additional medium containing radioactive label was added to the infected culture (making 5 ml total volume per T flask, 30 ml per roller bottle). [^{3}H]uridine and [^{3}H]adenosine were used at a concentration of 200 μCi/ml in T flasks and 50 μCi/ml in roller bottles. [^{14}C]uridine was added to the medium at 2 μCi/ml and $^{32}PO_4$ at 100 μCi/ml (medium containing 0.1 times the normal concentration of PO_4 was used for ^{32}P-labeling).

When intracellular viral RNA was being prepared, the cells were lysed and the RNA extracted 5-6 hr after infection by the Method C of Simmons and Strauss (1972a). When virion RNA was being isolated, the virus was harvested by the low salt-high salt reversal method from chick cells (Pierce et al., 1974). Virus grown in BHK cells or Aedes albopictus cells was concentrated from the culture fluid by precipitation with polyethylene glycol (Pierce et al., 1974). In both cases

the viral RNA was then purified by the method of Hsu, Kung, and Davidson (1973).

The viral single stranded intracellular RNA species were separated on sucrose gradients and occasionally were further purified on preparative agarose acrylamide gels as described by Simmons and Strauss (1972a). Viral double stranded RNA was separated from single stranded RNA on a 100 cm x 1.5 cm column of Sepharose 2B using 0.2 M NaCl, 0.01 M Na acetate (pH 5.5), 0.001 M EDTA, 0.2% SDS as a column buffer (Yogo and Wimmer, 1973). RFs were isolated from total RNA or double stranded viral RNA as previously described (Simmons and Strauss, 1972b) except that T-1 RNase (100 U/ml) was used instead of pancreatic RNase when the RFs were to be analyzed for poly U. The RFs were separated either on preparative 1.4% acrylamide 0.5% agarose gels (as described by Simmons and Strauss, 1972a) or by centrifugation on 15-30% sucrose gradients in 0.06 M NaCl, 0.01 M Tris, 0.001 M EDTA (pH 7.2) in an SW27 rotor (27,000 rpm for 13 hr at 23°) with the peaks being rerun to ascertain their purity. Formaldehyde denaturing gradients of viral RNA were run as described by Simmons and Strauss (1972a).

Isolation of poly A from viral RNA species. The RNA was diluted into 0.01 M Tris, 0.01 M EDTA (pH 7.2) for denaturation. Single stranded RNA was denatured at 60° for 5 min followed by rapid cooling. To double stranded RNA, 10 μg/ml of unlabeled poly A was added and the solution was taken up into capillary tubes and denatured at 120° for 30 sec in an ethylene glycol bath. In both cases, the ionic strength

of the solution was then brought to 0.2 M NaCl, 0.01 M Tris, 0.01 M EDTA (pH 7.2) by the addition of 0.1 volume of 2 M NaCl. Unlabeled poly A (10 µg/ml), pancreatic RNase (1 µg/ml) and/or T-1 RNase (100 U/ml) were added and the digestion was allowed to proceed for 40 min at 37°. The digestion was terminated by the addition of SDS to 0.2 % and the digest was immediately subjected to oligo dT cellulose chromatography.

Oligo dT cellulose columns 0.8 cm by 2 cm were poured in Dispo Columns (BioRad). The flow rate was kept constant by attaching the column to a proportioning pump. Before and between uses, the oligo dT cellulose was washed with 20 column volumes of 0.1 M NaOH, 5 column volumes 0.01 M Tris, 0.01 M EDTA containing 0.2% SDS and 20 column volumes of 0.2 M NaCl, 0.01 M Tris, 0.01 M EDTA containing 0.2% SDS. Samples were loaded onto the column in this final buffer at a flow rate of 0.1 ml/min and washed with at least 5 column volumes of the same buffer. Material was eluted from oligo dT cellulose at a flow rate of 0.5 ml/min with either the Tris/EDTA/SDS buffer or with 50% formamide in the NaCl/Tris/EDTA/SDS buffer. Using these conditions, we found oligo dT cellulose to quantitatively bind poly A greater than 10 nucleotides in length.

If material eluted from oligo dT cellulose columns was simply to be assayed for radioactivity, the sample was diluted in a scintillation vial with 1 ml of water and 10 ml of Aquasol-2 (New England Nuclear) and was counted in a Beckman Scintillation Counter. If the material was to be analyzed by gel electrophoresis, 100 µg of unlabeled poly A was added to the sample and RNA was precipitated by the addition of

0.1 volume of 2 M K acetate (pH 5.5) and 2.5 volumes of ethanol and stored at -20° for at least 2 hr. The precipitate was collected by centrifugation, resuspended in NaCl/Tris/EDTA/SDS buffer, phenol-chloroform extracted (Simmons and Strauss, Method C, 1972a), and reprecipitated with ethanol. The precipitate was again collected by centrifugation and resuspended in a small volume of 0.01 M Tris (pH 7.2) and gel electrophoresis was performed.

Isolation of poly U from viral double stranded RNA. Unlabeled poly U was added to double stranded RNA in 0.01 M Tris, 0.01 M EDTA at a concentration of 10 µg/ml and the solution was loaded into capillary tubes and denatured at 120° for 30 sec. T-1 RNase was added to 100 U/ml and the digestion proceeded at 37° for 90 min. The reaction was stopped by the addition of SDS to 0.2%. One-tenth volume of 2 M NaCl was added to bring the NaCl concentration to 0.2 M and the digestion mixture was immediately subjected to oligo dA cellulose chromatography. Oligo dA cellulose columns were prepared, run and maintained similarly to the oligo dT cellulose columns. Bound material was eluted from the columns in 0.01 M Tris, 0.01 M NaCl containing 0.2% SDS and either counted directly or prepared for gel electrophoresis as described for oligo dT cellulose bound material, except that 100 µg of unlabeled poly U was added as coprecipitant instead of unlabeled poly A.

Polyacrylamide gel electrophoresis. 1.8% acrylamide, 0.5% agarose gels were prepared and run as described by Simmons and Strauss (1972a). Preparative gel electrophoresis was done by fitting analytical tube gels with an adapter made from a syringe head connected to tubing

through which eluate from the gel was pumped (Lee and Sinsheimer, 1974). Poly A and poly U were examined on 10% acrylamide, 0.5% bis-acrylamide gels in 0.089 M Tris, 0.089 M boric acid, 0.003 M EDTA (pH 8.3) (Peacock and Dingman, 1967) and poured into 6.4 mm diameter vinyl tubes to a height of 10 cm. The electrode buffer was the same Tris-borate buffer made 0.2% in SDS. Gels were prerun at 3 mA/gel for 1 hr. The sample was applied in 100 µl of 0.01 M Tris (pH 7.2) to which had been added 10 µl of glycerol, 5 µl of 10% SDS and 10 µl of saturated bromophenol blue dye. The samples were electrophoresed at 3 mA/gel until the dye reached the bottom of the gel tube (2-3 hr). Occasionally samples were electrophoresed in 10% acrylamide gels run in 0.2 M sodium phosphate buffer (pH 7.8), 0.001 M EDTA. These gels were also run at 3 mA/gel, but it took 48 hr for the dye to reach the bottom of the gel tube. 10% acrylamide, 0.5% bis-acrylamide denaturing gels were made in 1.1 M formaldehyde according to the procedures of Boedtker (1971). Before application to the gel, RNA samples were reacted with 1.1 M formaldehyde at 60° for 15 min.

All gels were sliced on a Mickle gel slicer. If the RNA in the gels was to be counted, two 1 mm gel slices were placed in a scintillation vial, 6 ml of toluene based scintillation cocktail containing 5% NCS tissue solubilizer (Amersham-Searle) and 0.5% H_2O was added, and the vial was shaken overnight in the dark before counting. If RNA was to be eluted from the gel for further analysis, five 0.4 mm slices were placed in a 2 ml plastic minibeaker (Scientific Products) containing 1 ml of 0.2 M NaCl, 0.01 M Tris, 0.01 M EDTA, pH 7.2, containing 0.2% SDS, covered with parafilm, and shaken overnight at 37°.

The supernatant was freed of small gel particles by passing it through a small glass wool plug in the barrel of a Pasteur pipette.

Base composition analysis. RNA to be tested for base composition was digested to completion with 4 units of T-2 RNase in 0.1 ml of 0.04 M NH_4 acetate (pH 4.4), 0.001 M EDTA for 4 hr at 37°. The volume was reduced by flash evaporation, 50 µg of each nucleoside and nucleotide were added as markers, and nucleosides and nucleotides were separated by the high voltage paper electrophoretic method of Keith, Gleason and Fraenkel-Conrat (1974). After electrophoresis, the paper was dried thoroughly, the markers compounds were located under a UV light source and the paper was cut into strips and counted in the toluene-fluor-NCS scintillation cocktail described for acrylamide gels.

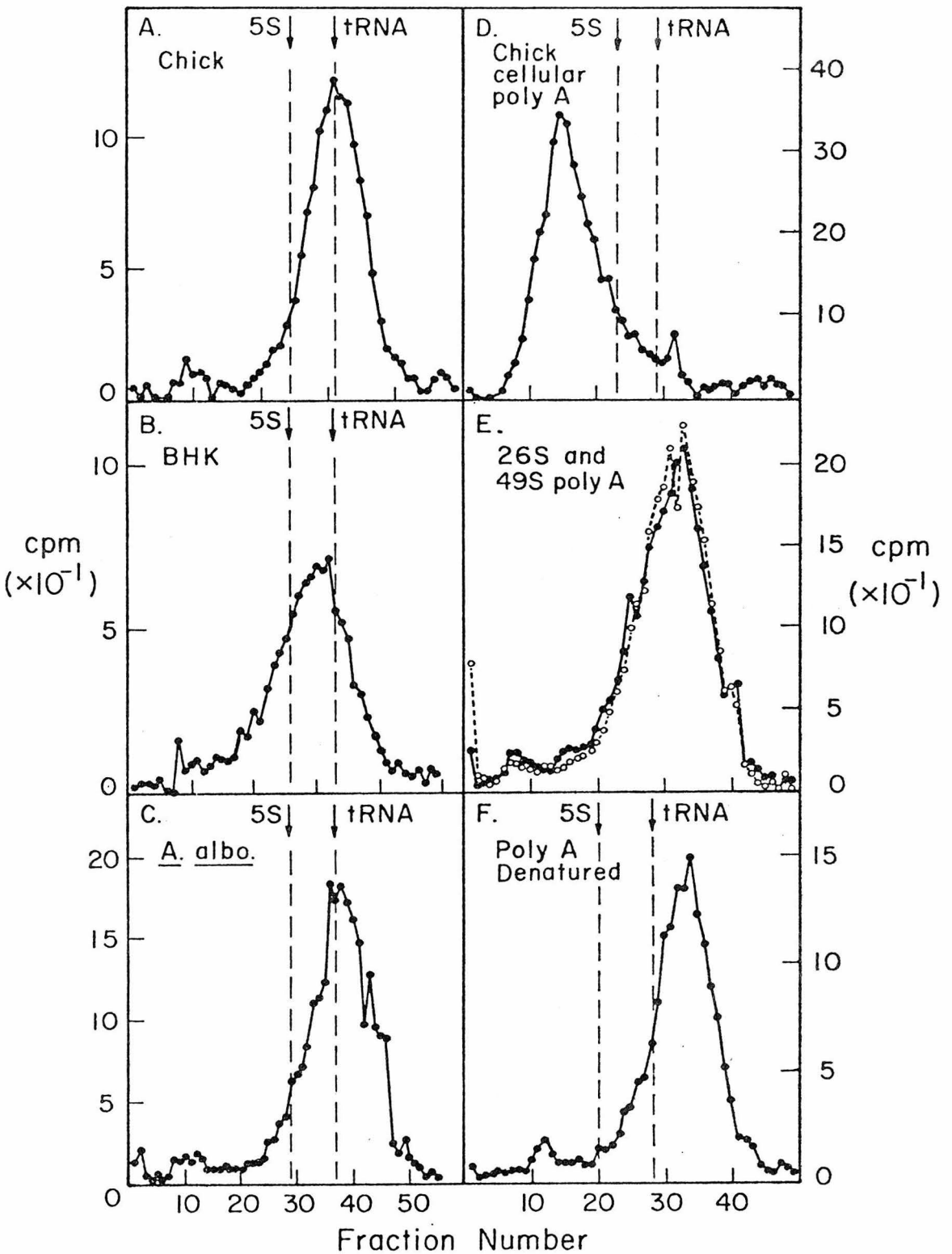
Assay for infectious RNA. Confluent monolayers of chick cells in small petri plates were soaked for 2 min in 0.6 M NaCl buffered with 0.04 M sodium phosphate (pH 7.4). The Sindbis 49 S RNA was then applied in 1 ml of 1.2 M NaCl containing the same buffer and allowed to adsorb at room temperature for 30 min. The cells were then washed for 2 min with the 0.6 M NaCl solution used above, washed two times with phosphate buffered saline (Dulbecco and Vogt, 1954) and then assayed for the formation of plaques as described by Strauss, Lenches and Strauss (1976). Within the range of RNA concentrations used (1×10^{-2} to 5×10^{-5} µg/ml) there was a linear relationship between the RNA concentration and number of plaques formed.

RESULTS

Size distribution of poly A isolated from Sindbis virus RNA. Poly A was isolated from the RNA of Sindbis virions grown in chick cells by ribonuclease digestion followed by oligo dT cellulose chromatography and analyzed by electrophoresis on 10% polyacrylamide gels. As shown in Fig. 1A, Sindbis virion poly A migrates as a heterodisperse peak, having a mean mobility similar to tRNA. Fig. 1B and 1C show gels of poly A isolated from the RNA from Sindbis virions grown in BHK cells and Aedes albopictus cells respectively. These results demonstrate that the length of Sindbis virion poly A shows little dependence on the host cell (although the poly A from BHK grown virus may be somewhat longer on average than that of chick and mosquito grown virus) and is markedly shorter than poly A stretches attached to the RNA of the uninfected host cell (a gel of uninfected chick cell poly A is shown in Fig. 1D). Poly A isolated from intracellular 26 S and 49 S RNA have a similar gel profile (Fig. 1E) to the virion RNA poly A.

To determine the actual size distribution of Sindbis virus poly A, samples of tRNA, 5 S RNA and Sindbis virion RNA poly A were combined, denatured with formaldehyde and run on a 10% acrylamide gel containing formaldehyde. As shown in Fig. 1F, the peak of Sindbis virus poly A migrates more rapidly than tRNA under denaturing conditions, which abolish the effect of secondary structure on mobility. Assuming a logarithmic relationship between mobility and size, and using values of 120 and 88 nucleotides for the lengths of E. coli 5 S and tRNA

Fig. 1. Gels of Sindbis virus poly A. [^{3}H]poly A was isolated from indicated species of RNA and analyzed on acrylamide gels as described in Materials and Methods. ^{14}C-labeled E. coli 5S RNA and tRNA were coelectrophoresed with the poly A sample where indicated. The direction of migration in all gels is from left to right. A, B and C) Poly A isolated from Sindbis virion 49S RNA grown in A) chick cells, B) BHK cells and C) Aedes albopictus cells. D) Poly A isolated from chick cell messenger RNA. E) Poly A isolated from Sindbis virus 26S and 49S intracellular RNA run on the same gel; (●—●—●), [^{3}H] adenosine labeled 26S poly A; (o---o) ^{32}P-labeled 49S poly A. F) A formaldehyde denaturing gel of poly A isolated from virion 49S RNA (grown in chick cells).


A.
Chick
5S
tRNA
B.
BHK
C.
A. albo.
D.
Chick
cellular
poly A
E.
26S and
49S poly A
F.
Poly A
Denatured
cpm
(×10⁻¹)
Fraction Number

respectively, the mean length of Sindbis virus poly A is calculated to be approximately 68 nucleotides. The bulk of the poly A has a size range from 45 to 120 nucleotides, but a small but reproducible fraction (8% of the total) of poly A molecules have lengths of up to 200 nucleotides.

As a confirmation to our poly A size distributions on acrylamide gels, [^{3}H]adenosine labeled poly A was extracted from gels, digested completely with T-2 nuclease and the ratio of adenosine phosphates to free adenosine molecules (from the 3' terminus of the poly A) was determined by paper electrophoresis. The terminal nucleoside to internal nucleotide ratio is approximately 1:67 for both 26 S and 49 S RNA poly A (data not shown), very close to the mean length of 68 determined on denaturing gels.

Base composition analysis of Sindbis virus poly A. ^{32}P-labeled poly A isolated from Sindbis virus 26 S RNA by digestion with pancreatic ribonuclease followed by oligo dT cellulose chromatography and size selection on acrylamide gels contains virtually 100% adenosine (Table 1). The residual cytidine in the pancreatic RNase derived poly A is probably due to an artifact caused by the isolation and analysis procedure (Molloy and Darnell, 1973). Poly A isolated similarly except for digestion with T-1 ribonuclease contains a significant proportion of pyrimidine residues. These residues are not internal to the poly A structure since poly A derived by T-1 RNase digestion migrates only slightly more slowly on acrylamide gels than poly A derived by pancreatic RNase treatment (data not shown).

TABLE 1

Base Composition of Sindbis Virus Poly A

Source of Poly A	Nuclease Used	% A	% C	% G	% U
26 S	Panc	98.4 (±0.2)[a]	1.4 (±0.0)	0.0	0.2 (±0.2)
26 S	T-1	90.4 (±4.3)	2.0 (±0.7)	0.3 (±0.6)	7.3 (±3.2)
49 S	T-1	88.0 (±2.6)	2.6 (±0.8)	0.2 (±0.2)	9.3 (±1.6)

[a]Ranges set at one standard deviation from the sample mean.

These residues are not on the 3' terminal end of the poly A since poly A derived by pancreatic RNase digestion contains terminal free adenosine (Deborde and Leibowitz, 1976, and this publication). Therefore, there must be a short stretch of uridine, cytidine, and possibly adenosine residues on the 5' end of the Sindbis virus poly A. There is at least one cytidine residue in this stretch since the cytidine levels in poly A from T1 RNase digestion of Sindbis virus RNA are above the background established by the pancreatic RNase digest. The 2.0% difference in uridine percentage between the 26 S and 49 S T-1 derived poly A is probably caused by variation in the analysis due to the low amount of uridine normally present (typically between 100 and 200 cpm). If there are 70 adenosine residues per stretch of poly A, then there are 6-7 uridine residues preceding it in its 5' end. Pancreatic RNase digestion of the T-1 derived poly A from both 26 S and 49 S RNA followed by analysis by paper electrophoresis reveals one AU dinucleotide per poly A (data not shown). From these data, the 3' sequence of Sindbis virus RNA is G (CAU_{5-6}) $A_{\overline{68}}$ A-OH (see also section on poly U composition of the minus strand). This sequence is different than the sequence reported for Semliki Forest virus of U_6 C_2 (AC) (AU) (AAU) $A_{\overline{40}}$ or $\overline{58}$ (Wengler and Wengler, 1976). This difference is undoubtedly due to sequence divergence between these two closely related alphaviruses.

Percentage of virion RNA molecules which contain poly A. To determine if all molecules of Sindbis virus RNA contain poly A, purified virion RNA was chromatographed through columns of oligo dT

cellulose. It was found that a reproducible fraction of Sindbis virion RNA failed to bind to oligo dT cellulose. As shown in Table 2, between 11 and 16% of the virion RNA was in this fraction. When the bound and unbound fraction of virion RNA were isolated and rechromatographed through oligo dT cellulose, they retained their binding characteristics (Table 2). When analyzed on agarose acrylamide gels (Fig. 2) the oligo dT cellulose unbound fraction of virion RNA migrates as a discrete peak one fraction (10%) more rapidly than total unfractionated RNA. Formaldehyde denaturing gradients of the bound and unbound fractions of virion RNA are shown in Fig. 3. Although the unbound fractions contain a high fraction of low molecular weight fragments, a significant proportion (27%) migrates in a discrete peak in the same location as the oligo dT bound fraction (63% of which migrates as if intact). These results indicate that there is a small fraction of virion RNA similar in size to full length virion RNA which is incapable of binding to oligo dT cellulose. Since this fraction migrates as a discrete peak under both non-denaturing and denaturing conditions, it is unlikely that the lack of binding is due to random degradation of the virion RNA during extraction procedures.

The fractionated Sindbis virion RNA was analyzed for poly A content by digesting it with ribonuclease and determining what percentage could be retained by oligo dT cellulose. As shown in Table 2, 1.70% of [^{3}H]adenosine virion RNA will bind to oligo dT cellulose after ribonuclease digestion and thus is determined to be poly A. Using a molecular weight for 49 S RNA of 4.3×10^6, an average molecular weight for nucleotides of 340 daltons, an adenosine content

TABLE 2

Oligo dT Cellulose Binding Characteristics and Poly A Percentages of Various Species of Sindbis Virus RNA

RNA Preparation	Binding to Oligo dT Cellulose: % Unbound	% Bound	% Poly A[a]
Virion 49 S	13.0 (±2.1)[b]	87.0 (±2.1)	1.70 (±0.07)
Virion 49 S Unbound fraction	96.0 (±2.6)	4.0 (±2.6)	0.28 (±0.09)
Virion 49 S Bound fraction	2.5 (±1.2)	97.5 (±1.2)	1.81 (±0.15)
Virion 49 S Gel purified	8.7 (±2.5)	91.3 (±2.5)	1.46 (±0.16)
Intracellular 49 S	13.4 (±0.9)	86.6 (±0.9)	1.66 (±0.01)
26 S	16.3 (±3.3)	83.7 (±3.3)	3.00 (±0.04)
26 S Unbound fraction	92.5	7.5	0.76
26 S Bound fraction	3.7	96.3	3.56 (±0.38)
Chick cell rRNA	96.4	3.6	0.13

[a] [^{3}H]adenosine labeled RNA was digested with ribonuclease and passed through columns of oligo dT cellulose. The radiolabel eluting from these columns in low salt buffer was considered to be poly A.

[b] Ranges are set at one standard deviation from the sample mean.

Fig. 2. Agarose acrylamide gel electrophoresis of oligo dT cellulose fractions of Sindbis virion RNA. [^{3}H]adenosine labeled virion RNA (●—●—●) was fractionated on oligo dT cellulose into bound and unbound fractions, and the two fractions were analyzed on 1.8% acrylamide 0.5% agarose gels with ^{14}C-labeled unfractionated virion RNA (o--o--o) added as a marker. A) Oligo dT cellulose bound virion RNA. B) Oligo dT cellulose unbound virion RNA. The direction of migration is from left to right.

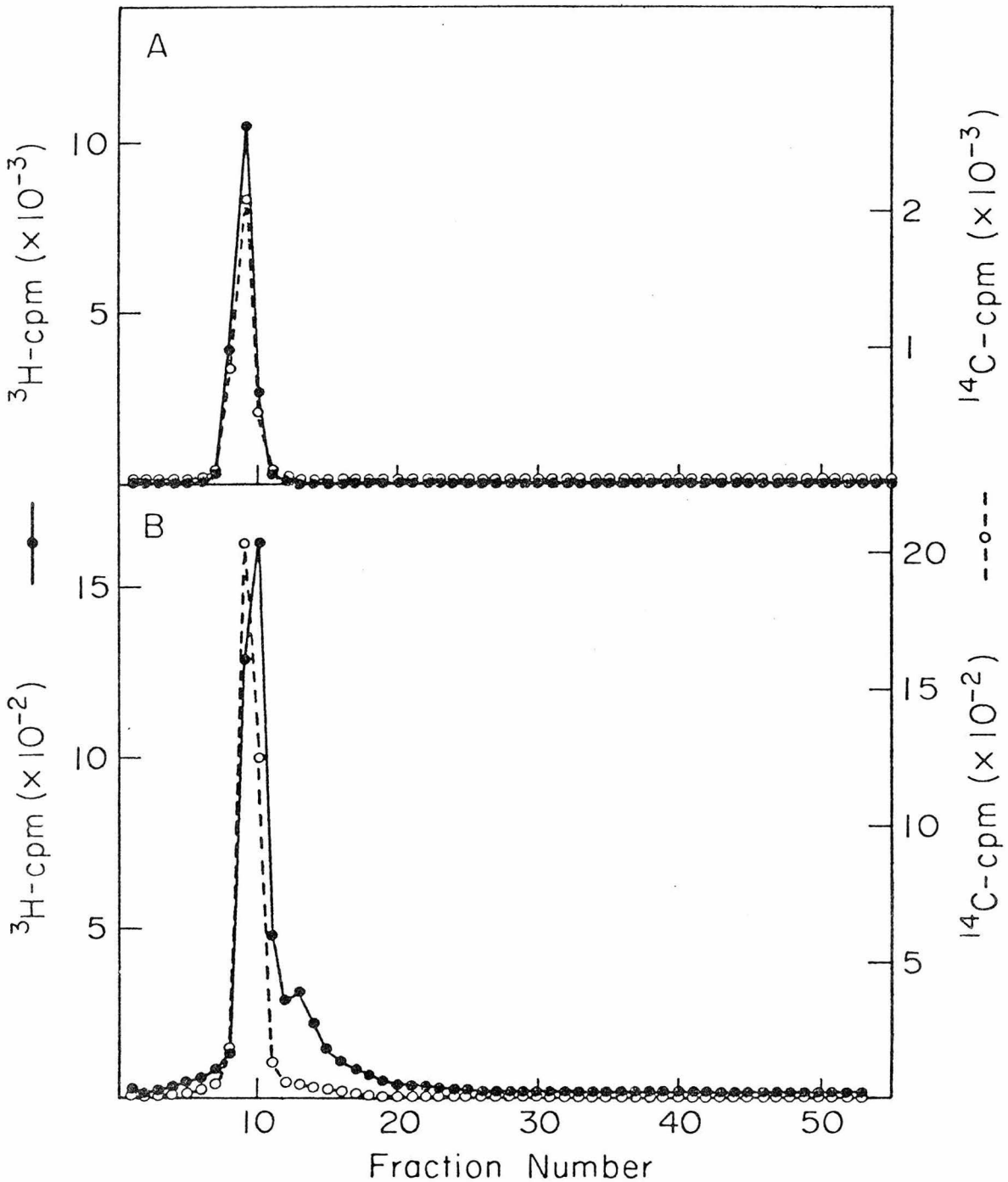
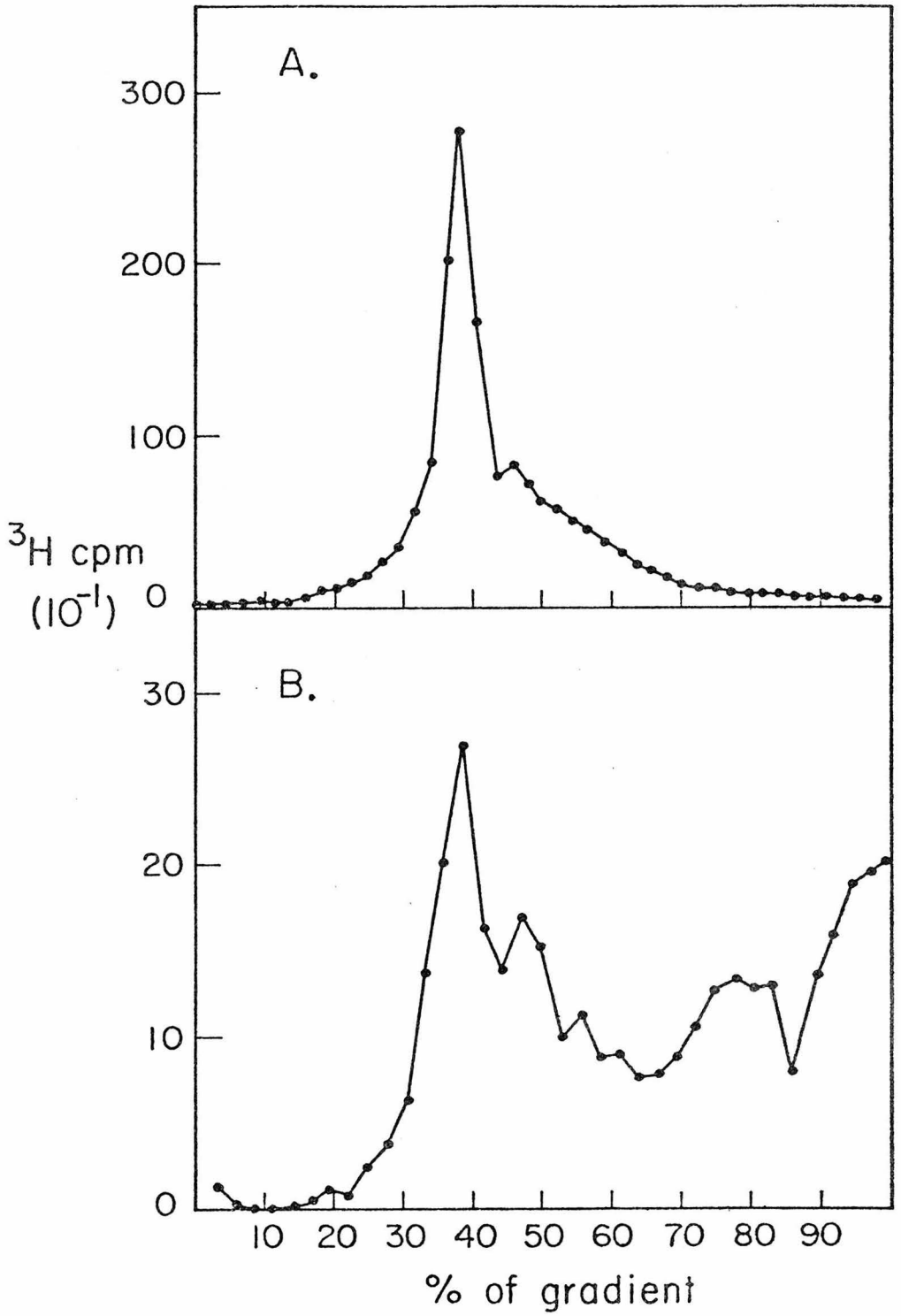

A
B
^{3}H-cpm (x10^{-3})
^{14}C-cpm (x10^{-3})
^{3}H-cpm (x10^{-2})
^{14}C-cpm (x10^{-2})
Fraction Number

Fig. 3. Formaldehyde denaturing gradients of oligo dT cellulose fractions of Sindbis virion RNA. [^{3}H]adenosine labeled virion RNA was fractionated on oligo dT cellulose, and the bound and unbound fractions were denatured with formaldehyde and analyzed on sucrose gradients containing formaldehyde. A) Oligo dT cellulose bound virion RNA. B) Oligo dT cellulose unbound virion RNA. The direction of sedimentation is from right to left.


A.
300
200
100
^{3}H cpm
(10^{-1})
0
B.
30
20
10
0
10 20 30 40 50 60 70 80 90
% of gradient

of 28.4% for Sindbis virus 49 S RNA, and taking into account the fact that 14% of the ^{3}H-label ends up as guanosine in the 49 S RNA (data not shown), this percentage corresponds to a poly A chain length of 70 nucleotides, close to the value calculated by denaturing gel electrophoresis. 1.81% of the oligo dT cellulose bound 49 S adenosine residues are present as poly A (corresponding to a chain length of 75 nucleotides). For the oligo dT cellulose unbound fraction of virion RNA, the percentage is 0.28% (corresponding to a chain length of 12 nucleotides) and thus this fraction contains a much reduced amount of poly A.

The poly A isolated from the oligo dT cellulose unbound fraction of virion RNA was further analyzed to determine if it was a short species of 3'-terminal poly A which was too short to bind the whole 49 S RNA molecule to oligo dT cellulose or was full length poly A arising from incomplete separation by the column. The fact that 0.13% of the ^{3}H adenosine from [^{3}H]-labeled ribosomal RNA isolated from chick cells binds to oligo dT cellulose after ribonuclease treatment (Table 2) indicates that at least part of the bound label in this type of poly A analysis arises from either internal polyadenylate or is due to nonspecific binding. When poly A isolated from the oligo dT cellulose unbound fraction of virion RNA was chromatographed on Sephadex G-50, it had a very broad size distribution, ranging from the length of full sized Sindbis virus poly A down to 10 nucleotides, the binding limit of oligo dT cellulose under our ionic conditions (data not shown). Approximately 70% of this poly A from the oligo dT cellulose unbound fraction migrated with full sized Sindbis virus poly A

(greater than 25 nucleotides) (data not shown). Thus the residual poly A derived from the oligo dT cellulose unbound fraction consists in part of full size poly A tracts arising from incomplete separation by the column and in part of smaller oligonucleotides (which could be derived from short 3'-terminal poly A, from internal polyadenylate, or be due to nonspecific contamination). From the fact that 0.13% of the bound label appears to be nonspecific or from the fact that 70% of the poly A from the oligo dT cellulose unbound fraction appears to be full sized, it can be calculated that 8-12% of the virion RNA molecules present in the oligo dT cellulose unbound fraction contain full length poly A.

Virion RNA freed of low molecular weight fragments by size selection on preparative acrylamide gels could again be separated into oligo dT cellulose binding and nonbinding fractions (Table 2). As expected, the elimination of such fragments increases the percentage of RNA bound to oligo dT cellulose from 87% to 91%. These fractions of gel purified virion RNA retained these binding characteristics when repassed through oligo dT cellulose and appeared equally intact on denaturing gradients (data not shown). The oligo dT cellulose bound fraction contained a normal amount of poly A while the unbound fraction contained little poly A (data not shown).

Poly A content of intracellular virus RNA. Similar oligo dT cellulose binding experiments were performed with the two major intracellular species of Sindbis virus RNA, 26 S and 49 S RNA. These species were isolated from infected cells and further purified by

preparative gel electrophoresis. As shown in Table 2 these RNAs could also be separated into unbound and bound fractions on oligo dT cellulose. The 26 S RNA fractions retained their binding characteristics when rechromatographed through oligo dT cellulose (Table 2), appeared intact when sedimented in denaturing gradients (data not shown); the unbound fraction contains a much reduced amount of poly A (Table 2). From these results it is clear that a small fraction of all the single stranded species of Sindbis virus RNA contain little or no poly A. Therefore, a random sample of the intracellular 49 S RNA pool is probably encapsidated to form Sindbis virions.

Infectivities of oligo dT cellulose bound and unbound virion RNA. The ability to separate virion 49 S RNA into fractions which bind and pass through oligo dT cellulose provides a means to test whether the presence of poly A in virion RNA is necessary for viral replication. Virion RNA fractionated on oligo dT cellulose was thus assayed for infectivity. As shown in Table 3, the oligo dT cellulose bound fraction of virion RNA was as infectious as unfractionated RNA. However, the unbound fraction of virion RNA is only 10% as infectious as unfractionated RNA. To reduce discrepancies due to the fact that the oligo dT cellulose unbound fraction of virion RNA contains low molecular weight fragments which would lower its specific infectivity (see Fig. 3), total virion RNA was first size selected on preparative agarose-acrylamide gels (see earlier section). Even when oligo dT cellulose unbound virion RNA was prepared in this manner, its specific infectivity was still only 18-20% of the infectivity of unfractionated

TABLE 3

Specific Infectivities of Total Sindbis Virion RNA and Virion RNA Fractions which Pass through and Bind to Oligo dT Cellulose

	Oligo dT Cellulose Binding Fraction		
RNA Preparation	Total	Unbound	Bound
Virion 49 S	4.1[a]	0.4	4,4
Gel purified[b] virion 49 S	6.8	1.1	5.0

[a]Expressed as plaque forming units/molecule x 10^7.

[b]Virion RNA was purified by preparative gel electrophoresis to remove small RNA fragments which would lower the specific infectivity of the RNA, especially the unbound fraction.

or oligo dT cellulose bound virion RNA. The simplest interpretation of this result is that the presence of poly A on virion RNA is necessary for replication.

Poly A in Sindbis virus double stranded RNA species. To determine whether Sindbis virus double stranded RNA contained poly A, [^{3}H]adenosine labeled viral double stranded RNA was heat denatured in the presence of unlabeled poly A (to complex any poly U that might be present in viral RNA), digested with pancreatic and T-1 ribonucleases, and chromatographed through columns of oligo dT cellulose. As shown in Table 4, 1.17% of the total [^{3}H]adenosine label in double stranded RNA bound to oligo dT cellulose. The binding of this material to oligo dT cellulose was inhibited by the addition of unlabeled poly U to the digestion mixture (after phenol extraction to remove RNase), indicating that the bound material is indeed poly A. When the material bound to oligo dT cellulose was examined on polyacrylamide gels (Fig. 4A), it gave a similar size distribution to poly A derived from Sindbis virus single stranded RNA (see Fig. 1). (Note, however, the somewhat higher content of longer poly A tracts in the poly A derived from double stranded RNA as compared with that derived from single stranded viral RNA.) Thus, the double stranded species of Sindbis virus RNA contain poly A of similar size to that found in single stranded viral RNA. These results are similar to findings with Semliki Forest virus (Bruton and Kennedy, 1975; Sawicki and Gomatos, 1976).

TABLE 4

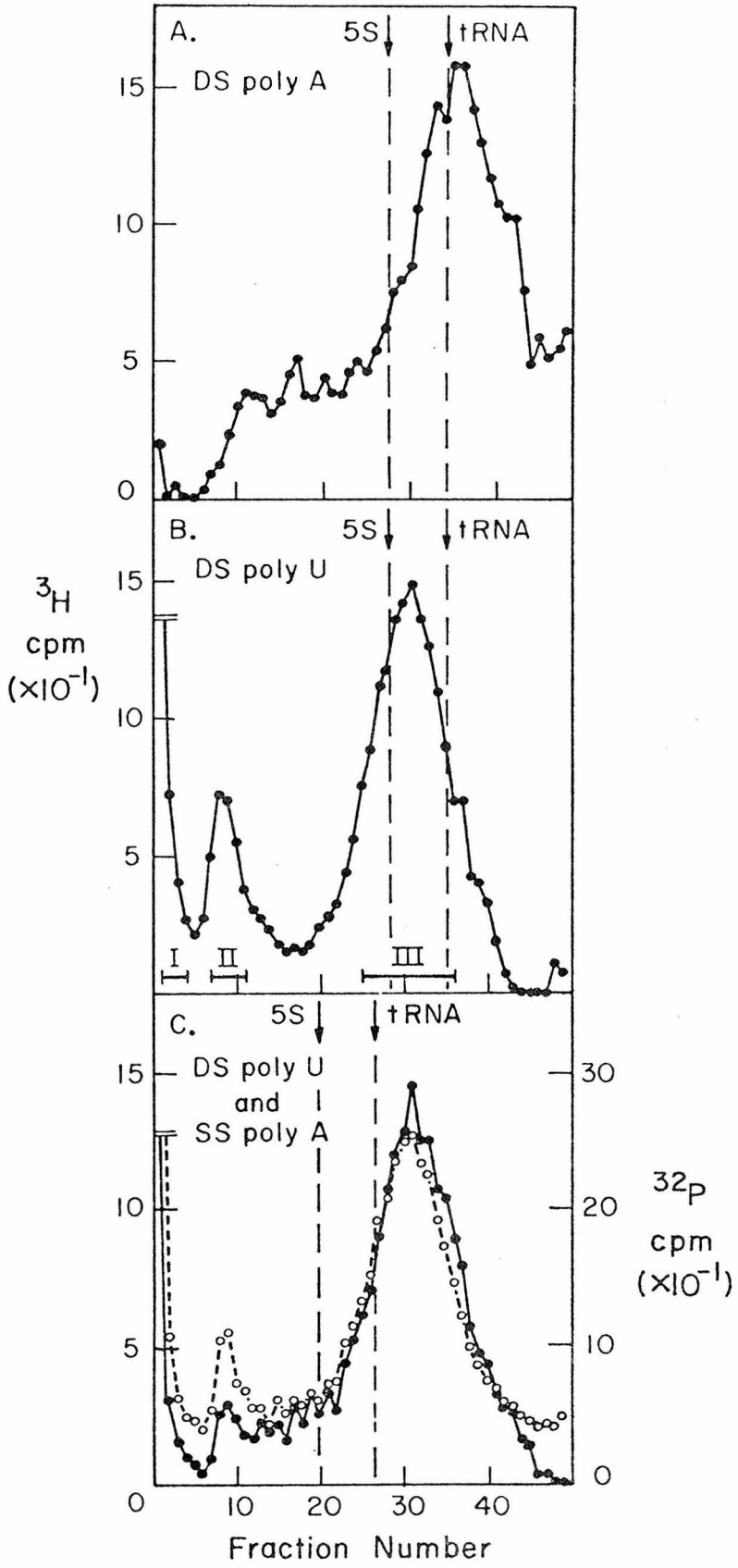
Percentage of ^{3}H-labeled Sindbis Virus RNA Species Which Bind to Columns of Oligo dT or Oligo dA Cellulose after Ribonuclease Digestion

RNA Preparation[a]	Column Used	Percent Bound[b]
^{3}H-adenosine DS RNA	Oligo dT	1.17 (±0.33)
^{3}H-adenosine DS RNA plus unlabeled poly U	Oligo dT	0.04
^{3}H-uridine DS RNA T-1 RNase	Oligo dA	0.96 (±0.19)
^{3}H-uridine DS RNA T-1 + panc RNase	Oligo dA	0.16 (±0.01)
^{3}H-uridine 49 S RNA T-1 RNase	Oligo dA	0.23

[a]RNA was labeled with [^{3}H]adenosine or with [^{3}H]uridine as indicated. DS = double stranded.

[b]Ranges are set at one standard deviation from the sample mean.

Fig. 4. Polyacrylamide gels of poly A and poly U isolated from Sindbis virus double stranded RNA. [^{3}H]poly A or [^{3}H]poly U was isolated from Sindbis virus double stranded RNA and analyzed on polyacrylamide gels as described in Materials and Methods. In Panel A and B, ^{14}C marker E. coli 5S RNA and tRNA were coelectrophoresed with the poly A or poly U. In Panel C, E. coli 5S RNA and tRNA markers were electrophoresed on a parallel gel. The direction of migration on all gels is from left to right. A) Poly A isolated from double stranded RNA. B) Poly U isolated from double stranded RNA. C) Formaldehyde denaturing gel of [^{3}H]poly U isolated from double stranded RNA (●——●) and [^{32}P]poly A isolated from 26S RNA (o---o).


A.
5S
tRNA
DS poly A
B.
DS poly U
I
II
III
C.
DS poly U
and
SS poly A
^{3}H
cpm
(×10^{-1})
^{32}P
cpm
(×10^{-1})
Fraction Number

Since Sindbis virus double stranded RNA contains poly A, it was of interest to determine which of the three species of Sindbis virus RFs contain poly A. [^{3}H]adenosine labeled RFs were isolated and separated by preparative gel electrophoresis. The presence of poly A in each RF was assayed by ribonuclease digestion, oligo dT cellulose chromatography and analysis of any bound material on polyacrylamide gels. We found that both RF I and RF III contain poly A while RF II does not. This result is consistent with the Simmons and Strauss (1972b) hypothesis that RF I and RF III are the templates for synthesis of the 49 S and 26 S viral RNA species. It also indicates that no species of poly A-containing viral RNA is synthesized from RF II.

Poly U on double stranded species of virus RNA. To search for a possible sequence of poly U in Sindbis virus double stranded RNA, [^{3}H]uridine labeled double stranded RNA was heat denatured in the presence of excess unlabeled poly U, digested completely with T-1 RNase and chromatographed through columns of oligo dA cellulose. As shown in Table 4, 0.96% of total [^{3}H]uridine label in viral double stranded RNA was bound to oligo dA cellulose after treatment with T-1. This percentage was substantially reduced when the material was digested with both T-1 and pancreatic ribonucleases. These results suggested the existence of a stretch of poly U in Sindbis virus double stranded RNA.

When [^{3}H]uridine material which bound to oligo dA cellulose was analyzed on polyacrylamide gels, three peaks of radioactivity were observed (Fig. 4B); peak I, the material at the top of the gel,

peak II, the peak migrating a few fractions into the gel, and peak III, the broad peak migrating between the 5 S and tRNA markers. To determine which peak was actually poly U, a sample of oligo dA cellulose bound, [^{3}H]uridine labeled material was treated with pancreatic ribonuclease and then analyzed on a similar polyacrylamide gel. After pancreatic ribonuclease treatment, peak I and peak II remained intact while peak III completely disappeared (data not shown). This result is interpreted to mean that peak III is actually the Sindbis virus poly U while peaks I and II are double stranded contaminants (either poly A:poly U hybrids that bind to oligo dA cellulose or reassociated double stranded RNA that binds to oligo dA cellulose nonspecifically).

Establishing a base composition for the poly U isolated from Sindbis virus double stranded RNA proved to be difficult. Poly A in the virus double stranded RNA copurifies with viral poly U since it is hybridized to the long stretches of unlabeled poly U added during preparation (Spector and Baltimore, 1975). We assumed that the viral poly A:unlabeled poly U complexes would migrate more slowly than free viral poly U on acrylamide gels and therefore the two species would be separated. However, when ^{32}P-labeled Sindbis virus double stranded RNA was digested with T-1 ribonuclease and chromatographed on oligo dA cellulose, the eluted material run on an acrylamide gel, the peaks eluted, and their base composition analyzed, peak III was found to contain equal amounts of ^{32}P-labeled adenosine and uridine. This was interpreted to mean that under the low ionic strength and elevated temperature conditions present in the 10% acrylamide gel, some of the labeled viral poly A denatured from the poly A:poly U hybrids and

migrated with the Sindbis virus poly U (obviously, this would not be detected when the material analyzed was labeled with [^{3}H]uridine). We tried several methods of separating viral poly U from the viral poly A:poly U hybrids including chromatography on cellulose, Sephadex G-200, and hydroxylapatite (Spector and Baltimore, 1975), all of which were unsuccessful in our hands. Finally, we ran 10% acrylamide gels in 0.2 M sodium phosphate buffer (pH 7.8), which should stabilize the viral poly A:carrier poly U double stranded structure. These gels gave a pattern similar to Fig. 4B, and when peak III was eluted and analyzed, it had the following base composition: adenosine, 7.1%; cytidine, 0.9%; guanosine, 3.0%; and uridine, 89.1%. This base composition is nearly complementary to the base composition for T-1 ribonuclease derived poly A from Sindbis virus single stranded RNA (Table 1). It is somewhat different than the base composition reported for Semliki Forest virus poly U of 0% adenosine, 1.2% cytidine, 3.5% guanosine, and 95.3% uridine (Sawicki and Gomatos, 1976).

If poly U is on the 5' end of the Sindbis virus minus strand RNA, as it is in Semliki Forest virus (Sawicki and Gomatos, 1976), then a few observations about the nucleotide sequence of both the Sindbis virus poly U and poly A can be made. The adenosine residues must be near the 3' end of the isolated poly U tracts since they should be complementary to the uridine residues on the 5' end of the (3'-terminal) poly A. Also, the cytidine residue in the pyrimidine tract in the 5' end of poly A nucleotide sequence must be very close to the 5' end of this pyrimidine tract. If it were in the middle or near the 3' end of these uridine residues, cleavage of the complementary G residue in

the poly U by T-1 ribonuclease would eliminate any adenosine residues on the 3' end of the poly U. Using this information, we can refine the sequence of the 3' end of 49 S RNA to read: G(UC) (AU_{4-5}) $A_{\overline{68}}$ AOH.

As shown in Fig. 4B, poly U isolated from Sindbis virus double stranded RNA migrates as a heterogeneous peak between the 5 S and tRNA markers. When denatured with formaldehyde and run in formaldehyde containing gels (data not shown), the mean size of the poly U was determined to be 72 nucleotides with a range of 40-110 nucleotides. This size distribution is very similar to the size distribution of Sindbis virus poly A. In fact when ^{3}H-labeled Sindbis virus poly U and ^{32}P-labeled poly A isolated from 26 S RNA were denatured with formaldehyde, mixed and run on a denaturing gel, they gave identical gel patterns (Fig. 4C). It is of interest then that poly A tracts and poly U tracts which migrate identically under denaturing conditions, appear to migrate slightly differently under nondenaturing conditions (Fig. 1 and Fig. 4).

[^{3}H]uridine labeled Sindbis virus RFs were isolated and separated by sucrose gradient sedimentation to determine which RF species contain poly U. When these RFs were digested with ribonuclease, chromatographed through oligo dA cellulose and the bound material was analyzed by polyacrylamide gel electrophoresis, poly U was found in RF I and RF III and not in RF II. This result indicates that the stretches of poly A and poly U are located in the same region of the Sindbis virus double stranded RNA and is similar to the results reported by Sawicki and Gomatos (1976) for Semliki Forest virus.

DISCUSSION

From analysis on acrylamide gels and determination of the ratio of terminal to internal nucleotide residues, we find the size distribution of poly A isolated from Sindbis virus RNA to be heterogeneous with a mean length of around 70 nucleotides. The bulk of the poly A has a length of from 40-120 nucleotides, but approximately 10% of the molecules are longer, reaching lengths of up to 200 nucleotides. These results are very similar to the lengths of Sindbis virus poly A reported by Eaton and Faulkner (1972) as determined by sucrose gradient sedimentation. Our size distribution for Sindbis virus poly A, however, is longer than the size range determined by Deborde and Leibowitz (1976) of 30-40 nucleotides. We see no particular reason for this difference, but it may be due to our use of different viral stocks.

Poly A isolated from Sindbis virus 26 S and 49 S RNA has an identical size distribution. We also find that the poly A isolated from virion RNA grown in culture cells of three divergent animal species, chicken, hamster, and mosquito, has a similar gel pattern. In all cases, the poly A on viral RNA is much shorter than the poly A on the host cell mRNA, which usually averages 150-250 nucleotides in length (review, Darnell, Jelinek, and Molloy, 1973), indicating that the virus itself must have some control over the length of poly A generated. These findings correlate well with a method of poly A addition to the viral RNAs by transcription of a minus strand poly U template by the viral replicase. Such transcription would produce

viral poly A of similar size distribution in different hosts and would not be influenced by possible host factors.

By studying the binding of whole RNA to oligo dT cellulose, we find that most Sindbis virus RNA contains poly A. However, a fraction (10-20%) of both 26 S and 49 S RNA will not bind to oligo dT cellulose and contains little poly A. We believe that this oligo dT cellulose unbound fraction of viral RNA is a definite species of RNA and is not due to random degradation during the extraction procedure. To ascertain this, we have done most of this work with the virion RNA. Virion RNA offers the advantage of not having to be purified by size selection, a procedure which can generate an artifactual poly A lacking species of RNA (King and Wells, 1976). The polyacrylamide gel pattern of the oligo dT cellulose unbound fraction of virion RNA shows a discrete RNA species and not random size fragments which would be generated by random degradation. It is interesting that the unbound fraction migrates slightly faster than total 49 S RNA in gels. It would not be expected that the absence of poly A, which comprises less than 0.5% of the 49 S RNA, would have that large an effect on gel mobility. Possibly the poly A does not fit nicely into the secondary structure of the 49 S RNA molecule and therefore exerts a dragging effect on the 49 S RNA molecule out of proportion to the size of the poly A. On denaturing gradients, the oligo dT cellulose unbound fraction of virion RNA runs as if it contains both a discrete species of RNA similar in size to oligo dT cellulose bound virion RNA (giving a pattern typical of a denatured RNA species with a tail of degraded material) and some low molecular weight fragments. As expected, size selection of virion

RNA on preparative gels eliminates these small molecular weight fragments and thus lowers the percentage of virion RNA which passes through oligo dT cellulose and yields an unbound fraction equally as intact as oligo dT cellulose bound RNA.

The oligo dT cellulose unbound fraction of virion RNA is much less infectious than the bound fraction of total virion RNA. The residual infectivity of the oligo dT cellulose unbound fraction is probably due to the fact that it is 10% contaminated with virion RNA molecules containing full size poly A. This result indicates that poly A is essential for the replication of Sindbis virus and coincides with the findings of Spector and Baltimore (1974) in poliovirus and Hruby and Roberts (1976) in encephalomyocarditis virus. Two reservations, however, must be made in this regard. First, the low in vivo infectivity of the oligo dT cellulose unbound virion RNA may not necessarily be indicative of an inability to replicate. Since the unbound virion RNA lacks significant poly A, it may simply be more sensitive to degradation upon entering the cell. Secondly, the genomic content of the unbound virion RNA is uncertain. Besides the absence of poly A, a small piece of genetic material could also be missing from the 3' end of the oligo dT unbound virion RNA molecule without affecting any of the data presented. Such an occurrence would have an adverse effect on infectivity.

The existence of two oligo dT cellulose binding classes of intracellular Sindbis virus RNA indicates that this property originates within the cell. Presumably the absence of poly A from a fraction of the viral RNA is due to either incomplete transcription or 3' terminal

degradation of the RNA in the cell. Since both intracellular and virion RNA species can be separated into oligo dT cellulose binding classes in approximately the same proportions, virion RNA represents a random sampling of the intracellular viral RNA pool with respect to poly A lengths. Thus poly A must have nothing to do with the encapsidation of the virion RNA. However, there is some selection in the encapsidation of the virion RNA, since even the incomplete 49 S RNA molecules which are encapsidated (the oligo dT cellulose unbound molecules) are nearly full sized. The only shorter species of viral RNA which is even encapsidated is defective interfering RNAs, short deleted species of viral RNA which all contain in common both the 5' and 3' terminal nucleotide sequences of the 49 S RNA (Guild, Flores, and Stollar, 1977; Kennedy, 1976; Kennedy et al., 1976). Thus, some structure near both ends of the 49 S RNA molecules appears to be essential for encapsidation. Such a structure could be the proposed inverted terminal repeats which allow the Sindbis 49 S RNA to form circles (Hsu, Kung, and Davidson, 1973). Therefore, it is possible that Sindbis virus RNA must be circular to be encapsidated.

We find that Sindbis virus double stranded RNA contains poly A with a size distribution similar to poly A on viral single stranded RNA. These results are similar to findings with Semliki Forest virus (Bruton and Kennedy, 1975; Sawicki and Gomatos, 1976). Thus poly A is added to Sindbis virus single stranded RNA at the site of viral RNA transcription. We find poly A specifically in RF I and RF III, but not in RF II. This location of poly A is consistent with the hypothesis of Simmons and Strauss (1972b) that RF I and RF III are the

templates for the synthesis of 49 S and 26 S RNA respectively. Also indicated is that no poly A containing species of viral RNA is synthesized from RF II. No such species of RNA has ever been found in infected cells and the reason for the labeling of the plus strand of RF II remains obscure (Simmons and Strauss, 1972b).

We also find stretches of poly U in Sindbis virus double stranded RNA, confirming the finding of poly U in Semliki Forest virus double stranded RNA by Sawicki and Gomatos (1976). We presume that this poly U is on the minus RNA strand, since none is found on plus stranded viral RNA. The poly U has an identical size distribution to Sindbis virus poly A, consistent with the idea that they are transcribed off each other. The percentage of [^{3}H]uridine labeled double stranded RNA which binds to oligo dA cellulose after RNase digestion indicates that there is one poly U stretch per double stranded RNA complex. This calculation is made using the following parameters: the molecular weight of Sindbis virus RF I is 8.6×10^6 daltons; viral double stranded RNA is 80% resistant to RNase digestion; 25% of the bases in double stranded RNA are uridine but 30% of the [^{3}H]uridine label ends up as cytidine; 0.96% of the ribonuclease digested [^{3}H]uridine labeled double stranded RNA binds to oligo dA cellulose, but only 68% of this actually migrates as poly U on gels. If there were one poly U per double stranded RNA molecule, that poly U is then calculated to be 73 nucleotides long, close to the length determined by denaturing gel electrophoresis. We find poly U in RF I and RF III, and not in RF II. This is similar to the location of double stranded poly A and consistent with the idea that poly A is transcribed off the poly U. The existence

of poly U in the double stranded RNA of both Sindbis virus and Semliki Forest virus strongly indicates that poly A synthesis in the alphaviruses is genetically coded. However, conclusive proof must await a rigorous purification of the viral transcriptase to analyze its synthetic capabilities.

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Chapter 2

Replication of Sindbis Virus: Location and Function of 5-Methyl Cytidine Residues in Virus Specific DNA

by

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SUMMARY

Sindbis virus intracellular 26S and 49S RNA contain internal 5-methyl cytidine (m^5C) residues. In [^{3}H-methyl] methionine labeled Sindbis virus RNA, an average of 19% of the [^{3}H] methyl label in 26S RNA is in m^5C while in 49S RNA an average of 8% of the [^{3}H] methyl label is in m^5C, the remainder of the label being in the cap. Sindbis virion 49S RNA contains much less m^5C than intracellular 49S RNA extracted from the same cells. In the 26S RNA, m^5C residues occur in five oligonucleotides, four of which have been identified as having base compositions of C_4A_4UG, C_6A_2UG, C_3A_2G, and C_2AG, which are found distributed between two locations, one approximately 4000 nucleotides from the 3' end and the other about 1200 nucleotides from the 3' end (out of a total length of 5000 nucleotides). The m^5C containing nucleotide C_2AG is found only at the former location and the oligonucleotide of composition C_4A_4UG is restricted to the latter location; 40 to 50% of the m^5C in 26S RNA is found in the oligonucleotide C_4A_4UG. The distribution of label between the two locations suggests that each region contains at least two of the methylated sequences. Thus it appears that there are five specific sites for methylation on the 26S RNA. Only a minority of these sites are modified, however, for each 26S RNA contains an average of less than one m^5C. Sindbis virus 26S RNA isolated from both polysomes and nonpolysomal ribonucleoproteins contain equal amounts of m^5C. 49S RNA isolated from polysomes contains 60 to 80% more m^5C than does 49S RNA which is isolated primarily from nucleocapsids.

INTRODUCTION

The occurrence of methylated nucleotides in the "cap" structure which resides on the 5' terminus of most eukaryotic messenger RNAs has been well documented (for review, see Shatkin, 1976). In addition, many eukaryotic messenger RNAs also contain internal methylated nucleotides. N^6-methyl adenosine (m^6A) is the most common internal methylated nucleotide (Shatkin, 1976) in eukaryotic messenger RNA although minor quantities of 5-methyl cytidine (m^5C) have also been reported (Dubin and Taylor, 1975; Sommer et al., 1976). m^6A residues are nonrandomly distributed in the messenger RNA of HeLa cells (Wei and Moss, 1977) and the virion RNA of Rous sarcoma virus (Beemon and Keith, 1977). m^6A residues are present in heterogeneous nuclear RNA (Salditt-Georgieff et al., 1976) and in the messenger RNAs of animal viruses which are dependent on the cell nucleus for replication but are absent from the messenger RNAs of viruses which replicate exclusively in the cytoplasm (Beemon and Keith, 1977; Sommer et al., 1976). This evidence suggests that m^6A is added to messenger RNA in the nucleus and may be functional in nuclear processing of messenger RNA (Beemon and Keith, 1977). Little is known about the occurrence or function of m^5C residues in eukaryotic messenger RNA. m^5C residues have been discovered in minor amounts (one fifth the quantity of m^6A residues) in the messenger RNA of hamster cells (Dubin and Taylor, 1975) and in the nuclear RNA and messenger RNA of adenovirus (Sommer, et al., 1976).

Cells infected with Sindbis virus, an alphavirus, contain two major species of virus messenger RNA (Mowshowitz, 1973; Simmons and Strauss, 1974b). One species, 49S RNA, has a molecular weight of

4.3×10^6 and is identical to the genomic RNA carried in the Sindbis virion (Simmons and Strauss, 1972; Wengler and Wengler, 1975). The 49S RNA serves as the messenger RNA for the translation of three or four nonstructural virus proteins (Simmons and Strauss, 1974a; Bracha, Leone, and Schlesinger, 1976; Lachmi and Kääriäinen, 1977; Strauss, Lenches, and Strauss, 1976). The second virus specific messenger RNA, 26S RNA, has a molecular weight of 1.6×10^6 and contains the 3' terminal third of the 49S RNA nucleotide sequences (Simmons and Strauss, 1972; Kennedy, 1976; Wengler and Wengler, 1976). The 26S RNA codes for the translation of the three virus structural proteins (Simmons and Strauss, 1974a; Cancedda and Schlesinger, 1974; Clegg and Kennedy, 1974). Although 49S RNA contains the genetic information for the virus structural proteins, it apparently cannot direct their translation (Simmons and Strauss, 1974a; Glanville et al., 1976).

Both 26S and 49S RNA of Sindbis virus contain the 5' terminal nucleotide sequence $m^7G(5')ppp(5')ApUp$ (Hsu-Chen and Dubin, 1976; Hefti et al., 1976). The Sindbis virus RNA cap is unusual in that it can contain the m^7G congeners $m_2^{2,7}G$ and $m_3^{2,2,7}G$ (Dubin et al., 1977; Hsu-chen and Dubin, 1976). The only other RNA species shown to contain these cogeners are small molecular weight nuclear RNAs of unknown function (Ro-Choi et al., 1975). These congeners of m^7G are present in the cap of Sindbis virus intracellular RNA, but the cap of the virion 49S RNA contains only m^7G (Dubin et al., 1977). The intracellular RNAs of Sindbis virus also contain internal m^5C residues (Dubin et al., 1977; Dubin and Stollar, 1975). m^5C accounts for 25% to 50% of the total methyl label in both intracellular 26S and 49S RNA extracted from infected hamster and chick cells (Dubin et al., 1977). The Sindbis

virion 49S RNA contains little m^5C, indicating that m^5C may serve an intracellular function, possibly in the translation of Sindbis virus messenger RNA (Dubin et al., 1977).

In this paper, we confirm the findings of Dubin et al. (1977) that the intracellular species of Sindbis virus RNA contain internal m^5C residues. We demonstrate that m^5C is present in five specific oligonucleotides which are found at two specific locations in the 26S RNA. We also provide some evidence that m^5C is functional in the translation of Sindbis virus messenger RNA.

MATERIALS AND METHODS

Materials: [^{3}H-methyl] methionine (10.5 Ci/mmole) was purchased from Amersham Searle and $^{32}PO_4$ (carrier free) was purchased from ICN. Ribonucleases T-1 and T-2 were obtained from Calbiochem. P-1 nuclease was a generous gift of Yamasa Shoyu Co., Ltd. (Japan). Carl Schleicher and Schuell supplied the cellulose acetate strips, Brinkman supplied DEAE cellulose coated polyethylene sheets (DE 300), and Eastman supplied plastic sheets coated with cellulose for thin layer chromatography. RNA (E. coli) for making homomixtures was purchased from BDH Biochemicals. 5-methyl cytidine and heparin were purchased from Sigma. 2-O-methyl cytidine was obtained from P-L Biochemicals. Dimethylsulfoxide (spectro-quality grade) was supplied by Matheson, Coleman and Bell. Collaborative Biochemicals supplied oligo dT cellulose (Type T-3). 1 M triethyl-ammonium bicarbonate (pH 8.6) made from redistilled triethylamine was given to us by Dr. Gene R. Peterson.

Cell Culture, Virus Infections and Preparation of Virus Specific RNA

Primary cultures of chicken embryo fibroblasts were maintained at 37°C in Eagle's minimal essential medium (Eagle, 1959) containing 2% fetal calf serum (Pierce, Strauss and Strauss, 1974). Twelve hours prior to infection, the medium over confluent monolayers of chick cells in 75 cm^2 T-flasks or 800 cm^2 roller bottles was changed to Eagle's medium containing one-tenth the normal concentration of phosphate and 3% dialyzed fetal calf serum. Two hours prior to infection, the medium was changed to Eagle's medium containing 3% dialyzed fetal calf serum, one-tenth the normal concentration of phosphate, and one-twentieth the normal concentration of essential amino acids. The cells were infected at 37°C in this medium containing 1 µg/ml actinomycin D and 0.1 mM adenosine and guanosine (to suppress ring labeling of these compounds by [^{3}H-methyl] methionine (Dubin, 1974)) with the HR strain of Sindbis virus (Burge and Pfefferkorn, 1966) at a multiplicity of 35 plaque forming units per cell. One hour after infection, the inoculum was removed and the monolayers were washed three times with Eagle's medium containing 3% dialyzed fetal calf serum, one-tenth the normal concentration of phosphate, no methionine, one-twentieth the normal concentration of the other essential amino acids, 0.1 mM adenosine and guanosine, and 1 µg/ml actinomycin D. Each infected monolayer then received [^{3}H-methyl] methionine at 50 µCi/ml (which makes one-twentieth of the normal concentration of methionine in the medium) and $^{32}PO_4$ at 100 µCi/ml in this medium. Five ml of labeling medium was used for T-flasks and 30 ml was used for roller bottles.

Six to eight hours after infection, the vessel containing the infected cells was immersed in an ice water bath, the labeling medium was removed, and the cells were washed twice with cold phosphate buffered saline (Dulbecco and Vogt, 1954) and once with cold 0.15 M KCl, 0.01 M Tris (pH 7.2), 0.0015 M $MgCl_2$ (Mowshowitz, 1973). The cells were then lysed in 0.15 M KCl, 0.01 M Tris (pH 7.2), 0.0015 M $MgCl_2$ containing 0.5% NP40 and 500 µg/ml heparin as a ribonuclease inhibitor. One ml of this solution was used to lyse a T-flask of cells and 10 ml was used to lyse a roller bottle. Nuclei and membranous debris were removed by centrifugation at 2000 rpm for 5 minutes in a Sorvall centrifuge. At this point, if polysomes from the infected cells were to be analyzed, one ml of the cell lysate supernatant was layered over a 7% to 47% sucrose gradient in 0.15 M KCl, 0.01 M Tris (pH 7.2), 0.0015 M $MgCl_2$, 50 µg/ml heparin, made in an SW40 or SW41 cellulose nitrate tube (Mowshowitz, 1973). Centrifugation was for 110 minutes at 40,000 rpm at 4°C. The optical density profile of the gradient was analyzed on an ISCO gradient fractionator. If RNA was to be prepared from the infected cells, the cell lysate supernatant was made 1.0% in SDS and extracted by the phenol-chloroform method of Simmons and Strauss (1972, Method C). Virus 49S and 26S intracellular RNA were separated on sucrose gradients as described by Simmons and Strauss (1972). To prepare Sindbis virion 49S RNA labeled with [^{3}H-methyl] methionine and $^{32}PO_4$, infected cells were labeled in the same manner, virus was harvested 11 hours after infection by the low salt-high salt reversal method (Pierce, Strauss and Strauss, 1974) and virion RNA was purified using the methods of Hsu, Kung and Davidson (1973).

Analysis of nucleotides. To analyze a sample of [^{3}H-methyl] methionine labeled RNA for the presence of methylated nucleotides, the RNA sample (containing up to 100 µg of RNA) was dissolved in 0.1 ml of 0.04 M ammonium acetate (pH 4.4), 0.001 M EDTA and digested with 4 units of T-2 ribonuclease for 4 hours at 37°C. The digested sample was then flash evaporated to dryness, resuspended in 10 µl of 0.01 M Tris (pH 7.2) containing 5 µg of P-1 nuclease, and digested again for 1 hour at 37°C. The digest was spotted directly on a sheet of Whatman 3MM paper on which 50 µg of each nucleoside and nucleotide had been spotted as markers. Nucleotides and nucleosides were then separated by the high voltage electrophoretic method of Keith, Gleason and Fraenkel-Conrat (1974). After electrophoresis, the paper was dried thoroughly, the marker compounds were located under a UV light source, the paper was cut into strips and placed in scintillation vials, 6 ml of a liquid scintillation cocktail consisting of toluene fluor, 5% NCS tissue solubilizer (Amersham-Searle) and 0.5% H_2O was added to each vial, and the vials were shaken overnight in the dark before counting.

Separation of cytidine and 5-methyl cytidine by cellulose thin layer chromatography was done as described by Randerath and Randerath (1974) using butanol:isopropanol;concentrated NH_3:water (3:3:1:1) as a developing solvent. For production of T-1 oligonucleotides of Sindbis virus RNA, the RNA was suspended in 2 µl of 0.01 M Tris (pH 7.4), 0.01 M EDTA containing 50 units of T-1 ribonuclease, drawn into a capillary tube, and digested for 2 hours at 37°C. The T-1 ribonuclease digest was fractionated by the fingerprinting techniques described by

Barrell (1971) which employ ionophoresis on cellulose acetate as the first dimension and homochromatography (using homomixture C) as the second orange (Barrell, 1971) was included with every ionophoresis and homochromatography for mobility markers. Homochromatography spots were scraped from the polyethylene sheets into a suction device (Brownlee and Sanger, 1969), washed well with 95% ethanol, and eluted with 1 M triethylammonium bicarbonate (pH 8.6). Oligonucleotides were removed from cellulose acetate strips by blotting them onto DEAE cellulose coated polyethylene sheets with pads of Whatman 3MM paper soaked with water and eluting them from the DEAE cellulose sheets as described from homochromatography spots.

Mapping m^5C sites in 26S RNA. Partial alkali degradation of the 26S RNA was done by the procedure of Wengler and Wengler (1976), although incubation times in 0.1 M Na_2CO_3 (pH 11) were extended to 20 minutes to produce smaller fragments of RNA. After partial degradation, the RNA was denatured by making it 80% in DMSO and RNA fragments containing the 3' terminus were isolated by oligo dT cellulose chromatography. The poly A containing RNA fragments eluted from oligo dT cellulose were precipitated with ethanol in the presence of 50 µg of carrier E. coli ribosomal RNA, resuspended in 90% DMSO, 0.1 M LiCl, 0.005 M EDTA, 0.001 M Tris (pH 6.5), 0.2% SDS, and fractionated by size on a sucrose gradient containing 50% DMSO (Bantle, Maxwell and Hahn, 1976). A hole was punched in the bottom of the gradient tube, fractions were collected, and the RNA in each fraction was precipitated with ethanol in the presence of 50 µg of carrier E. coli ribosomal RNA.

Each fraction was assayed for the presence of methylated nucleotides as described above.

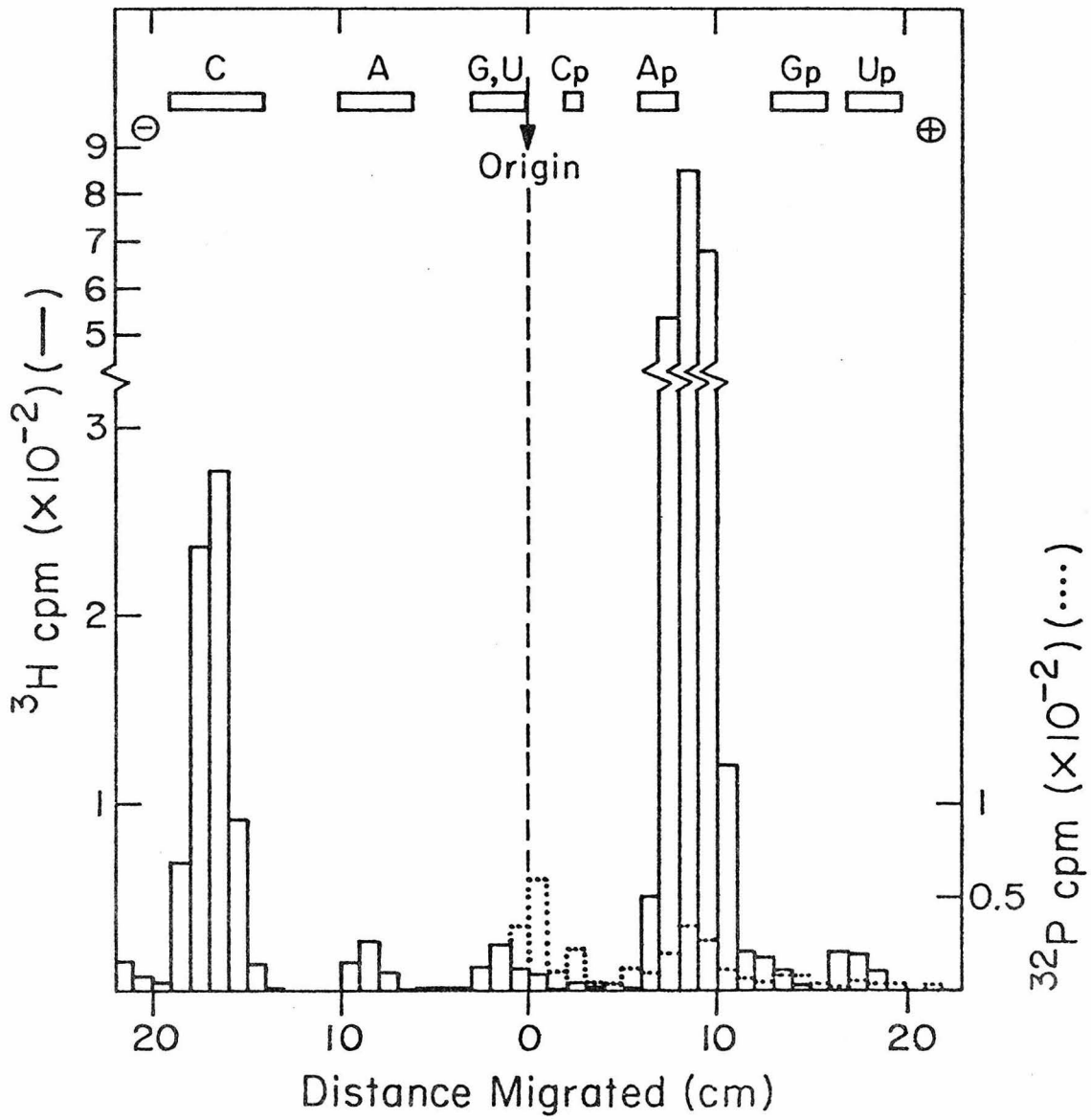
RESULTS

Occurrence of 5-Methyl Cytidine in Sindbis Virus RNAs

To analyze the distribution of [^{3}H] methyl label in Sindbis virus RNA, [^{3}H-methyl] methionine labeled RNA was digested to completion with T-2 ribonuclease and 3' phosphate groups on the resulting mononucleotides were then removed with P-1 nuclease. Using this digestion procedure, internal nucleotides are digested to nucleosides while caps retain the two 5' terminal nucleotides connected by their triphosphate bridge. These species can then be separated by one dimensional paper electrophoresis. Such an analysis of 26S RNA is shown in Figure 1. The largest peak of [^{3}H] methyl label migrates towards the anode slightly more rapidly than the adenosine monophosphate marker. Since this nucleotide species is negatively charged and contains $^{32}PO_4$ label, it is identified as the cap. All experiments we conducted on the cap were in agreement with the findings of Dubin et al. (1977) that both 26S and 49S RNA contain a cap of sequence $m^7GpppAp$ and that there is one cap per 26S or 49S RNA molecule.

A second large peak of [^{3}H] methyl label coelectrophoreses with the cytidine marker. As shown in Figure 2, with both 26S and 49S RNA, when the cytidine marker was eluted from the paper electropherogram and analyzed by cellulose thin layer chromatography, the [^{3}H] methyl labeled species comigrated with 5-methyl cytidine. This result confirms

Fig. 1. Electropherogram of [^{3}H] methyl labeled nucleosides in Sindbis virus RNA. [^{3}H-methyl] methionine and [$^{32}PO_4$] labeled Sindbis virus 26S RNA was digested and electrophoresed as described in Materials and Methods. The positions of the unlabeled nucleoside and nucleotide markers coelectrophoresed with the sample are noted at the top of the Figure. In this particular sample of 26S RNA, 22.3% of the [^{3}H] methyl label is in cytidine, 1.6% is in adenosine, 1.6% is in guanosine-uridine, and 74.5% is in the cap. (——), [^{3}H] methyl label; (---), $^{32}PO_4$ label.


C
A
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Origin
9
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7
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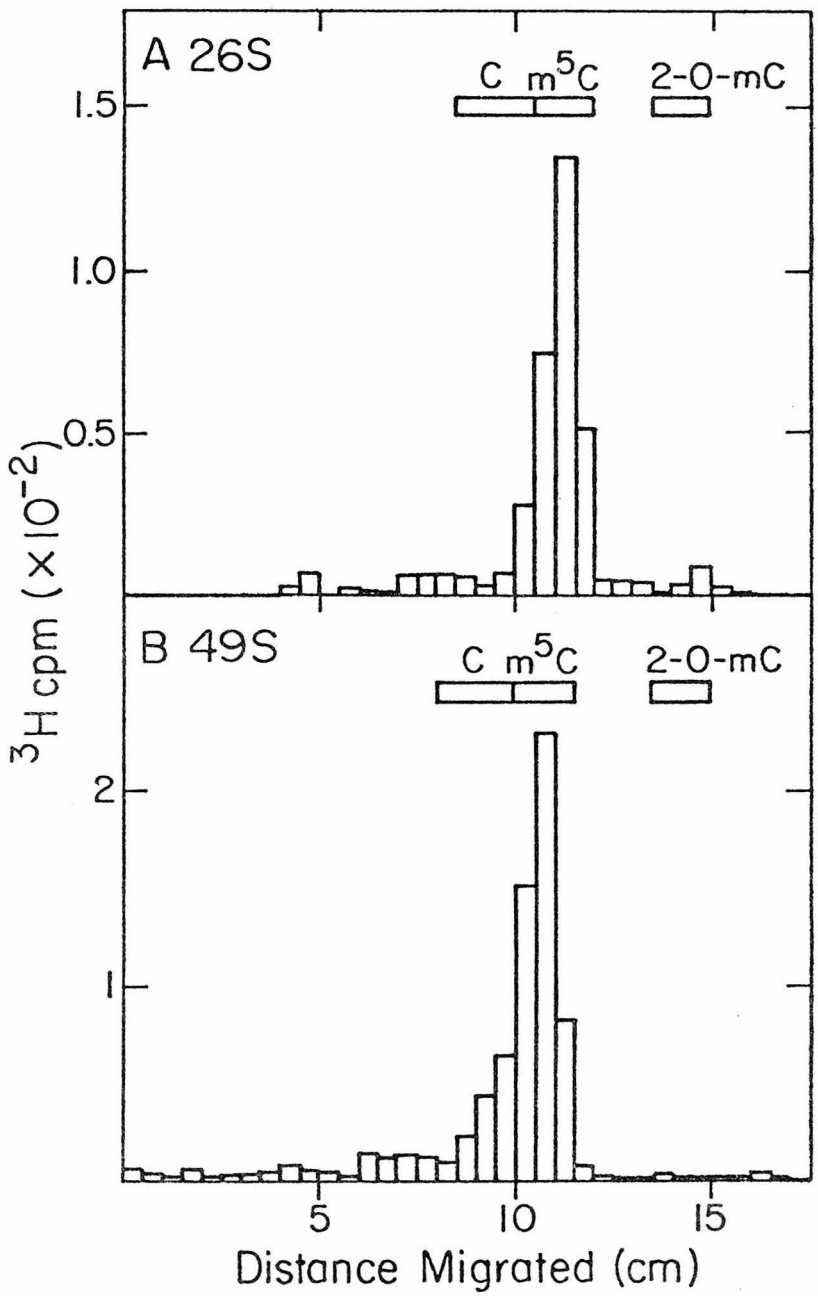
^{3}H cpm (X10^{-2}) (—)
1
0.5
^{32}P cpm (X10^{-2}) (....)
20
10
0
10
20
Distance Migrated (cm)

Fig. 2. Analysis of [^{3}H-methyl] cytidine found in Sindbis virus RNA. Material in the region containing the cytidine marker on paper electropherograms of ribonuclease digested [^{3}H-methyl] methionine labeled Sindbis virus 26S and 49S RNA was eluted and analyzed by thin layer cellulose chromatography as described in Materials and Methods. Unlabeled cytidine (C), 5-methyl cytidine ($m^{5}C$), and 2-O-methyl cytidine (2-O-mC) were added to each analysis as markers and their positions on the chromatogram were located under a UV light source.


A 26S
C m5C
2-O-mC
1.5
1.0
0.5
B 49S
C m5C
2-O-mC
2
1
3H cpm (X10-2)
5
10
15
Distance Migrated (cm)

the findings of Dubin et al. (1977) that both Sindbis virus 26S and 49S RNA contain internal m^5C residues.

As shown in Figure 1, small amounts of [^{3}H] methyl label also coelectrophorese with the adenosine and the guanosine-uridine markers. The amounts of [^{3}H] methyl label in both of these peaks can be decreased by adding unlabeled adenosine and guanosine to the culture medium 2 hours before infection instead of at infection. When the [^{3}H] methyl labeled species which coelectrophoreses with adenosine was eluted from electropherograms of digests of both 26S and 49S RNA and analyzed by cellulose thin layer chromatography, it was found to consist of 70% adenosine and 30% N^6-methyl adenosine. Since 1.7% and 2.1% of the [^{3}H] methyl label in 26S and 49S RNA, respectively, coelectrophoreses with adenosine (see Table I), between 0.5% and 0.6% of the [^{3}H] methyl label in 26S and 49S RNA is in m^6A. Using the fact that there is one cap per 26S or 49S RNA molecule and using the data of Dubin et al. (1977) on the distribution of [^{3}H] methyl label in the three methylated guanosine congeners in the cap, this percentage of m^6A corresponds to one molecule of m^6A per 110 molecules of 26S or 49S RNA. Thus, m^6A is not a regular component of Sindbis virus RNA and is possibly a cellular contaminant.

Table I presents the distribution of [^{3}H] methyl label in Sindbis virus 26S and 49S RNA. These distributions are an average taken from eight separate virus RNA isolations made from seven separate primary chick cell preparations over a period of 5 months. As shown in Table I, the percentage of [^{3}H] label in m^5C in 26S RNA varies widely. This wide variation is due to the fact that in three of the 26S RNA preparations, the percentage of [^{3}H] methyl label in m^5C was between 9.4% and 12.4%,

TABLE I

Distribution of [^{3}H] methyl label
in Sindbis virus intracellular RNAs

	Percentage of [^{3}H] methyl label in:			
RNA	C	A	G,U	Cap
26S	19.1[a](± 6.8)[b]	1.7 (± 1.0)	1.6 (± 0.9)	77.7 (± 6.7)
49S	8.0 (± 2.9)	2.1 (± 1.6)	1.6 (± 1.2)	88.3 (± 2.7)

[a]Average obtained from eight different RNA preparations.

[b]Range set as one standard deviation from the sample mean.

while in the other five preparations, the percentage was between 22.4% and 26.3%. The 26S RNA preparations which had the low percentages of [^{3}H] methyl label in m^5C were isolated consecutively over a 2 month period. 26S RNA preparations isolated either before or after this 2 month period had a percentage of [^{3}H] methyl label in m^5C in the higher range. During this study, the virus stock, media, and growth and labeling conditions were not changed, and therefore this depression in percentage of [^{3}H] label in m^5C in 26S RNA isolated during this 2 month period was probably due to the state of the chicken embryos used to prepare primary cultures. There is less m^5C in Sindbis virus 49S RNA than in 26S RNA, the percentage of [^{3}H] methyl label in m^5C in 49S RNA always being between 30% and 55% of the percentage of [^{3}H] methyl label in 26S RNA from the same RNA preparation. The percentage of [^{3}H] methyl label in m^5C in 49S RNA ranged from 4.0% to 12.9%.

To exclude the possibility that m^5C is actually present in cellular RNA species which copurify with the Sindbis virus RNA species, the following experiments were done. Denaturation in 90% DMSO at 37°C followed by sedimentation in a sucrose gradient containing 50% DMSO did not alter the percentage of [^{3}H] methyl label in m^5C in 26S RNA (Table II). Difficulties in sedimenting 49S RNA in gradients containing DMSO (Simmons and Strauss, 1972) precluded a similar experiment on 49S RNA. When 26S and 49S RNA were subjected to two cycles of oligo dT cellulose chromatography with a denaturation in 90% DMSO at 55°C prior to the second cycle (Bantle, Maxwell and Hahn, 1976), the percentage of [^{3}H] methyl label in m^5C actually increased in both 26S and 49S RNA (Table II). This is due to the fact that if the RNA samples were partially degraded, the cap, being located at the 5' end of the RNA molecule, has

TABLE II

[^{3}H] methyl label in Sindbis virus intracellular RNAs after various treatments

RNA and Treatment	Percentage of [^{3}H] methyl label in: C	A	G, U	Cap
26S				
Control	25.0	1.7	1.6	71.7
DMSO gradient[a]	23.9	0.6	0.4	75.1
Oligo dT cellulose[b]	32.3	1.2	0.8	65.8
49S				
Control	7.8	2.6	3.1	86.6
Oligo dT cellulose	15.9	3.1	4.8	76.2

[a]The RNA was denatured in 90% DMSO at 80°C for 5 minutes and sedimented through a sucrose gradient containing 50% DMSO (see Materials and Methods). The fractions of the gradient containing the peak of RNA were pooled and analyzed for [^{3}H] methyl labeled nucleotides.

[b]The RNA was subjected to oligo dT cellulose chromatography. RNA bound to oligo dT cellulose was analyzed for [^{3}H] methyl labeled nucleotides.

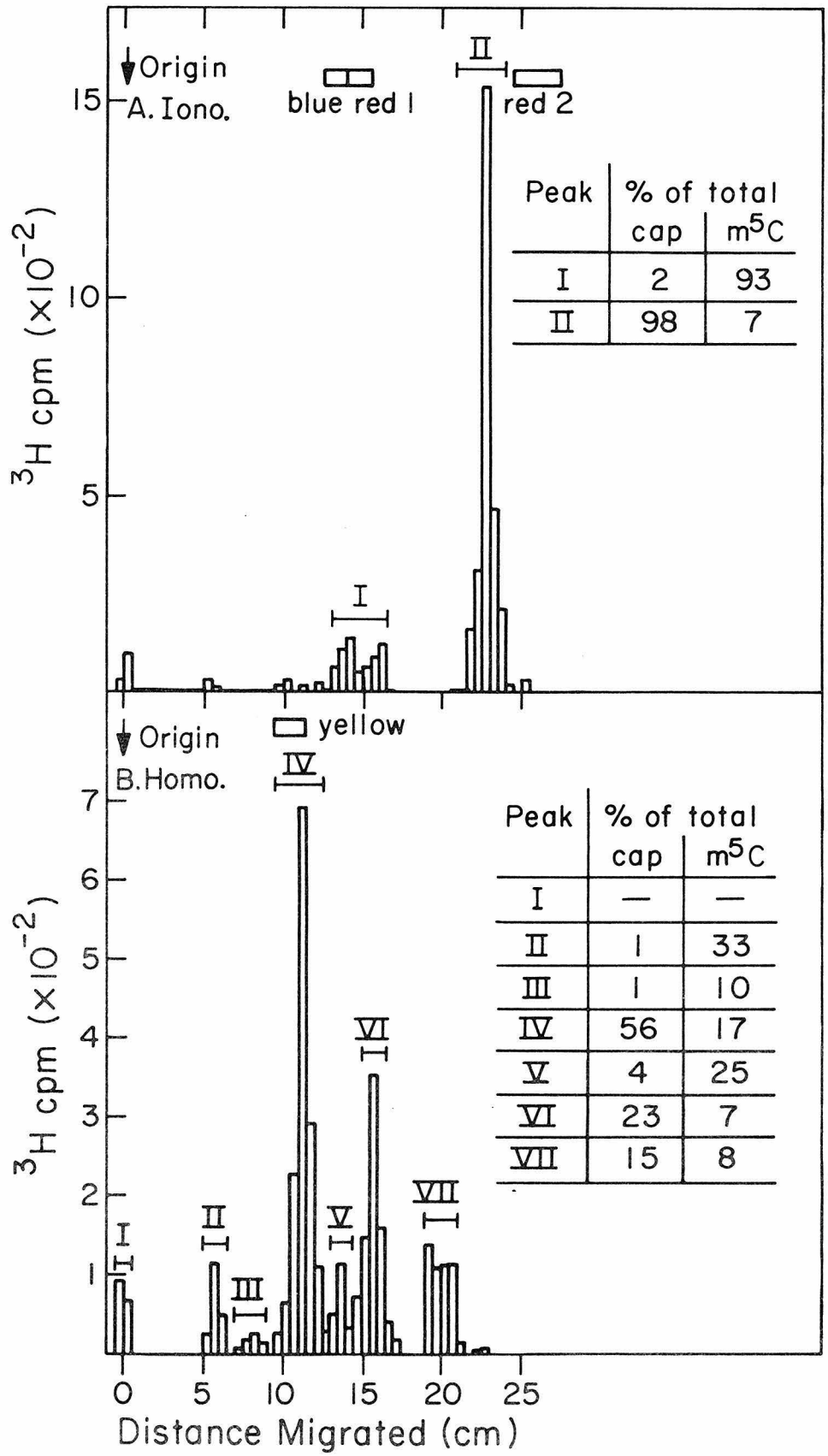
a smaller chance of being bound to oligo dT cellulose than do internally located m^5C residues. The results of these two experiments indicate that m^5C is not a cellular contaminant located in the ribosomal RNA species which copurify with Sindbis virus RNA or in species of RNA which would aggregate to 26S RNA during isolation procedures. m^5C could be located on cellular messenger RNA species which cosediment with Sindbis virus RNA. However, when infected and mock infected cells were labeled with [^{3}H-methyl] methionine, RNA was extracted, and equal amounts of RNA from each preparation were run on sucrose gradients, the 26S region of the gradients run with RNA from mock infected cells contained only 6% of the m^5C found in the same region of the gradients run with material from infected cells. It is thus concluded that m^5C is a component of Sindbis virus specific RNA and not a cellular contaminant.

Sindbis virion 49S RNA was also analyzed for the presence of m^5C. Eleven hours after infection, virus was harvested from an infected roller bottle labeled with [^{3}H-methyl] methionine and the intracellular virus RNAs were then isolated from the same infected cell monolayer. In the virion 49S RNA, 4.7% of the total [^{3}H] methyl was in m^5C, while in intracellular 49S RNA isolated from the same infected cells, 24.4% of the [^{3}H] methyl label was in m^5C (in 26S RNA from the same preparation, 34.1% of the [^{3}H] methyl label was in m^5C). This result confirms the findings of Dubin *et al.* (1977) that Sindbis virion 49S RNA contains much less m^5C than Sindbis virus intracellular 49S RNA.

Location of m^5C Residues in Specific T-1 Oligonucleotides

To determine whether m^5C residues in Sindbis virus RNA are randomly distributed or are located in specific oligonucleotides, a sample of [^{3}H-methyl] methionine and $^{32}PO_4$ labeled Sindbis virus 26S RNA was digested completely with T-1 ribonuclease and the oligonucleotides produced were analyzed in one dimension by both ionophoresis at pH 3.5 on cellulose acetate and by homochromatography. As shown in Figure 3A, when the T-1 ribonuclease digest of 26S RNA (in which 22.4% of the [^{3}H] methyl label was in m^5C) was fractionated by ionophoresis, three large peaks of [^{3}H] methyl label are observed. When the material in regions corresponding to these peaks on a parallel ionopherogram was eluted and analyzed, the majority of the m^5C was found in the two smaller, more slowly migrating peaks (combined as peak I in Fig. 3A) although a small amount was found in the larger, more rapidly migrating peak (peak II). Cap was found almost exclusively in peak II. As shown in Figure 3B, a one-dimensional analysis by homochromatography of a similar T-1 ribonuclease digest of 26S RNA is more complex, with seven peaks (labeled I to VII in Fig. 3B) of [^{3}H] methyl label being detected. The material in regions corresponding to these seven peaks on a parallel homochromatogram was eluted and analyzed. No [^{3}H] methyl labeled material could be eluted from peak I at the origin, probably because material in this peak is very large (it could be undigested double stranded RNA) and thus binds tenaciously to DEAE cellulose. Cap was found in peaks IV, VI and VII. This is probably due to the presence in Sindbis virus RNA cap of the congeners of methylated guanosine (Dubin et al., 1977) which would be differentially charged at pH 8.7 during the homochromatography.

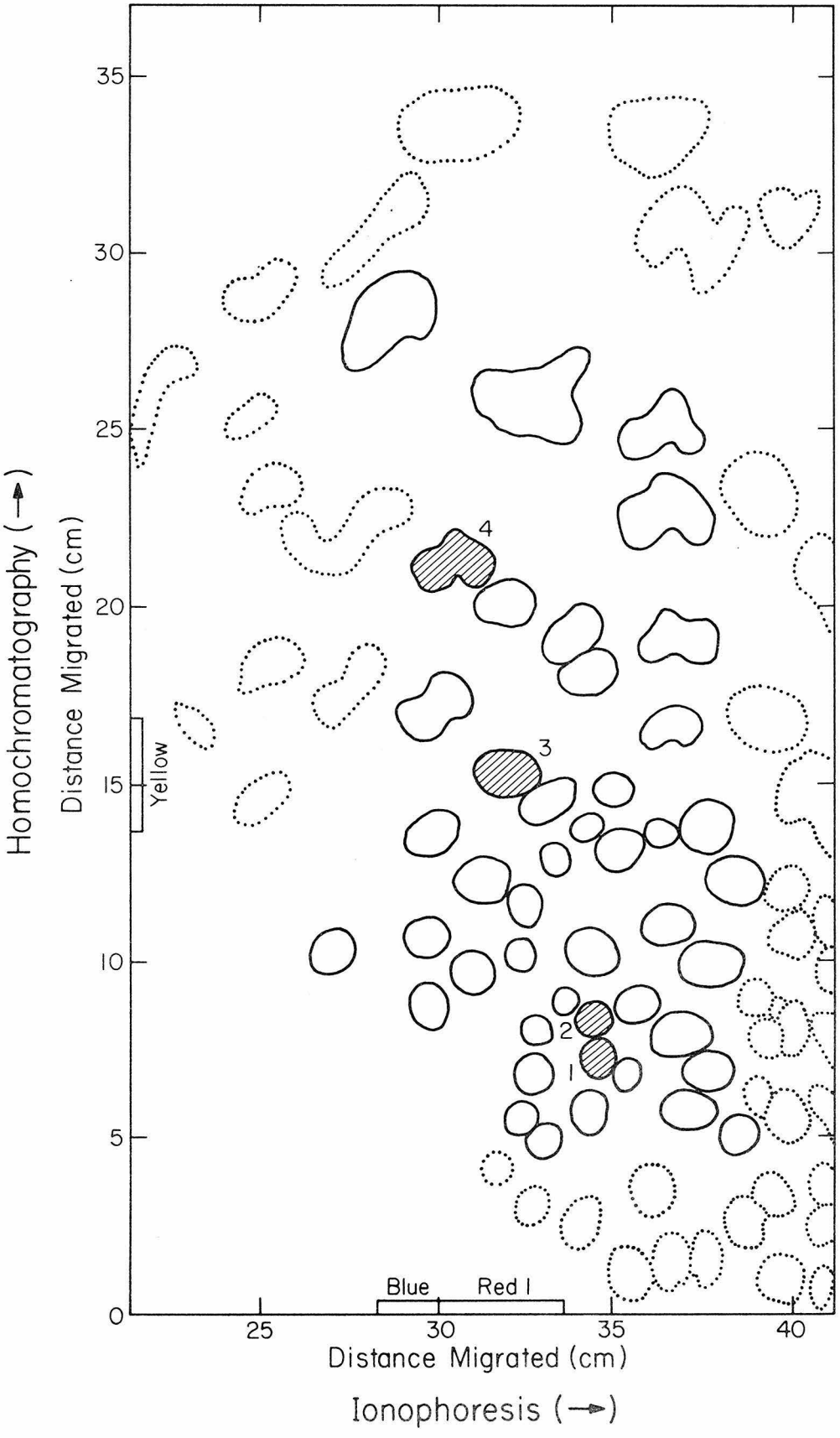
Fig. 3. Analysis of a T-1 ribonuclease digest of 26S RNA in one dimension by ionophoresis and by homochromatography. $^{32}PO_4$ and [^{3}H-methyl] methionine labeled samples of 26S RNA were digested completely with T-1 ribonuclease and fractionated in one dimension by A) ionophoresis, or B) homochromatography. The position of migration of marker dyes in each analysis is shown at the top of each graph; the positions of the Xylene cyanol FF (blue) and two bands of acid fuchsin (red 1 and red 2) being noted in the ionophoresis and the position of methyl orange (yellow) being noted in the homochromatography. The distribution of $^{32}PO_4$ label in these analyses is not shown. Material in regions of a parallel ionopherogram and homochromatogram corresponding to the peaks of [^{3}H] methyl label shown in these figures was eluted and analyzed for the presence of m^5C and cap. The table in each graph shows the distribution of total m^5C and cap among the peaks of [^{3}H] methyl label shown on that graph.


Origin
A. Iono.
blue
red 1
red 2
II
I
^{3}H cpm (×10^{-2})
15
10
5
Peak
% of total
cap
m^{5}C
I
2
93
II
98
7
Origin
B. Homo.
yellow
IV
VI
VII
III
V
7
6
5
4
3
2
1
0
5
10
15
20
25
Distance Migrated (cm)
Peak
% of total
cap
m^{5}C
I
—
—
II
1
33
III
1
10
IV
56
17
V
4
25
VI
23
7
VII
15
8

m^5C was found in six peaks, peaks II through VII. Since material on the homochromatogram from which material was eluted for analysis ran 5% slower than material on the parallel homochromatogram which was assayed for total radioactivity, the m^5C found in peak VI is probably spillover from material in peak V. None of the m^5C in the other peaks could be accounted for by similar spillover. Thus, it appears that m^5C residues are present in five oligonucleotides, which migrate similarly during ionophoresis.

To identify which oligonucleotides contain m^5C, a T-1 ribonuclease digest of [^{3}H-methyl] methionine and $^{32}PO_4$ labeled 26S RNA (from the same 26S RNA preparation used in the one-dimensional analyses) was fingerprinted by the methods of Barrell (1971) (the first dimension is cellulose acetate ionophoresis and the second dimension is homochromatography). The one-dimensional analyses described in the previous paragraph were used as a guide to locate m^5C containing oligonucleotides in the fingerprint. Since in ionophoresis the m^5C containing peaks co-electrophorese with Xylene cyanol FF (blue) and slowest band of the acid fuchsin (red) dyes (Fig. 3A), the first dimensional ionophoresis was run until these dyes neared the end of the cellulose acetate strip to achieve maximum separation of the oligonucleotides, and only material in the region of the strip containing these dyes was transferred to a DEAE cellulose coated plastic sheet for homochromatography. A line drawing of the autoradiograph of the fingerprint is shown in Figure 4. Selected oligonucleotides (circled with solid lines) were recovered from the fingerprint and examined for m^5C. These spots were chosen on the basis of the mobilities of the m^5C oligonucleotides relative to the

Fig. 4. Location of m^5C oligonucleotides on a fingerprint of 26S RNA. [^{3}H-methyl] methionine and $^{32}PO_4$ labeled 26S RNA was digested with T-1 ribonuclease and fingerprinted by the methods of Barrell (1971). This diagram was drawn from an autoradiograph of that fingerprint. The spots surrounded by dotted lines (····) were present in the autoradiograph but were not analyzed for m^5C, the spots surrounded by solid lines (——) were analyzed for m^5C, m^5C being found in the cross-hatched spots labeled 1 through 4. The boxes on the margin indicate the position of the marker dyes Xylene cyanol FF (blue), acid fuchsin (red 1), and methyl orange (yellow) in both dimensions of the fingerprint.


Homochromatography (→)
Distance Migrated (cm)
35
30
25
20
15
10
5
0
Yellow
4
3
2
1
Blue
Red I
25
30
35
40
Distance Migrated (cm)
Ionophoresis (→)

dye markers as determined in the experiment shown in Figure 3. m^5C was found in four oligonucleotides, shown cross-hatched in Figure 4 and numbered 1 through 4; from their mobilities we assume they correspond to peaks II through V, respectively, in Figure 3B. Recovery of m^5C in these four oligonucleotides was 90% of the amount of [^{3}H] 5-methyl cytidine monophosphate (produced by T-2 digestion of the same 26S RNA preparation) recovered from a parallel fingerprint. From the relative recoveries of these four oligonucleotides and taking them to represent 90% of the m^5C in 26S RNA, oligonucleotide 1 contained 49% of the total m^5C, oligonucleotide 2 contained 17%, and 3 and 4 both contained 13%. These estimates are probably more accurate than those of Figure 3B because of the technical difficulties encountered in matching the preparative and analytical homochromatograms of this figure as noted above. We could find no spot containing m^5C with the same mobility as peak VII of Figure 3B. This oligonucleotide may migrate differently in the ionophoresis dimension than the other four oligonucleotides, possibly accounting for the m^5C found comigrating with the cap during ionophoresis.

From the $^{32}PO_4$ label, the base composition of the four m^5C containing oligonucleotides was determined by digestion with T-2 ribonuclease and separation of the resulting mononucleotides by paper electrophoresis. The relative frequency of occurrence of each oligonucleotide in 26S RNA was also determined by comparing the $^{32}PO_4$ label present in that spot to the total $^{32}PO_4$ labeled 26S RNA applied to the fingerprint. Thus oligonucleotide 1 has a base composition of approximately C_4A_4UG and occurs once per 26S RNA, and is therefore unique; oligonucleotide 2 has

a base composition of approximately C_6A_2UG and is also unique; oligonucleotide 3 has a base composition of C_3A_2G and occurs six times per 26S RNA; and oligonucleotide 4 has a base composition of C_2AG and occurs twelve times per 26S RNA.

In a second experiment, a T-1 ribonuclease digest of a sample of $^{32}PO_4$ and [^{3}H-methyl] methionine labeled 26S RNA in which 12.4% of the [^{3}H] methyl label was in m^5C was fingerprinted and analyzed, m^5C was found in oligonucleotides in similar positions as oligonucleotides 1, 2 and 4 on the fingerprint shown in Figure 4 (data not shown). The base compositions of these oligonucleotides were found to be C_4A_3UG, C_5A_2UG, and C_2AG, respectively. We assume that these oligonucleotides are identical to oligonucleotides 1, 2 and 4 and that the slight differences in base compositions is because only small amounts of ^{32}P label were available in this experiment for the base composition analyses (less than 200 counts per minute for oligonucleotides 1 and 2) and also because on this fingerprint the spots corresponding to oligonucleotides 1 and 2 were not well resolved. In this experiment, 41% of the [^{3}H] m^5C was found in oligonucleotide 1, 32% was found in oligonucleotide 2, and 27% was found in oligonucleotide 4. No m^5C was found in oligonucleotide 3, which partly explains why some 26S RNA preparations contain smaller amounts of m^5C.

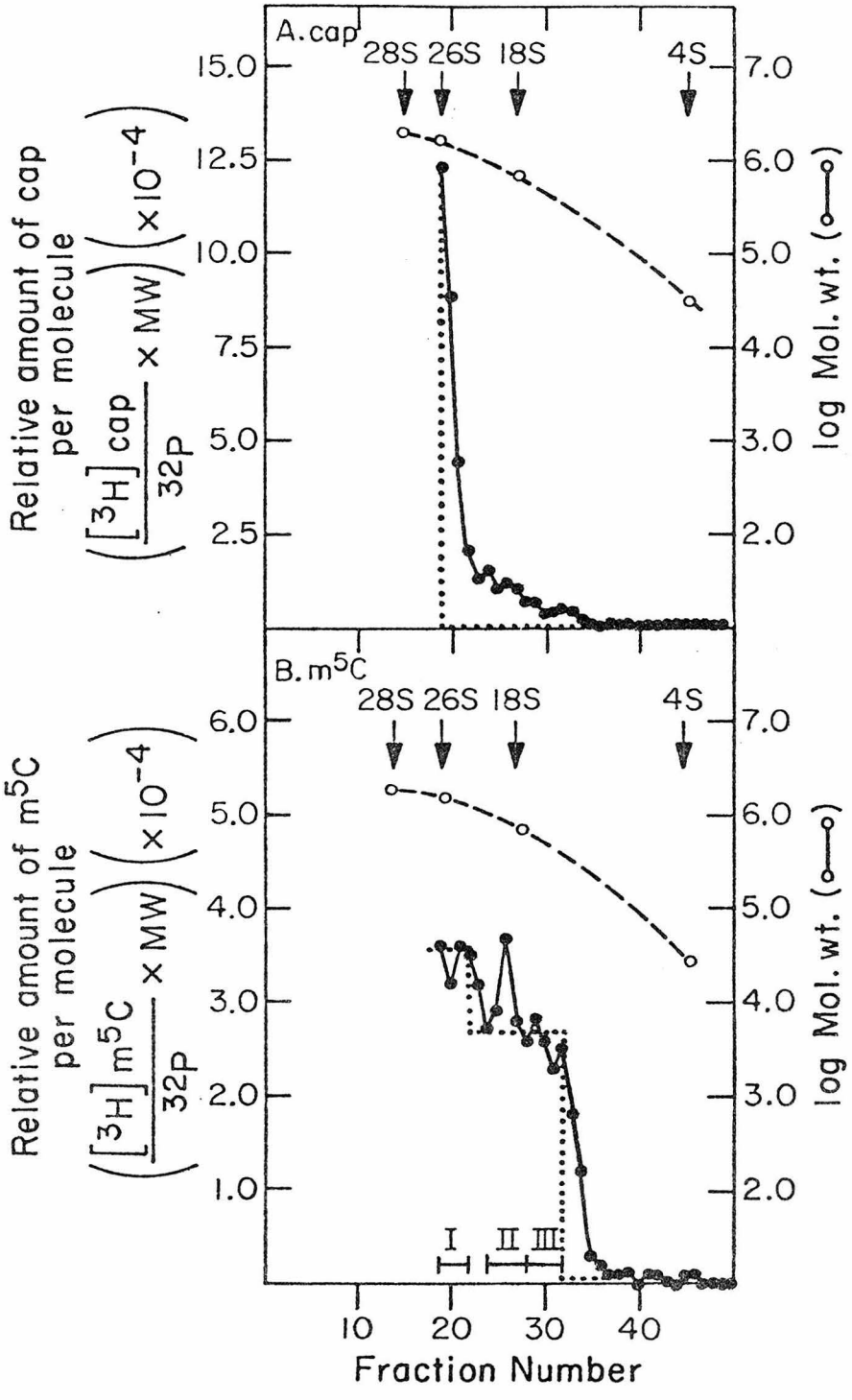
Location of m^5C Residues on 26S RNA

To map the locations at which m^5C residues occur on the 26S RNA, a preparation of $^{32}PO_4$ and [^{3}H-methyl] methionine labeled 26S RNA (in which 24.0% of the [^{3}H] methyl label was in m^5C) was randomly fragmented

by mild alkali treatment, fragments containing the 3' terminus (poly A) were selected by oligo dT cellulose chromatography, and these were then separated by size in a denaturing sucrose gradient. Using this technique, a map of the 26S RNA is generated because, starting at the top and moving down the gradient, each successive fraction contains a longer set of 3' terminal fragments of 26S RNA. A landmark (such as a specific oligonucleotide or a m^5C residue) which is a certain distance from the 3' end of the 26S RNA will not be present in fractions of the gradient which contain 3' terminal fragments shorter than this distance but will be present in all fractions containing longer fragments. Thus, moving down from the top of the gradient, the first fraction in which the landmark is present contains 3' terminal fragments equal in length to the distance from the landmark to the 3' end of the 26S RNA molecule. Therefore, the amount of [^3H] m^5C and cap in each fraction of the gradient was determined. To correct for the fact that some fractions of the gradient have more material than others, the amounts of [^3H] m^5C and cap in each fraction was divided by the total amount of [^{32}P] label in the fraction. Then to get a figure which represents the relative number of m^5C residues and caps per molecule of RNA, the ratio of the [^3H] m^5C and cap to [^{32}P] label is multiplied by the apparent molecular weight of material in that fraction. Molecular weights were approximated from standards run on a parallel gradient.

The results of this gradient are presented in Figure 5. The only molecules containing the cap are full length 26S RNA, as expected since the cap and the poly A are at opposite extremities of the molecule. This distribution also demonstrates that the fraction of the gradient

Fig. 5. Mapping the location of cap and m^5C residues on Sindbis virus 26S RNA. [^{3}H-methyl] methionine labeled Sindbis virus 26S RNA was fragmented with mild alkali treatment and 3' terminal containing fragments were selected by oligo dT cellulose chromatography and separated by size on denaturing sucrose gradients. Each fraction from the gradient was analyzed for the presence of cap and m^5C. In this figure, the relative number of cap and m^5C residues per molecule of RNA in each fraction of the gradient is presented (as described in the text). Migration in the gradient is from right to left; undegraded 26S RNA is found in Fraction 19. The molecular weight of material sedimenting in each fraction is estimated from RNA markers of known molecular weight which were run on a parallel gradient. Noted at the top of the figure are the positions of these markers, 28S and 18S ribosomal RNAs and 4S transfer RNA from chick cells (molecular weights of 1.9×10^6, 7×10^5 and 3×10^4 daltons, respectively). (●—●—●), relative concentration of cap or m^5C per molecule; (o--o--o), molecular weight; (·····), author's interpretation of the cap and m^5C map.


A. cap
28S 26S 18S
4S
B. m5C
28S 26S 18S
4S
Relative amount of cap per molecule
Relative amount of m5C per molecule
log Mol. wt. (o—o)
I
II
III
Fraction Number

used to determine the distance from a landmark to the 3' end of the 26S RNA should be the fraction just before the relative concentration of the landmark begins to decrease. As shown in Figure 5B, it appears that m^5C residues occur at two locations on the 26S RNA. As shown by the dotted lines on Figure 5B, we estimate that 25% of the m^5C occurs at a location approximately 4000 nucleotides from the 3' end of the 26S RNA and 75% of the m^5C occurs at a location approximately 1200 nucleotides from the 3' end of the 26S RNA (26S RNA contains approximately 5000 nucleotides). Taking into account inaccuracies in molecular weight measurement and analysis of m^5C, we estimate that these distances are accurate to within 10%.

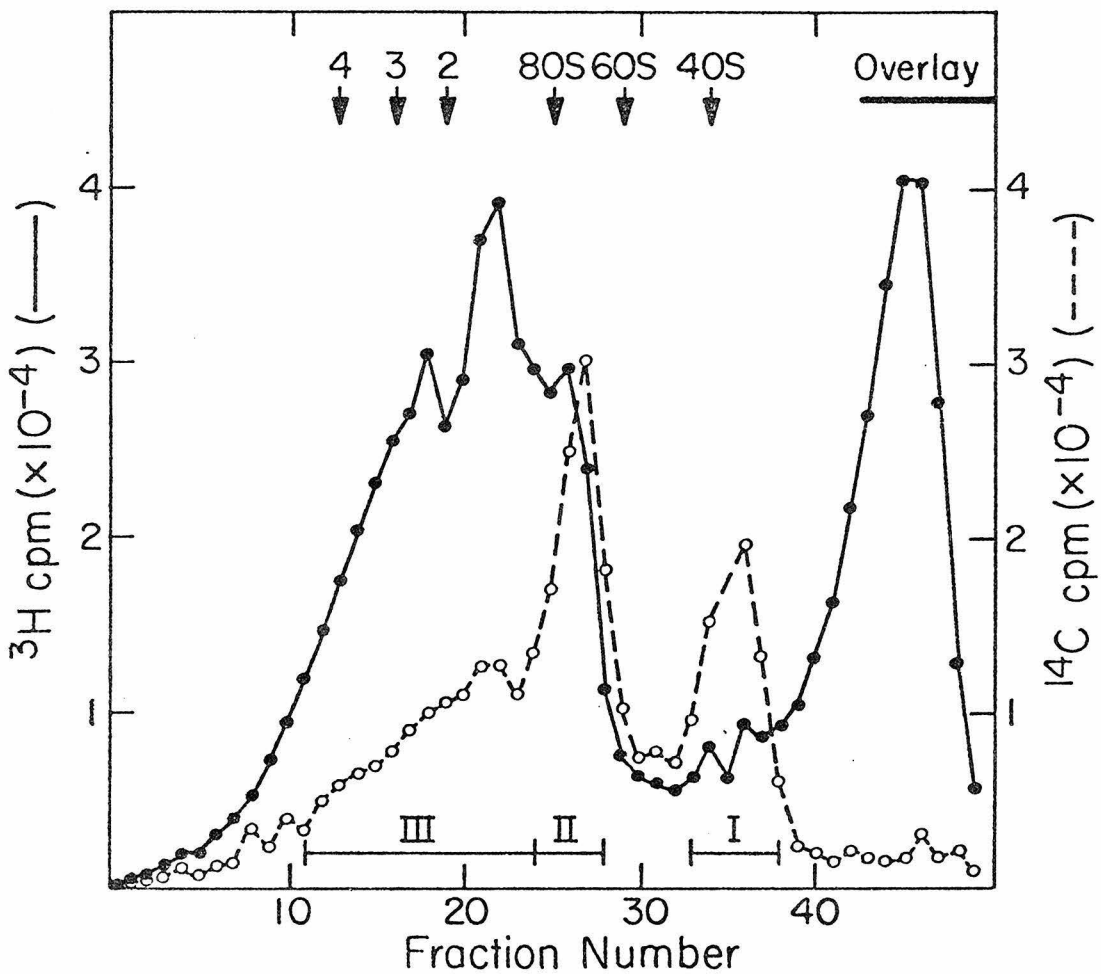
To determine which m^5C containing oligonucleotides are present at each of the two m^5C locations on the 26S RNA, material from each of the three regions of the gradient indicated in Figure 3B was pooled, digested with T-1 ribonuclease, and analyzed by one-dimensional homochromatography (data not shown). One-dimensional homochromatography was used because not enough label was present to do a complete fingerprint. Oligonucleotide 1 (C_4A_4UG) is present in all three regions and therefore must occur at the location 1200 nucleotides from the 3' end of the 26S RNA. Oligonucleotide 4 (C_2AG) is present in Region I but not in Regions II and III and therefore occurs at the location 4000 from the 3' end of the 26S RNA. The presence of oligonucleotides 2 and 3 (C_3A_2G and C_6A_2UG) on these one-dimensional homochromatograms was obscured by residual cap so a definite conclusion as to their presence in any of the regions could not be made. However oligonucleotide 1

contains 49% of the total m^5C in 26S RNA but the m^5C location at which it is found (1200 nucleotides from the 3' end) contains 75% of the m^5C; similarly oligonucleotide 4 contains 13% of the m^5C but the m^5C location 4000 nucleotides from the 3' end contains 25% of the m^5C. Thus it seems likely that each location contains in addition at least one of the two unplaced oligonucleotides (2 and 3) or the unidentified oligonucleotide.

Presence of m^5C in Sindbis Virus Polysomal RNA

Since from the data presented in Table I, it is apparent that m^5C is present in 26S RNA at a concentration of less than 1 residue per 26S RNA molecule, it was of interest to determine if Sindbis virus RNA from different size classes of polysomes or from nonpolysomal ribonucleoprotein contained different amounts of m^5C. Figure 6 shows a gradient of polysomes isolated from infected cells which had been labeled from 1 to 6 hours after infection with [^{14}C] uridine and then exposed to a short pulse of [^{3}H] leucine (2 minutes) before the cells were lysed. Most of the [^{3}H] leucine labeled acid precipitable material sediments either at the top of the gradient or with polysomes, and not with the ribosomal subunits, and thus little degradation of the polysomes had occurred during the extraction procedure. Figure 6 also shows that Sindbis virus specific RNA sediments in three regions of this polysome gradient, at approximately 40S (Region I), at 70 to 80S (Region II), and with polysomes greater than 80S (Region III). When the RNA isolated from these regions was analyzed on sucrose gradients, the 40S peak was found to consist solely of 26S RNA and therefore must be 26S

Fig. 6. Sucrose gradient sedimentation of ribosomes from Sindbis-infected chick cells. A culture of chick cells infected with Sindbis virus was labelled with [^{14}C] uridine from 1 to 6 hours after infection, followed by a 2 minute pulse of [^{3}H] leucine. The cells were lysed and the ribosomes and polyribosomes separated by sucrose gradient sedimentation as described in Materials and Methods. The gradient was analyzed on an ISCO gradient fractionator equipped with a flow cell absorbance monitor. The locations of the optical density peaks corresponding to the major ribosomal species are indicated at the top of the graph. An aliquot of each fraction was analyzed for acid-precipitable [^{3}H] and [^{14}C] material. Sedimentation was from right to left. The bars indicate pools of material for subsequent analysis as in Table III. (●—●—●), [^{3}H] leucine label; (o--o--o), [^{14}C] uridine label.


4
3
2
80S
60S
40S
Overlay
^{3}H cpm (x10^{-4}) (——)
^{14}C cpm (x10^{-4}) (----)
III
II
I
10
20
30
40
Fraction Number

ribonucleoprotein. The 70S to 80S peak contained both 26S and 49S RNA in a molar ratio of 1.8 to 1.0. This peak probably contains 26S RNA from monosomes and 49S RNA from ribonucleoprotein, monosomes and from nucleocapsids (nucleocapsids are destabilized by heparin and NP40, resulting in most of the capsid protein migrating at the top of the gradient and the nucleocapsid 49S RNA migrating as an RNA-protein complex at 70 to 80S. Approximately 20% of nucleocapsids still sediment at 140S, as can be seen from the peak in the ^{3}H-leucine label at that position). The polysomal RNA consisted of 26S RNA and 49S RNA in a molar ratio of 12 to 1. A second cell lysate, labeled with [^{3}H-methyl] methionine and $^{32}PO_4$, was fractionated in the same way and RNA was isolated from these same three regions of the gradient. 26S RNA from all three regions contained similar amounts of m^5C (Table III). However, 49S RNA from polysomes contained 60 to 80% more m^5C than did 49S RNA from the 70 to 80S region of the gradient (Table III).

DISCUSSION

In this paper, we have confirmed and extended the findings of Dubin et al. (1977) that the RNA species of Sindbis virus contain internal 5-methyl cytidine residues. Although our findings on the distribution of [^{3}H] methyl label in Sindbis virus RNAs are in general agreement with those of Dubin et al. (1977), there are some differences. We find lower amounts of [^{3}H] methyl label in m^5C in virus intracellular RNA species (19% in 26S RNA, 9% in 49S RNA) than reported by

TABLE III

[^{3}H] methyl label in Sindbis virus polysomal RNAs

RNA and Region of Polysomal Gradient	Experiment 1 Percentage of [^{3}H] methyl label in: m^5C	Cap	Experiment 2 m^5C	Cap
26S				
Unfractionated[a]	24.7	75.3	27.3	72.7
40S[b]	27.3	72.7	32.4	67.6
70-80S	25.1	74.9	26.0	74.0
Polysomes	24.4	75.6	29.2	70.8
49S				
Unfractionated	9.4	90.6	13.1	86.9
70-80S	10.2	89.8	11.4	88.6
Polysomes	16.2	83.8	20.4	79.6

[a]Before the polysome gradient was run, RNA was extracted from a portion of the infected cell lysate and the [^{3}H] methyl label in 26S and 49S RNA was analyzed.

[b]40S, 70-80S and polysomes refer to material sedimenting in Regions I, II and III respectively of the polysome gradient shown in Fig. 6.

Dubin et al. (1977) (34% in 26S RNA, 39% in 49S RNA). We also find that intracellular 49S RNA contains about half as much m^5C as is found in 26S RNA from the same RNA preparation, in contrast to the finding of Dubin et al. (1977) that intracellular 49S RNA contains equal or greater amounts of m^5C than 26S RNA. These differences could possibly be due to the different strains of virus or the different cell preparations used. Similar to the results of Dubin et al. (1977), we find that Sindbis virion 49S RNA contains much less m^5C than intracellular 49S RNA isolated from the same infected cell culture. We have also established that m^5C residues are an integral part of the Sindbis virus RNA and not a cellular contaminant.

We find that m^5C occurs in as many as five different oligonucleotides in 26S RNA, four of which have been identified as having base compositions of C_1A_4UG, C_6A_2UG, C_6A_2G and C_2AG. We have also found that m^5C occurs at two locations on the 26S RNA, one approximately 4000 nucleotides (± 10%) and the other approximately 1200 nucleotides (± 10%) from the 3' end of the molecule. m^5C in the C_2AG oligonucleotide occurs at the first location and m^5C in the C_1A_4UG oligonucleotide occurs at the second location. These results indicate that there are probably five specific sites on the 26S RNA at which cytidine residues can be methylated. The C_1A_4UG and C_6A_2UG oligonucleotides occur only once per 26S RNA molecule. Although there are 12 C_2AG oligonucleotides per 26S RNA molecule, m^5C in this oligonucleotide occurs only within a restricted region of several hundred nucleotides on the 26S RNA, and it seems likely that only one specific C_2AG oligonucleotide on the 26S RNA is methylated. Similarly we assume the same is true for the C_3A_2G

and the unidentified oligonucleotide. From the distribution of m^5C among the four identified oligonucleotides and between the two locations, it seems likely that there are at least two m^5C sites at each location. Due to the small amount of m^5C in some of the oligonucleotides, it is possible that one of these m^5C containing oligonucleotides occurs at a location on the 26S other than these two major locations.

What feature of these sites is recognized by a methylating enzyme is unclear, but it could be a specific nucleotide sequence (the C_2AG and the C_3A_2G oligonucleotides being part of longer sequences). The base composition of the m^5C containing oligonucleotides which have been identified are remarkably similar, and thus possibly the sites have common nucleotide sequences recognized by such a methylating enzyme. However, there appear to be at least two cytidine methylating activities since one preparation of 26S RNA lacked m^5C at the C_3A_2G site.

It is apparent that our preparations of 26S RNA contain less than one m^5C residue per molecule. We have no indication of the distribution of m^5C residues on individual 26S RNA molecules, but a single 26S RNA molecule could contain up to five m^5C residues. Since the m^5C specific oligonucleotides contain m^5C in unequal proportions and the m^5C site 1200 nucleotides from the 3' end is twice as likely to contain m^5C as the site 4000 nucleotides from the 3' end, methylated 26S RNA molecules which contain less than five m^5C residues must exist. In calculating the percentage of 26S RNA molecules that contain m^5C, the fact that [^{3}H] methyl label in the Sindbis virus cap is present in 3 congeners of m^7G must be taken into account. Using the data of Dubin et al. (1977)

on 26S RNA extracted from chick cells 8 hours after infection that 15% of the caps contain $m_3^{2,2,7}G$, 36% contain $m_2^{2,7}G$, and 49% contain m^7G, we calculate that for preparations of 26S RNA containing 73% of [^{3}H] methyl label in the cap and 24% in m^5C that 6% of the 26S RNA molecules contain m^5C in the C_2AG oligonucleotide, 6% contain m^5C in the C_3A_2G oligonucleotide, 7% contain m^5C in the C_6A_2UG oligonucleotide, and 22% contain m^5C in the C_4A_4UG oligonucleotide. Thus 42% of the 26S RNA molecules could contain m^5C if there were only one m^5C per molecule. Only 6% of the 26S RNA molecules could contain m^5C in all four oligonucleotides. Thus, only a minority of the 26S RNA molecules contain m^5C.

Both polysomal and nonpolysomal 26S RNA contain equal amounts of m^5C. It is still likely that m^5C has a function in translation, however, since the 26S RNA in ribonucleoproteins is most likely only momentarily dissociated from polysomes and is translated at some time during infection. Our finding that 49S RNA isolated from polysomes has more m^5C than 49S RNA isolated from virions or from an RNA-protein peak derived primarily from nucleocapsids suggests that the methylation of the RNA is involved in translation. This finding also suggests that cytidine is methylated on Sindbis virus RNA after the RNA has been transcribed (possibly after the RNA has left the transcription complex), and that much of the 49S RNA is sequestered into nucleocapsids quickly after transcription where it cannot be methylated. Our data that the amount of m^5C in Sindbis virus RNA depends partly on the state of the cells imply that host cell enzymes are responsible for methylation in Sindbis virus RNA, as would be expected from the limited genome size of

the virus. Dubin et al. (1977) found that Sindbis virus intracellular RNA species isolated from infected chick and hamster cells contain roughly equal amounts of m^5C; however, low amounts of m^5C are found in the cellular RNAs of both cell species.

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Chapter 3

Biophysical Studies on Circle Formation by Sindbis Virus 49S RNA

by

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SUMMARY

Sindbis virus 49S RNA extracted from virions is completely in the form of hydrogen-bonded circles, which are separable from linear 49S RNA on sucrose gradients containing 0.01 M NaCl. The melting temperature (T_m) of the circles is 39.5°C in 0.023 M NaCl and 53.5°C in 0.1 M NaCl. The ΔH for circularization is -160 kcal/mole and the ΔS for circularization is approximately 500 eu. These parameters indicate that the length of the double-stranded region which is formed upon circularization of the molecule is most likely short, on the order of 10-20 nucleotides. Our data indicate that extensive mismatching in this double stranded region is unlikely. Intact linear 49S RNA molecules readily renature to form circles under appropriate conditions, the energy of activation for this process being 42.6 kcal/mole. From the measured rate constants for circularization it is clear that Sindbis RNA will circularize readily under physiological conditions of temperature and ionic strength. The virion RNA from Semliki Forest virus also forms circles whose T_m is very similar to that of Sindbis RNA circles, suggesting that the sequences involved in circularization have been conserved.

INTRODUCTION

The genomic RNA of Sindbis virus, the prototype alphavirus, is a single stranded RNA which is infectious (and therefore of plus polarity) (Wecker, 1959) and has a molecular weight of 4.3×10^6 daltons (14,000 bases) (Simmons and Strauss, 1972). This RNA, which sediments at 49S, has a cap of sequence ^{m7}GpppApUpGp... as its 5' terminus (Hefti et al., 1976) and a stretch of polyadenylic acid (poly A) 40 to 120 nucleotides long on its 3' end (Eaton and Faulkner, 1972). Sindbis virus 49S RNA serves as a messenger RNA during infection (Mowshowitz, 1973; Simmons and Strauss, 1974b), directing the translation of three or four virus-specific nonstructural polypeptides (Lachmi and Kääriäinen, 1977; Brzeski and Kennedy, 1977; Strauss, Lenches and Strauss, 1976; Simmons and Strauss, 1974a).

Hsu, Kung and Davidson (1973) demonstrated that Sindbis virus 49S RNA can circularize. When 49S RNA was treated with a denaturing agent (glyoxal or formamide) to abolish most secondary structure, then spread by Kleinschmidt techniques and examined under an electron microscope, up to 50% of the 49S RNA molecules appeared circular. (Hsu, Kung and Davidson, 1973). The circular 49S RNA molecules retained some secondary structure since their mean length (2.76 μm) was only 80% of the mean length of fully extended, linear 49S RNA molecules (3.45 μm). Many circular 49S RNA molecules appeared to contain a panhandle averaging 0.083 μm in length (corresponding to a nucleotide sequence of 250 ± 50 base pairs), to which stretches of poly dT could be bound. Sindbis virus 49S RNA circles could be denatured into linear RNA molecules by treatment

with 50% formamide or extended incubation with glyoxal; circles could be reformed under the appropriate renaturing conditions. These results indicate that Sindbis virus 49S RNA is held in the circular configuration by hydrogen bonded, complementary nucleotide sequences located near the 5' and 3' ends of the RNA molecule. The ability of poly dT to bind to the panhandles indicates that these panhandles are near the 3' terminal poly A stretch and that the poly A is not involved in the base pairing.

The genomic RNAs of other alphaviruses and of the bunyaviruses are also capable of forming circles. When examined by electron microscopy, a fraction of the RNA of Semliki Forest virus was found to be circular (Kennedy, 1976). Members of the bunyavirus group contain three circular, helical nucleocapsids which exhibit RNA transcriptase activity (Obijeski et al., 1976; Pettersson and von Bonsdorff, 1975; Samso, Bouloy and Hannoun, 1975). When the RNAs of two bunyaviruses, Uukuniemi virus and Lumbo virus, were extracted from their nucleocapsids and analyzed, all three species of RNA were found to be capable of circularization, apparently due to the presence of complementary nucleotide sequences near the ends of the RNA molecules (Hewlett, Pettersson and Baltimore, 1977; Samso, Bouloy and Hannoun, 1976).

The RNA isolated from four series of defective interfering particles of Sendai virus (a paramyxovirus) have been shown to be capable of circularization (Kolakofsky, 1976), due to the hydrogen bonding of terminal, complementary nucleotide sequences about 110 nucleotides in length (D. Kolakofsky, personal communication). Similarly, RNA isolated from defective interfering particles of vesicular stomatitis virus (a rhabdovirus) have also been found to be circular (A. Huang, personal

communication). It is of interest in this regard that the genomic RNAs of neither of these viruses have been shown to be circular.

The genomic DNA's of two groups of DNA-containing animal viruses are also capable of assuming a hydrogen-bonded circular configuration. When the double stranded genomic DNA of adenovirus is denatured, a fraction of the single stranded DNA molecules form circles due to the hydrogen bonding of terminal complementary nucleotide sequences which are between 100 and 140 nucleotides in length (Garon et al., 1972; Roberts, Arrand and Keller, 1974; Wolfson and Dressler, 1972). Adeno-associated virus single stranded genomic DNAs of both plus and minus polarity are capable of being circularized by terminal complementary nucleotide sequences 200 to 500 nucleotides in length (Berns and Kelly, 1974; Koczot et al., 1973).

It is known that the ring form and the linear form of the single stranded DNA of bacteriophage ØX174 differ in sedimentation coefficient by 10% to 15% when sedimentation is conducted in solutions of low ionic strength, although the two forms sediment almost identically in 0.2 M NaCl (Sinsheimer, 1959; Fiers and Sinsheimer, 1962). Similarly, configurational variants of the RNA of phage MS2 are separable when sedimentation is in low salt (Strauss and Sinsheimer, 1968). This led us to examine the sedimentation properties of the circular and linear forms of Sindbis virus 49S RNA in solutions of low salt, and we have developed sucrose gradient conditions such that the two forms are separable. Using this technique, we have studied the kinetics of denaturation and reformation of circular Sindbis virus RNA. From these kinetic measurements, we can

estimate the length of the base paired region which holds Sindbis virus RNA in a circular configuration.

MATERIALS AND METHODS

(a) Materials

[5,6-^{3}H]uridine (58 Ci/mmole) and [2-^{14}C]uridine (50 mCi/mmole) were purchased from Amersham-Searle. [^{32}P]phosphoric acid (carrier free) was obtained from ICN. Ultrapure sucrose (density gradient grade) was supplied by Schwarz-Mann and pancreatic ribonuclease (Type X-A) was supplied by Sigma. Hae III restriction endonuclease was obtained from Bethesda Biochemicals. Liquified phenol from Mallinckrodt was redistilled and stored at -20°C. Glass tubes were siliconized by placing them in a desicator with a small volume of dichloro-dimethyl silane (Aldrich), evacuating the desicator for 1 min followed by slow release of the vacuum, and then baking the tubes overnight at 60°C (K. Grohman, personal communication). Semliki Forest virus was obtained from Dr. Judith Levin.

(b) Purification of Virus RNA

Primary cultures of chicken embryo fibroblasts were maintained at 37°C in 800 cm^{2} roller bottles in Eagle's minimal essential medium (1959) containing 2% fetal calf serum (Pierce, Strauss and Strauss, 1974). At least 6 hours after adding fresh medium, confluent cell monolayers at 37°C were infected with the HR strain of Sindbis virus (Burge and Pfefferkorn, 1966) at a multiplicity of 35 plaque forming units per cell in 10 ml of low salt Eagle's medium (ionic strength = 0.105) containing 3% dialyzed fetal calf serum and 1 µg/ml actinomycin D (Pierce, Strauss

and Strauss, 1974). One hour after infection, an additional 20 ml of low salt medium containing radioactive label was added to the infected culture. [^{3}H]uridine was used at a concentration of 50 μCi/ml, [^{14}C]-uridine at 2 μCi/ml, and $^{32}PO_4$ at 100 μCi/ml (medium containing one-tenth the normal concentration of PO_4 was used for ^{32}P-labeling). Eleven hours after infection, the medium was discarded and the virus was harvested by adding small volumes of high salt Eagle's medium (ionic strength = 0.2) to the cells (Pierce, Strauss and Strauss, 1974). The virus was purified by velocity and isopycnic sedimentation and the virion RNA was extracted within 24 to 48 hours of virus harvest by the methods of Hsu, Kung and Davidson (1973). Two modifications of the published procedure were used to improve the quality of the final virus RNA preparations: phenol was added to the pelleted virus before adding SDS-containing buffer (Hsu, Kung and Davidson, 1973); and the final aqueous phase of the phenol-chloroform treatment was extracted three times with ether to remove traces of phenol. Ethanol precipitated virus RNA was redissolved in 0.1 M NaCl, 0.01 M Tris (pH 7.4), 0.001 M EDTA and stored at -80°C.

(c) Denaturation and Renaturation Determinations

For denaturation of virus RNA at lower salt concentrations, 5 μl of ^{32}P-labeled virus RNA in 0.1 M NaCl/Tris/EDTA (0.1 M NaCl, 0.01 M Tris (pH 7.4), 0.001 M EDTA) was diluted with either 100 μl or 60 μl of 0.01 M NaCl/Tris/EDTA (0.01 M NaCl, 0.01 M Tris (pH 7.4), 0.001 M EDTA) (making a final NaCl concentration of 0.014 M or 0.023 M respectively) in a small siliconized test tube. For RNA denaturation in 0.10 M NaCl, the

^{32}P virus RNA was diluted into 50 µl of 0.10 M NaCl/Tris/EDTA. The samples were denatured by placing the tubes in a Haake circulating water bath set at the appropriate temperature and holding them for 5 minutes. The tubes were then immediately plunged into an ice bath and 0.5 ml of ice cold 0.01 M NaCl/Tris/EDTA was quickly added to each sample (this cooling process usually took less than 10 sec). For RNA renaturation experiments, 5 µl of ^{32}P virus RNA in 0.1 M NaCl/Tris/EDTA was diluted to 50 µl in 0.01 M NaCl/Tris/EDTA, denatured at 45°C for 5 minutes, and cooled in an ice bath. 5 µl of 1 M NaCl was added (making a final NaCl concentration of 0.1 M) and the sample was incubated in a water bath at the appropriate temperature for the desired amount of time and then quickly cooled as described for denatured samples. After denaturation or renaturation, marker ^{3}H-labeled undenatured virus 49S RNA was added to each sample. The samples were then layered on 15 to 30% sucrose gradients made in 0.01 M NaCl/Tris/EDTA containing 0.2% SDS in an SW40 or SW41 cellulose nitrate tube. Centrifugation was for 6.5 to 7 hours at 40,000 rpm at 23°C. Gradients were fractionated by punching a hole in the bottom of the centrifuge tube and collecting drops directly into scintillation vials. To each fraction, 1 ml of water and 10 ml of Aquasol-2 (New England Nuclear) were added and the sample was counted in a Beckman liquid scintillation counter.

(d) Electron Microscopy

5 µl of virus RNA samples at a concentration of 10 µg/ml in 0.023 M NaCl, 0.01 M Tris (pH 7.4), 0.001 M EDTA were mixed with 5 µl of 1 M glyoxal, 0.01 M sodium phosphate buffer (pH 7.0) and incubated for 30 or

40 minutes at 35°C (Hsu, Kung and Davidson, 1973). The samples were then spread, stained, shadowed and visualized on an electron microscope using the methods of Davis, Simon and Davidson (1971).

(e) Isolation and Analysis of Double Stranded RNA Regions

Four samples of ^{32}P-labeled Sindbis virus 49S RNA in 50 µl of 0.1 M NaCl/Tris/EDTA were diluted with 200 µl of 0.01 M NaCl/Tris/EDTA. Two of these samples were denatured at 60°C for 5 minutes and quickly cooled on ice. To all four samples were then added 5 µg of unlabeled E. coli ribosomal RNA, 30 µl of 4 M NaCl (making a final NaCl concentration of 0.4 M), and 5 µg of pancreatic ribonuclease. Two samples (one undenatured and one denatured) were digested for 10 minutes at room temperature (22°C) and the other two samples were digested at 0°C in an ice bath for 10 minutes. The digestions were terminated by adding 15 µl of 10% SDS, 0.5 ml of phenol, and 0.5 ml of chloroform to each sample and mixing the sample briefly on a rotary shaker. 25 µg of unlabeled carrier E. coli tRNA (Boehringer) were added to each sample and each sample was then extracted once more with phenol and chloroform, and once with chloroform alone, the organic phase being removed after each extraction. RNA was precipitated from the final aqueous phase by the addition of 0.1 volume of 2 M potassium acetate (pH 5.5) and 2.5 volumes of cold ethanol and storage at -20°C for 12 hours. The precipitate was collected by centrifigation and redissolved in 5 µl of 0.0089 M NaCl, 0.0003 M EDTA, 0.0089 M boric acid (pH 8.3) containing 10% glycerol and 5% saturated bromophenol blue dye. The digests were analyzed by electrophoresis in a 15 cm x 15 cm x 0.2 cm slab gel of 7.5% acrylamide, 0.375% bisacrylamide made in

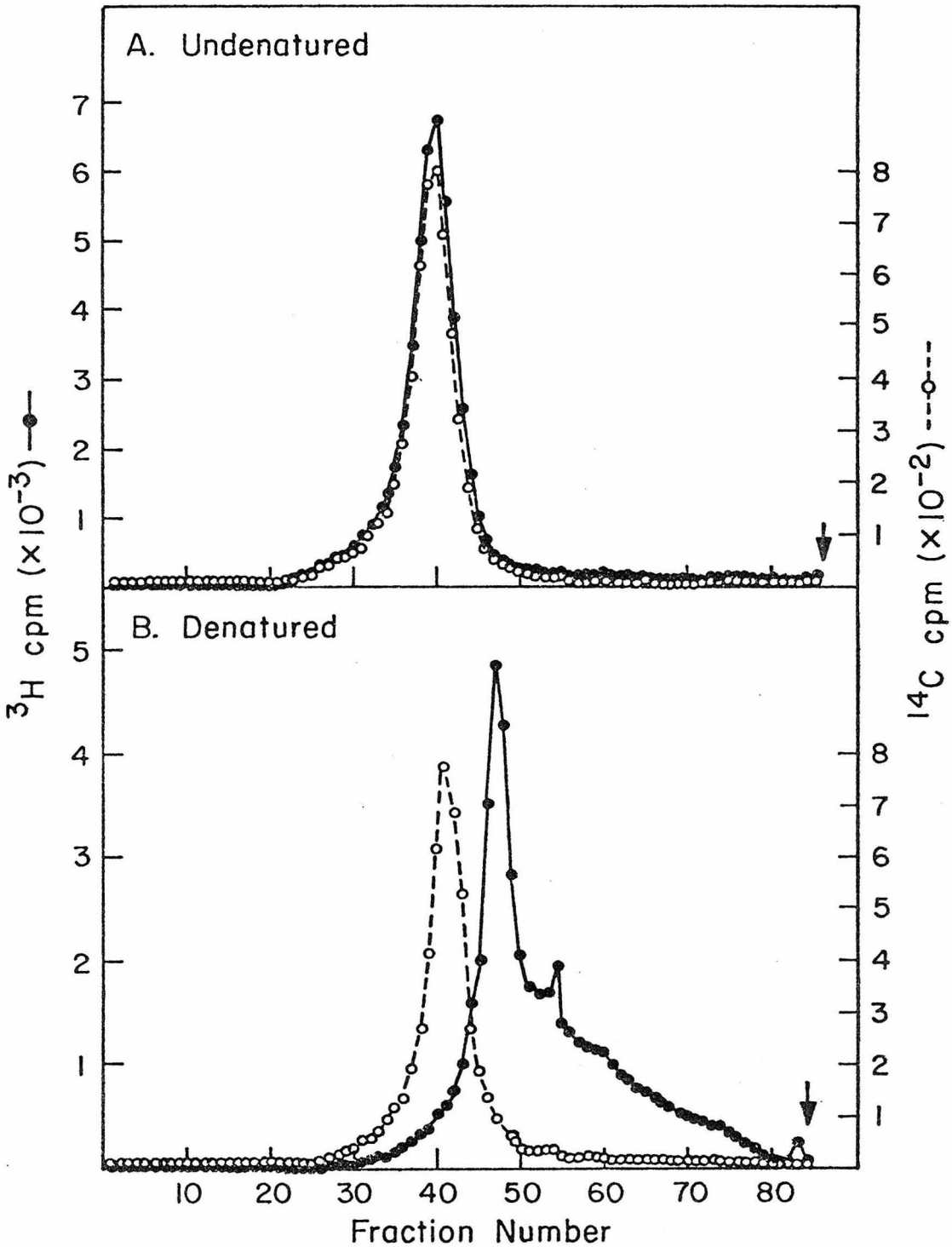
0.089 M Tris, 0.003 M EDTA, 0.089 M boric acid (pH 8.3) (Peacock and Dingman, 1967). Electrophoresis was at 10 V/cm for 3.75 hours. For molecular weight markers, a Hae III restriction enzyme digest of ØX174 RF DNA (kindly provided by Richard Parker and Brian Seed) was run in a parallel lane in the same gel. The Hae III restriction fragments were localized by ethidium bromide staining before the gel was dried and analyzed by autoradiography.

RESULTS

(a) Sucrose Gradient Sedimentation

The configurational restraint placed on a circular molecule by its ends being joined together results in the circular form of a molecule assuming a more compact configuration than the corresponding linear form of the same molecule. Thus, the circular form of an RNA molecule will sediment more rapidly than the linear form of the same RNA molecule. This difference in configuration is especially pronounced in solutions in which the structure of the RNA is extended, such as in solutions of low ionic strength. As shown in Fig. 1, when Sindbis virus 49S RNA is denatured in 0.01 M NaCl at 60°C for 3 minutes, mixed with undenatured 49S RNA and sedimented through sucrose gradients, the denatured 49S RNA does indeed migrate more slowly than undenatured 49S RNA. The trail of small molecular weight RNA fragments which sediments behind the denatured 49S RNA peak in Fig. 1B, is due to the denaturation of nicked 49S RNA molecules which were held together by the secondary structure of the 49S RNA before the molecules were denatured. Denatured and undenatured 49S

Fig. 1. Sucrose gradients of undenatured and denatured 49S RNA. Two samples of [^{3}H-uridine] labeled 49S RNA in 0.01 M NaCl, 0.01 M Tris (pH 7.4), 0.001 M EDTA were heated for 3 minutes, one at room temperature (A) and the other at 60°C (B). Unheated [^{14}C-uridine] labeled 49S RNA was added to each sample as a marker and both samples were then sedimented through sucrose gradients containing 0.01 M NaCl. The direction of sedimentation is from right to left. The end of the gradient is marked by an arrow (↓). (●—●—●), [^{3}H-uridine] labeled 49S RNA; (o--o--o), [^{14}C-uridine] labeled 49S RNA.


A. Undenatured
B. Denatured
^3H cpm ($\times 10^{-3}$)
^{14}C cpm ($\times 10^{-2}$)
Fraction Number
10
20
30
40
50
60
70
80

RNA separate by 3.5% in gradients containing 0.1 M NaCl, by 5.0% in gradients containing 0.05 M NaCl, by 11% to 13% in gradients containing 0.01 M NaCl, and by 11% in gradients containing 0.005 M NaCl. Therefore, gradients containing 0.01 M NaCl were used for all further analyses reported in this paper.

When Sindbis virus 49S RNA is heated in 0.015 M NaCl at various temperatures, the change in sedimentation rate on sucrose gradients occurs between 35°C and 41°C, as shown in Fig. 2. When 49S RNA is heated at 37°C and 39°C the presence of the two differentially sedimenting species can be observed on the same gradient (Fig. 2B and 2C). Heating to higher temperatures (temperatures to 80°C have been tested) produces no further change in the sedimentation rate. When the 49S RNA is heated in 0.023 M NaCl and in 0.1 M NaCl, the change in sedimentation rate on sucrose gradients occurs at 39.5°C and 53.5°C respectively (data presented in "Denaturation Measurements" section).

(b) Electron Microscopy

Circular structures were easy to observe when Sindbis virus 49S RNA was treated with glyoxal, spread and examined under an electron microscope by the methods of Hsu, Kung and Davidson (1973) (Fig. 3). Our methods differed slightly from those of Hsu, Kung and Davidson (1973) in that we found treatment of the RNA with 0.5 M glyoxal at pH 7.0 for 30 to 40 minutes at 35°C produced the best spreads. After 30 minutes treatment with glyoxal, between 20% and 45% of the full length molecules observed were circular and after 40 minutes glyoxal treatment, the figure was 15% to 25% circles. Under our conditions, both circular and linear 49S RNA molecules exhibited secondary structure in the form of knobs and panhandles (Fig. 3).

Fig. 2. Sucrose gradients of 49S RNA heated to various temperatures. [^{32}P] labeled samples of 49S RNA in 0.015 M NaCl, 0.01 M Tris (pH 7.4), 0.001 M EDTA were heated for 5 minutes at A) 35°C, B) 37°C, C) 39°C, D) 41°C and then analyzed on sucrose gradients. Unheated [^{3}H-uridine] labeled 49S RNA was included in each gradient as a marker; its position on the gradient is shown by the arrow (↓) and vertical dashed line. The direction of sedimentation is from right to left.

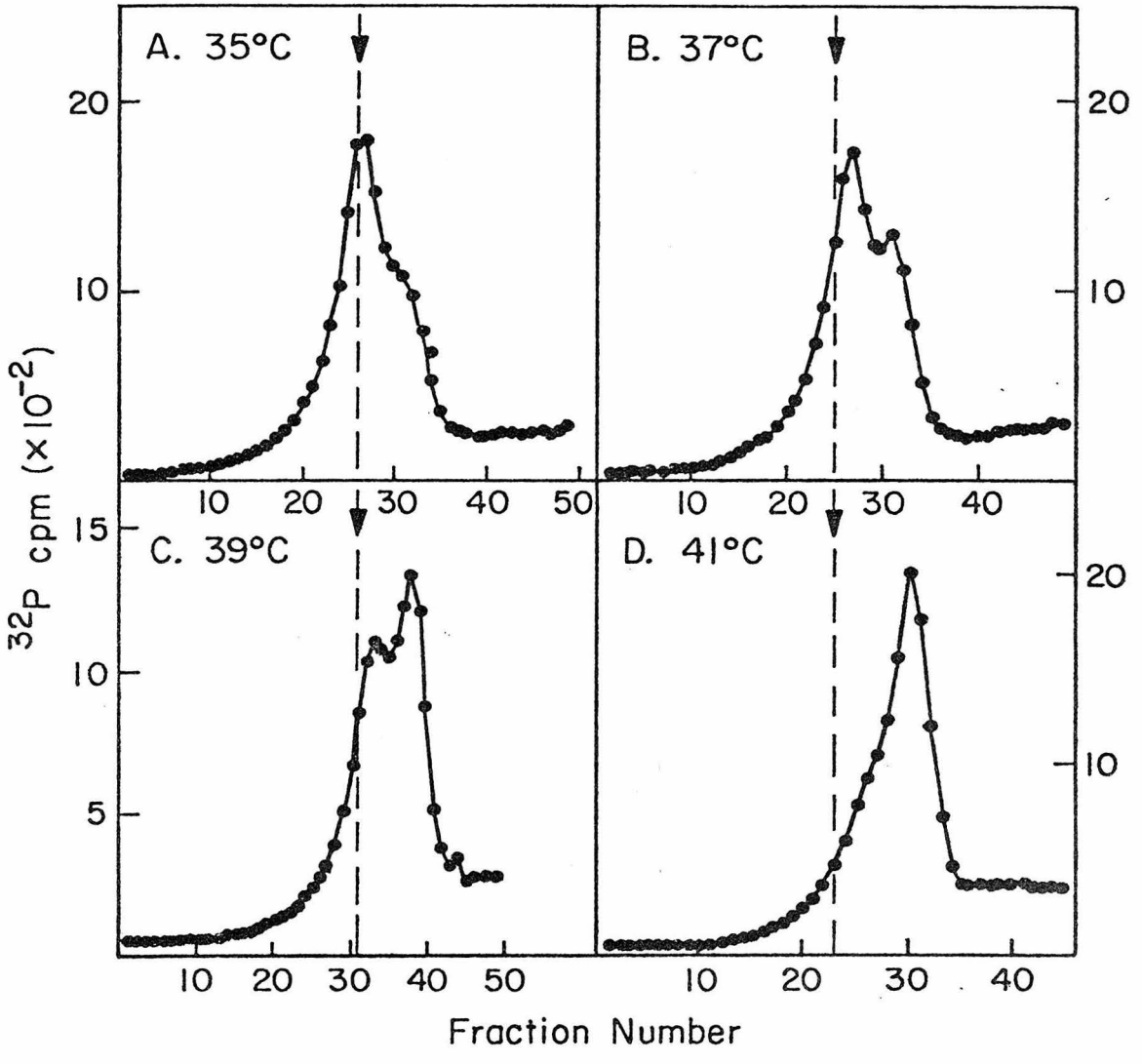
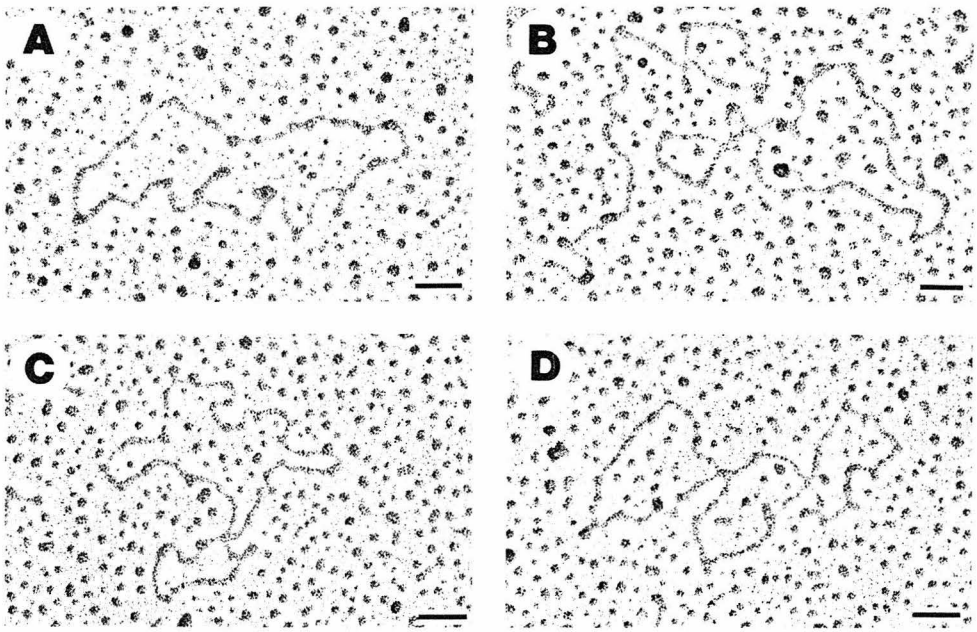

A. 35°C
B. 37°C
C. 39°C
D. 41°C
^{32}P cpm (×10^{-2})
Fraction Number

Fig. 3. Electron micrographs of 49S RNA. Sindbis virus 49S RNA was treated with 0.5 M glyoxal in 0.01 M sodium phosphate (pH 7.0) for either 30 minutes (panel A) or 40 minutes (panels B, C and D) at 35°C before spreading. The scale bar is 100 nm.


A
B
C
D

The mean lengths of circular 49S RNA molecules were 2.12 μm (± 0.18 μm) and 2.26 μm (± 0.24 μm) after 30 and 40 minutes of glyoxal treatment, respectively. These lengths are 77% and 82% of the mean length of circular 49S RNA molecules reported in Hsu, Kung and Davidson (1973) (2.76 μm) indicating that RNA molecules exposed to our spreading conditions were not as fully denatured. We could not study the presence of panhandles on circular RNA, since with the secondary structure still present on molecules spread by our conditions, most circular RNA molecules contained more than one panhandle-like region (Fig. 3).

To demonstrate that the two differentially sedimenting species of Sindbis 49S RNA observed on sucrose gradients are the circular and linear forms of the 49S RNA, the temperature at which the change in sedimentation rate of the 49S RNA is observed was compared to the temperature at which circular 49S RNA molecules could no longer be observed by electron microscopy. When 49S RNA was heated to 35°C in 0.023 M NaCl for 5 minutes it contained 28% circular forms when examined by electron microscopy, while a similar sample heated to 45°C contains no circular forms (Table I). Thus, at this salt concentration, the 49S RNA circular form melts at approximately the same temperature at which the change in sedimentation rate of 49S RNA on sucrose gradients occurs. In a second experiment, samples of 49S RNA in 0.023 M NaCl were heated at 35°C, 39°C and 43°C for 5 minutes. A portion of each of these RNA samples was analyzed on a sucrose gradient and the remainder of the sample was examined by electron microscopy. As shown in Table I, as a sample of 49S RNA is heated at higher temperatures, the decrease in the percentage of circular forms observed by electron microscopy correlates well with the conversion of

TABLE I

Circular 49S RNA sediments more rapidly than linear 49S RNA

Temperature of Denaturation[a]	% Faster Sedimenting RNA[b]	Circles in EM[c] 30 Min Glyoxal % Circles	30 Min Glyoxal Molecules Counted[d]	40 Min Glyoxal % Circles	40 Min Glyoxal Molecules Counted
Experiment 1					
35°C	--	28.0	50	--	--
45°C	--	0.0	75	--	--
Experiment 2					
35°C	100	20.6	107	15.0	100
39°C	76	13.0	100	6.0	251
43°C	3	1.7	181	0.5	215

Table I (Continued)

[a]49S RNA in 0.023 M NaCl, 0.01 M Tris (pH 7.4), 0.001 M EDTA was heated at the indicated temperature for 5 minutes.

[b]Where indicated, a sucrose gradient was run on a portion of the denatured 49S RNA sample and the percentage of the more rapidly sedimenting species present on the gradient was calculated.

[c]A portion of the denatured 49S RNA sample was analyzed for the presence of circular and linear forms by electron microscopy.

[d]Only full length molecules were counted.

the more rapidly sedimenting form to the more slowly sedimenting form of 49S RNA in sucrose gradients run on the same sample. The fact that less than half of the 49S RNA molecules are circular when examined by electron microscopy is due to the partially denaturating conditions which must be employed during preparation of an RNA sample for electron microscopy in order to extend the molecule on the grid; otherwise a tangled mass is observed. We conclude that the two differentially sedimenting species of Sindbis virus 49S RNA observed on sucrose gradients are the circular and linear forms of the 49S RNA. We also note that the change in sedimentation rate occurs at 53.5°C in 0.1 M NaCl (see below) and this correlates well with the findings of Hsu, Kung and Davidson (1973) that circular forms of 49S RNA cannot be observed by electron microscopy when the RNA is spread with formamide concentrations of 50% or greater in 0.1 M Tris at room temperature. Formamide lowers the melting temperature of double stranded nucleic acid by 0.6°C per percent formamide (Bluthmann et al., 1973; Tubbetts et al., 1973) and thus the data of Hsu et al. (1973) predict a melting temperature of approximately 55°C in 0.1 M Tris.

From the appearance of the undenatured peaks of Sindbis virus 49S RNA on the sucrose gradients shown in Fig. 1, it is apparent that most of the 49S RNA as isolated under our conditions is in a circular configuration. However, in the case of some undenatured samples of 49S RNA, a distinct shoulder of linear forms can be observed (Fig. 2A). This shoulder becomes more pronounced with repeated usage of the RNA sample and therefore is probably due to the denaturation or nuclease nicking of some of the circular forms in the sample. A nicked circular form should be held

together by its cohesive ends and thus will sediment as a linear form instead of as two fragments.

As shown in Fig. 2B and 2C, when circular 49S RNA is heated to temperatures approaching the melting point of the circular structure and examined on sucrose gradients, not only is the peak of linear 49S RNA present, but the peak of the remaining circular RNA sediments slightly more slowly (two fractions) than the undenatured control 49S RNA. This is probably due to the fact that at temperatures approaching the melting temperature of the circular form of the 49S RNA, the structure of the circular form becomes extended because of denaturation of other intramolecular hydrogen bonding and therefore its rate of sedimentation decreases.

(c) Denaturation Measurements

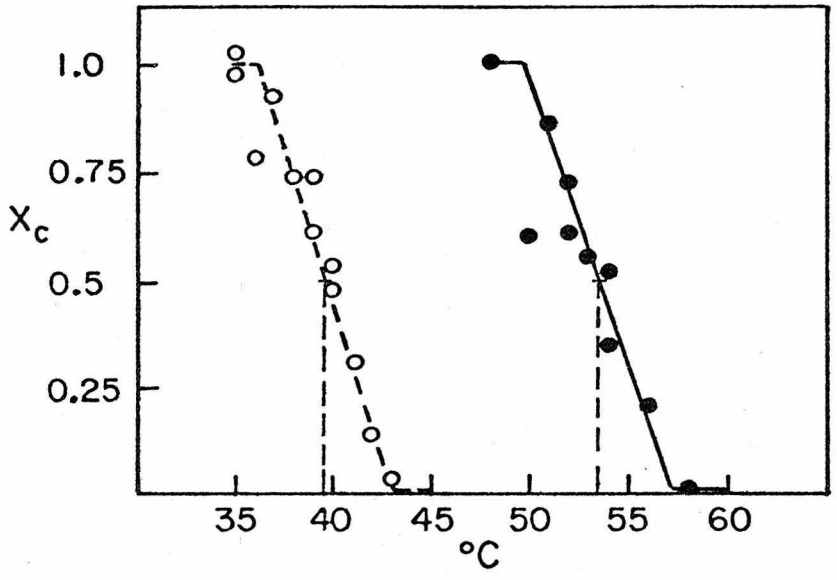
The percentage of circular and linear forms of 49S RNA present in samples of RNA heated to various temperatures was calculated by simply drawing a vertical line on the graph of the sucrose gradient data which appeared to separate the circular and linear forms of RNA. The material present on either side of the vertical line was then summed. The undenatured 49S RNA standard which was included in every gradient often contained a shoulder of linear RNA (see Fig. 2A and the discussion in an earlier paragraph) which was useful in positioning this vertical line. The corrected mole fraction of circular forms (X_c) remaining in the sample was calculated by using the formula

$$X_c = \frac{C_t - C_o}{C_\infty - C_o} \qquad (1)$$

where C_∞ is the percentage of circular forms in the unheated 49S RNA preparation, C_o is the percentage of circular forms in the same preparation after heating to temperatures well above the T_m, and C_t is the percentage of circular forms in the sample heated to temperature t. Use of this formula corrects for the fact that the circular and linear forms of 49S RNA overlap on sucrose gradients, that some of the preparations of 49S RNA were not completely circular initially, and that when samples of 49S RNA in 0.1 M NaCl are heated to temperatures 5 to 10° above the T_m and analyzed on sucrose gradients, approximately 25% of the RNA is circular. Since, as will be shown, the rate of renaturation of the 49S RNA circular form is very rapid at 50°C in 0.1 M NaCl, this background is probably due to the reformation of circular forms while the sample is being cooled.

The denaturation of Sindbis virus 49S RNA circular molecules into linear molecules as a function of temperature at two different salt concentrations is displayed as a melting curve in Figure 4. The temperature at which the mole fraction of circular forms in 49S RNA (X_c) is 0.5 defines the "melting temperature" or T_m. The T_m of the circular form of Sindbis virus 49S RNA is 39.5°C in 0.023 M NaCl and 53.5°C in 0.10 M NaCl. An indication of the size of the double stranded region which holds the 49S RNA in a circular configuration can be obtained by comparing the T_m of the 49S RNA circular form to the T_m of long double stranded RNA. The T_m of the double stranded RNA of bacteriophage MS2, which has a GC content of 52% (Billeter et al., 1966), is 87°C in 0.015 M NaCl and 103°C in 0.15 M NaCl (Billeter et al., 1966). Using the fact that the change in T_m is proportional to the change in the logarithm of the salt concentration, we estimate the T_m of MS2 double stranded RNA to be 88°C in 0.023 M NaCl and

Fig. 4. Melting curves of the circular form of 49S RNA. The mole fraction of circles (X_c) present in sucrose gradients run on samples of 49S RNA which had been heated at various temperatures either in 0.023 M NaCl, 0.01 M Tris (pH 7.4), 0.001 M EDTA (o--o--o) or in 0.10 M NaCl, 0.01 M Tris (pH 7.4), 0.001 M EDTA (●—●—●) was calculated as described in the text. The vertical dashed lines show the T_m's of the circular form at the two NaCl concentrations.

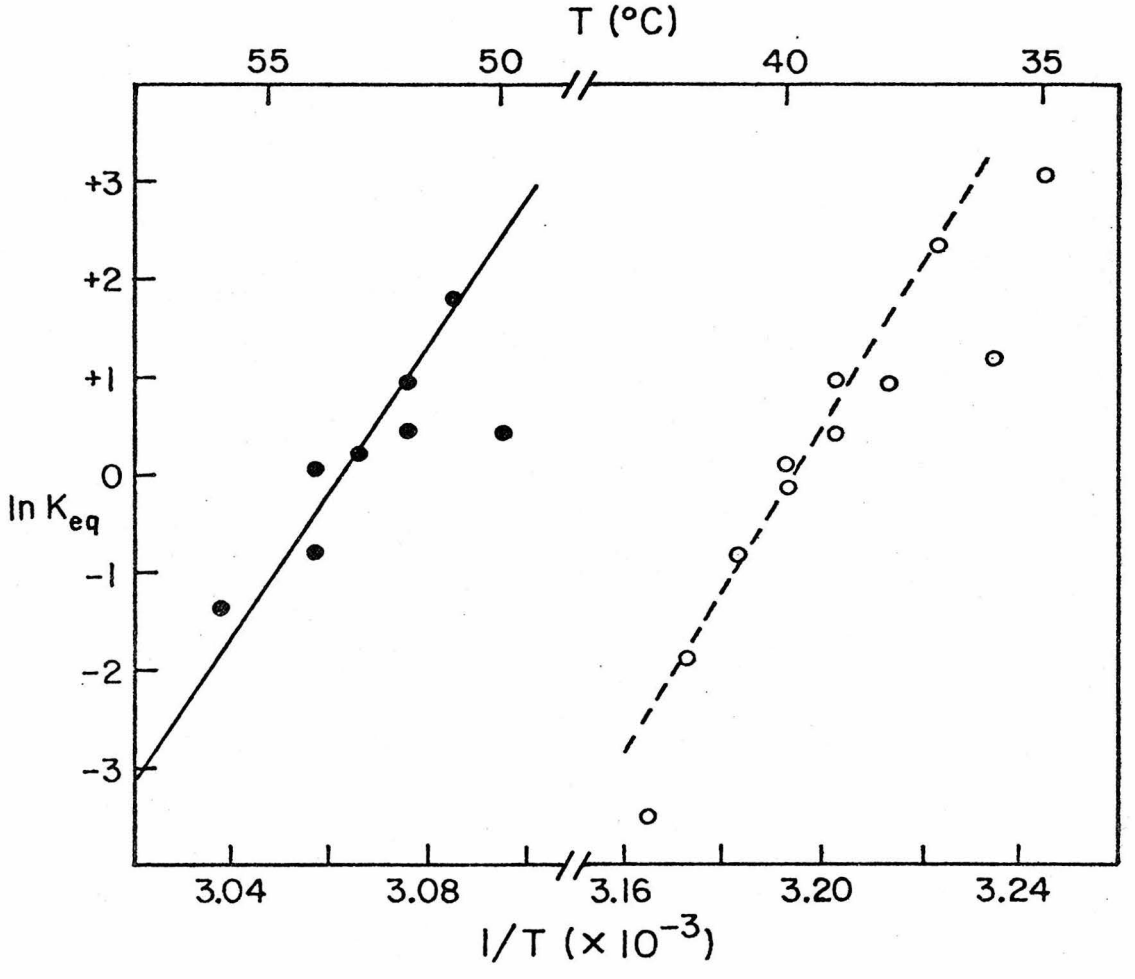

1.0
0.75
X_c
0.5
0.25
35
40
45
50
55
60
°C

99°C in 0.100 M NaCl. Thus the T_m of Sindbis virus 49S RNA circular form is approximately 46°C to 48°C below the T_m of long double stranded RNA of 50% GC content. This indicates that the double stranded region which holds the 49S RNA molecule in a circular configuration is either very short, heavily base mismatched, or destabilized by the large mass of RNA it holds together. In the Discussion section, we will show that kinetic data favor the idea that this double-stranded region is very short and not greatly mismatched.

The length of a short stretch of double stranded nucleic acid can be calculated by the depression in its T_m compared to the T_m of an infinitely long nucleic acid of similar nucleotide composition. T_m depression factors of between 470/n (Burd et al., 1975) and 600/n (Britten, Graham and Neufeld, 1974), where n is the nucleotide length, have been derived. Using these factors and the T_m depression of 46°C to 48°C, we can estimate that the length of a short, perfectly base paired, double stranded region of 50% GC which would hold Sindbis virus RNA in a circular configuration would be between 10 and 13 nucleotides.

In establishing the T_m of the circular form of Sindbis virus 49S RNA in 0.1 M NaCl (Fig. 4), 49S RNA samples were held at room temperature between 48°C and 58°C for 5 minutes. Since, as will be shown in the next section, the renaturation of 49S RNA linear forms into circular forms in 0.1 M NaCl at these temperatures is complete in one minute or less, in these samples of 49S RNA a state of equilibrium between circular and linear forms has been reached. In Figure 5 the natural logarithm of the equilibrium constant, $K_{eq} = X_c/X_L$, where X_L is the mole fraction

Fig. 5. Natural logarithm of the equilibrium constant (K_{eq}) of the 49S RNA circular form as a function of the inverse of the temperature. The equilibrium constant ($K_{eq} = X_c/X_L$, where X_c the mole fraction of circles, is calculated as described in the text and X_L, the mole fraction of linears, is given by $1 - X_c$) was calculated for samples of 49S RNA which had been heated to various temperatures, either in 0.023 M NaCl, 0.01 M Tris (pH 7.4), 0.001 M EDTA (o--o--o); or in 0.10 M NaCl, 0.01 M Tris (pH 7.4), 0.001 M EDTA, (●—●—●).


T (°C)
55
50
40
35
+3
+2
+1
0
-1
-2
-3
ln K_{eq}
3.04
3.08
3.16
3.20
3.24
1/T (× 10^{-3})

of linear forms in a 49S RNA sample and X_c the fraction of circles, is plotted as a function of the inverse of the temperature for these samples heated in 0.1 M NaCl (solid line). From the slope of this plot, the changes in enthalpy and entropy (ΔH and ΔS) for the circularization of Sindbis virus 49S RNA under these conditions can be calculated from the classical equations

$$\frac{d \ln K}{d(1/T)} = -\Delta H/R \qquad (2)$$

$$\Delta S = R \ln K + \Delta H/T = \Delta H/T_m \qquad (3)$$

ΔH is calculated to be -155 kcal/mole ($\pm$ 25 kcal/mole) in 0.1 M NaCl and ΔS is -475 eu ($\pm$ 80 eu).

We have also plotted the data for the samples heated in 0.023 M NaCl (Fig. 5, dashed line). We have no renaturation data under these conditions to assess whether equilibrium has been reached, but it appears from the fact that the slope of this line is nearly identical to that for the data in 0.1 M NaCl that the sample is very close to equilibrium. Assuming a state of equilibrium has been reached, ΔH for denaturation in 0.023 M NaCl is -165 kcal/mole ($\pm$ 10 kcal/mole) and the entropy change is -527 eu ($\pm$ 40 eu).

By assuming that the ΔH for circularization of Sindbis virus 49S RNA is due solely to the process of hydrogen bonding, the number of base pairs involved in the circularization of the molecule can be calculated. The ΔH for the formation of an adenosine-uridine base pair has been calculated to be -8.2 kcal/mole (Krakauer and Sturtevant, 1968)

and the ΔH for the formation of a guanosine-cytosine base pair has been calculated to be 3 to 4 kcal/mole higher (Uhlenbeck, Martin and Doty, 1971). Using these figures, the circularization of Sindbis virus 49S RNA is calculated to be due to the hydrogen bonding of from 11 to 20 base pairs, depending upon the GC content. This is in good agreement with the estimate of 9 to 12 nucleotides for the length of this double stranded region derived using the T_m of the circular structure (calculated for 50% GC).

(d) Renaturation Measurements

We have examined the renaturation of Sindbis virus 49S RNA linear forms into circular forms. Shown in Figure 6 are sucrose gradients of samples of 49S RNA which had been heated above the T_m in 0.01 M NaCl, made 0.1 M in NaCl, and allowed to renature for various times at 45°C. As seen in Figure 6, samples renatured for increasing periods of times contain a higher percentage of circular forms, renaturation being complete within 10 minutes under these conditions. Since the completely renatured 49S RNA sample in Figure 6E sediments identically to the undenatured marker 49S RNA, all intact Sindbis virus 49S RNA molecules are capable of reforming circles.

From such data, the percentage of circular forms in these samples was calculated as described above. In Figure 7 is plotted the function $\ln[(C_\infty - C_o)/(C_\infty - C_t)]$, where C_∞ is the fraction of circular forms in completely renatured sample of 49S RNA, C_o is the fraction of circular forms in a sample of denatured 49S RNA, and C_t is the percentage of circular forms in a sample of 49S RNA renatured for time t, for samples

Fig. 6. Sucrose gradients of denatured 49S RNA allowed to renature at 45°C. Samples of [^{32}P] labeled 49S RNA in 0.01 M NaCl, 0.01 M Tris (pH 7.4), 0.001 M EDTA were denatured at 45°C, made 0.1 M in NaCl, and then allowed to renature at 45°C for A) 0 minutes, B) 1 minute, C) 2 minutes, D) 3 minutes, E) 5 minutes, or F) 10 minutes before being examined on sucrose gradients. [^{3}H-uridine] labeled undenatured 49S RNA was included in each gradient as a marker; its position is shown by the arrow (↓) and vertical dashed line. Direction of sedimentation is from right to left.

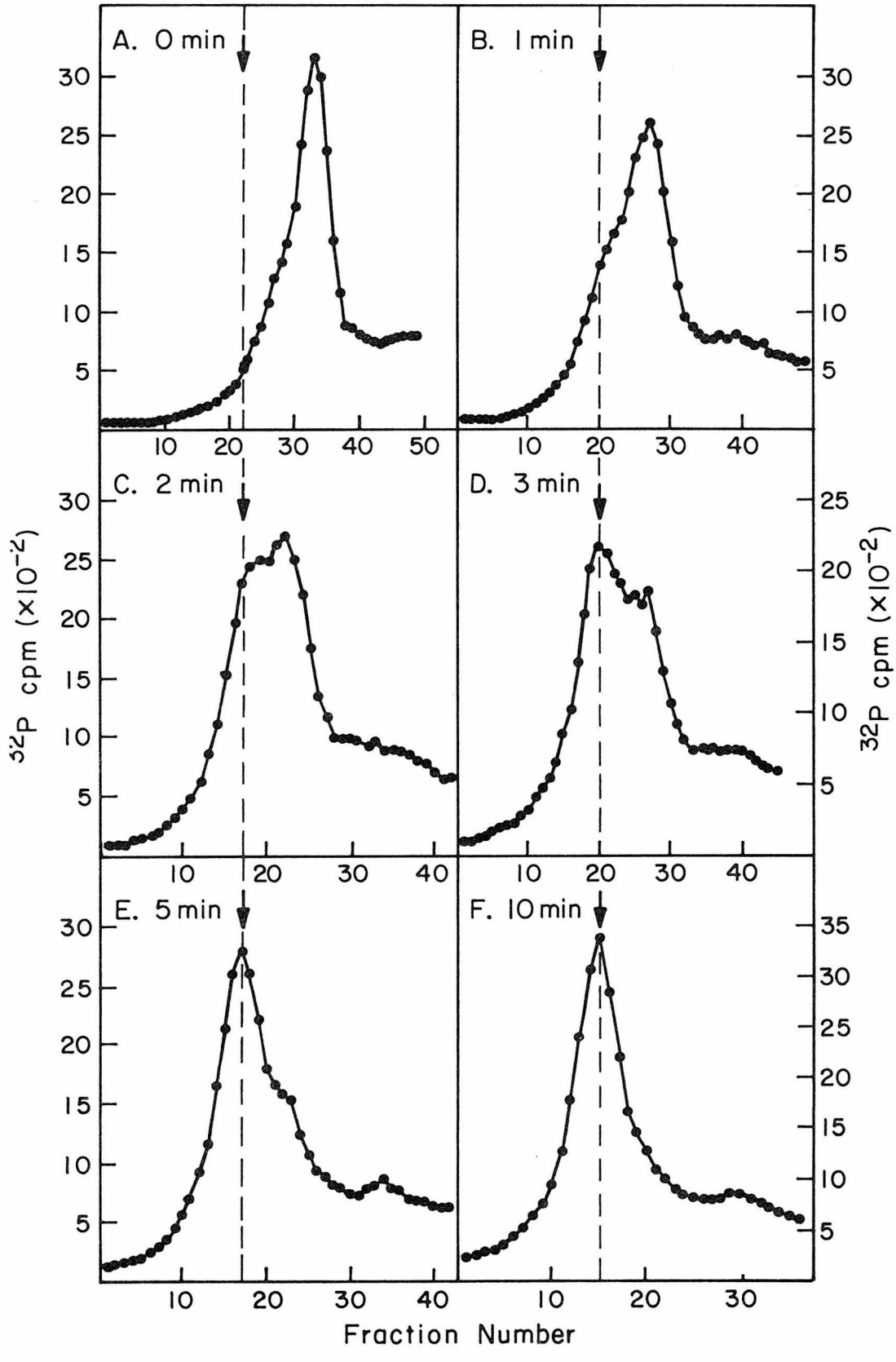
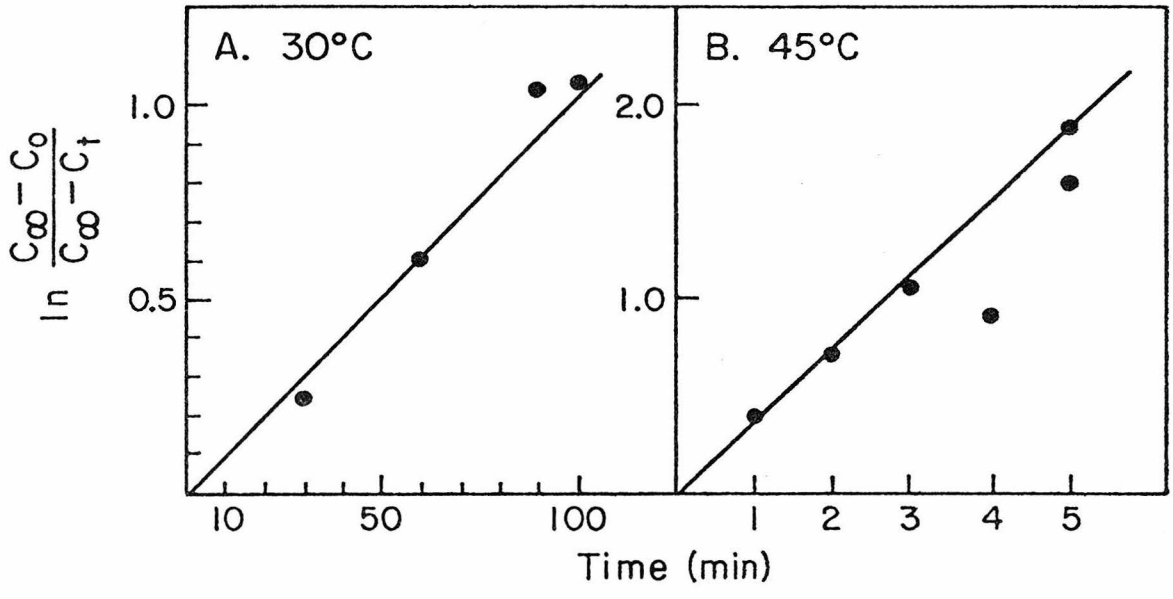

A. 0 min
B. 1 min
C. 2 min
D. 3 min
E. 5 min
F. 10 min
^{32}P cpm (×10^{-2})
Fraction Number

Fig. 7. Course of renaturation of 49S RNA linear form into circular form. Denatured 49S RNA was allowed to renature in 0.1 M NaCl, 0.01 M Tris (pH 7.4), 0.001 M EDTA for various periods of time at either A) 30°C or B) 45°C and the amounts of circular and linear RNA was assayed on sucrose gradients. The natural logarithm of the functions $(C_\infty - C_o)/(C_\infty - C_t)$, where C is the fraction of circles at 0 time, infinite time, or time t, has been plotted as a function of the time of renaturation.

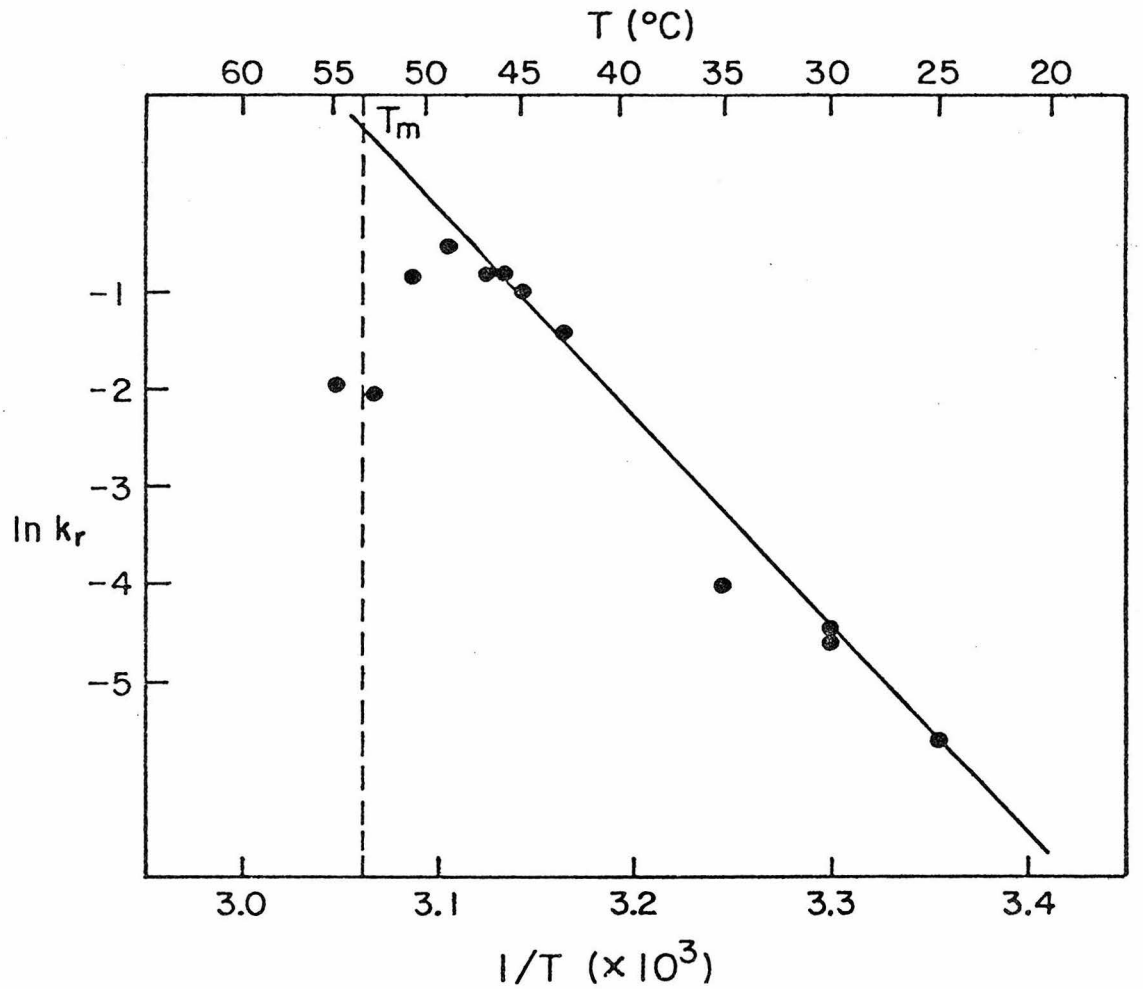

A. 30°C
B. 45°C
$\ln \frac{C_\infty - C_0}{C_\infty - C_t}$
1.0
0.5
2.0
1.0
10
50
100
1
2
3
4
5
Time (min)

of 49S RNA renatured in 0.1 M NaCl at 30°C or 45°C for various periods of time. Straight lines were obtained showing that, as expected, the reformation of 49S RNA circular forms follows first order kinetics. The slopes of the line in Figure 7 are the renaturation rate constants (k_r) under these conditions. Renaturation rate constants at several other temperatures in 0.1 M NaCl were determined from single renaturation measurements at each temperature in which between 30% and 70% of the circles had been reformed, and an Arrhenius plot ($\ln k_r$ vs. $1/T$) is shown in Figure 8; from this, the rate of renaturation under these conditions can be determined at any temperature. For example, reformation of circles in 0.1 M NaCl would be 50% complete in approximately 40 days at 0°C, in approximately 60 minutes at 30°C, in about 6 minutes at 40°C, and in about 0.7 minutes at 50°C. As shown in Figure 8, the optimum temperature for reformation of 49S RNA circles in 0.1 M NaCl is approximately 49°C, 5°C below the T_m. At temperatures above 49°C and below the T_m, the rate of renaturation decreases (Fig. 8) because the rate of denaturation becomes significant resulting in the samples reaching equilibrium during the experiment; thus true renaturation rate constants were not measured at these temperatures.

The rate of renaturation of the linear form to the circular form of Sindbis virus 49S RNA is highly dependent on temperature. From the slope of the Arrhenius plot in Figure 8 and the classical equation

$$\frac{d(\ln k)}{d(1/T)} = \frac{-E_a}{R} \qquad (4)$$

Fig. 8. Arrhenius plot of the rate constant for renaturation of 49S RNA circular form in 0.1 M NaCl. The rate constant for renaturation of 49S RNA, defined by the function $C/C_o = \exp(-k_r t)$, where C is the concentration of linear at 0 time or at time t, in 0.1 M NaCl, 0.01 M Tris (pH 7.4), 0.001 M EDTA, was determined at several different temperatures and its natural logarithm is plotted as a function of the inverse of the temperature of renaturation. The dashed vertical line is the T_m of the 49S RNA circular form in 0.1 M NaCl (53.5°C).


T (°C)
60
55
50
45
40
35
30
25
20
T_m
-1
-2
-3
ln k_r
-4
-5
3.0
3.1
3.2
3.3
3.4
1/T (x 10^3)

where R is the gas constant, the energy of activation (E_a) for the circularization of 49S RNA is 42.6 kcal/mole (± 3.0 kcal/mole), a high value. Expressed in a different way, the Q_{10} for the circularization reaction is 9. In this regard we might note that the optimum temperature for renaturation of denatured double stranded nucleic acids is 25°C to 30° below the T_m (Britten, Graham and Neufeld, 1974), as contrasted to 5° below the T_m for 49S RNA circle formation.

(e) Attempts to Isolate the Double Stranded Region of 49S RNA Responsible for Circularization

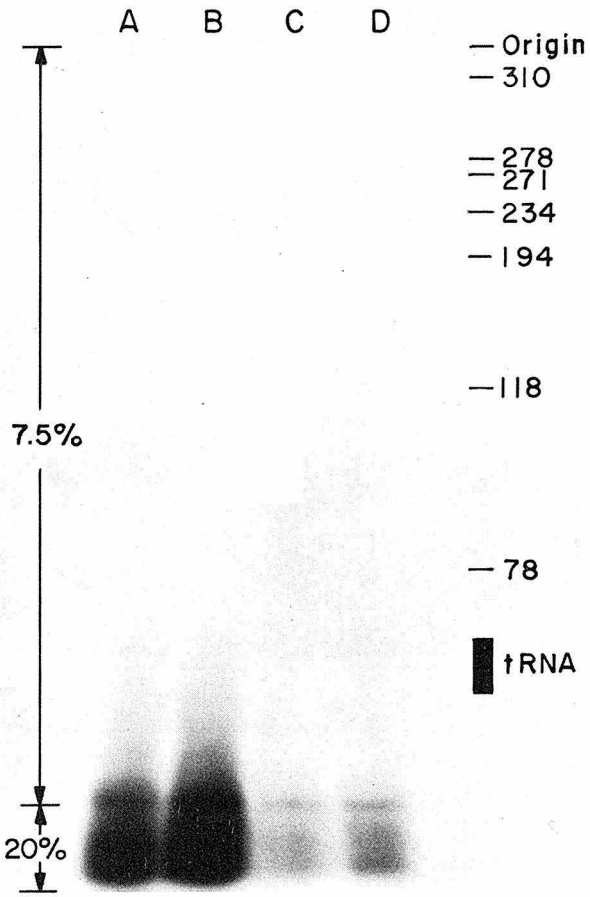
In order to determine the length of the base paired region which holds Sindbis virus 49S RNA in a circular configuration, we have attempted to isolate this double stranded region by digestion with RNase. Samples of ^{32}P-labeled 49S RNA, either undenatured or denatured to form linear molecules, were digested in 0.4 M NaCl with pancreatic RNase at an enzyme to substrate ratio of one for 10 minutes at either room temperature (23°C) or at 0°C. The more stringent conditions of digestion (23°C) have been used by D. Kolakofsky (personal communication) to isolate the double stranded regions of approximately 100 nucleotides which are responsible for circle formation by the RNAs of defective interfering particles of Sendai virus. After phenol extraction, the digests were analyzed by electrophoresis in 7.5% polyacrylamide slab gels using Hae III restriction fragments of ØX174 DNA as molecular weight markers. If the double stranded species of RNA responsible for circularization of the 49S RNA molecule resist nuclease digestion under these conditions, they will be present in the digest of undenatured 49S RNA

but absent in the digest of denatured 49S RNA. As shown in Figure 9, in digests of 49S RNA done at either 23°C or 0°C, no species of double stranded RNA greater than approximately 50 nucleotides in length could be detected. Enough labeled 49S RNA was added to each digest to yield 5000 cpm in a band of double stranded RNA 50 nucleotides in length, an amount of label which should be clearly visible after the 5 days autoradiography used in the exposure presented in Figure 9. The streaking observed in all channels is probably due to the 49S RNA poly A, which migrates as a broad band centered near the carrier tRNA. In material on the gel less than 50 nucleotides in length, no bands were present in the undenatured samples which were absent from the denatured samples. However, since the bands in this region of the gel are faint and not well resolved, no conclusion can be drawn as to whether the double stranded species responsible for circularization migrates in this region of the gel. The fact that we could find no double stranded RNA region greater than 50 nucleotides in length in Sindbis virus 49S RNA is consistent with our other results indicating that this region is either very short or very mismatched.

(f) Circularization of Semliki Forest Virus RNA

Kennedy (1976) reported that the genomic 49S RNA of another alphavirus, Semliki Forest virus, is capable of assuming a circular configuration. When we examined Semliki Forest virus 49S RNA under the electron microscope, using conditions identical to those we used for Sindbis virus 49S RNA, approximately 10% of the full length RNA molecules observed were circular, confirming the findings of Kennedy (1976). Similar to

Fig. 9. Gel to examine double stranded regions in 49S RNA. A pancreatic ribonuclease digest of ^{32}P-labeled 49S RNA was examined in a 7.5% acrylamide gel as described in the text. Lane A, denatured 49S RNA digested at 0°C; Lane B, undenatured 49S RNA digested at 0°C; Lane C, denatured 49S RNA digested at 22°C; Lane D, undenatured 49S digested at 22°C. The boundaries of the 7.5% acryamide gel and the 20% support gel are shown on the left margin. On the right margin are shown the positions of Hae III restriction fragments of ØX174 double stranded DNA of indicated lengths (in base pairs) which were run in a parallel lane. The position of the tRNA added as a carrier to all four samples is also noted.


A
B
C
D
Origin
310
278
271
234
194
118
78
tRNA
7.5%
20%

our findings with Sindbis virus 49S RNA, Semliki Forest virus 49S RNA which was heated to temperatures greater than 40°C in 0.023 M NaCl migrates 10% more slowly on sucrose gradients than does undenatured Semliki Forest virus 49S RNA (data not shown). As is the case for Sindbis RNA, this transition occurs between 35° and 40°C. The similarity in T_m for conversion of circles to linears in the two RNAs indicates that the double stranded regions responsible for circularization are similar with respect to base sequence, length, and degree of mismatching. Possibly this double stranded region has been conserved in the two viruses.

DISCUSSION

Sindbis virus 49S RNA as isolated from the virion with our extraction procedures is almost completely in the circular form. This 49S RNA within the virion is circular, since from our study of renaturation kinetics it would take months for linear RNA to circularize under our conditions of RNA extraction and storage. The $S_{20,w}$ coefficient of the alphavirus virion RNA has been reported to be variously 49S, 45S and 42S (Simmons and Strauss, 1972; Levin and Friedman, 1971; Friedman, Levy and Carter, 1966; Sonnabend, Martin and Mecs, 1967). This range of values for the sedimentation coefficient of the RNA could be due in part to the differences in sedimentation of circular and linear forms. However, variability in the $S_{20,w}$ coefficient of this RNA species is

also probably due in part to the ionic strength of the solution through which the RNA is being sedimented. In our hands, Sindbis virion RNA sediments at 49S in solutions containing 0.2 M NaCl and 0.06 M NaCl (Strauss and Strauss, 1977; Simmons and Strauss, 1972) but sediments at 42S to 43S in solutions containing 0.01 M NaCl (Hsu, Kung and Davidson, 1973; T. Frey, unpublished observations).

The 42S and 38S species of alphavirus RNA detected in infected cells (Levin and Friedman, 1971) which have been shown to be conformational variants of each other (Kennedy, 1976), are likely to be linear and circular forms of the alphavirion RNA. The assignment of a sedimentation coefficient to the "38S" RNA was based on its migration in acrylamide gels relative to markers of known S value (Levin and Friedman, 1971). Although molecules migrating more rapidly in gels usually have smaller sedimentation coefficients, in the case of configurational variants having the same molecular weight, the assignment of an S-value to a species from its rate of migration in gels leads to confusion. A circular molecule will migrate more rapidly in a gel than the linear form of the same molecule, but in the centrifuge the circular form also sediments more rapidly. Therefore, "38S" RNA must be the circular form (and its actual sedimentation coefficient will be greater than that of "42S" RNA); indeed, Kennedy (1976) reported that he found 8 times as many circles in the electron microscope from "38S" RNA than from "42S" RNA.

The T_m of the circular form of 49S RNA is 46° to 48°C below the T_m of long, double-stranded RNA of 50% GC. Thus the base-paired regions which hold the RNA in a circle are either short or heavily mismatched.

If the T_m depression were due entirely to unpaired bases in a long, otherwise complementary sequence, then a 40 to 80% mismatch would be required, since it has been shown experimentally that each 1% mismatch lowers the T_m of double-stranded nucleic acids by 0.6 to 1.2°C (Gralla and Crothers, 1973; Dodgson and Wells, 1977). It seems very unlikely that this degree of mismatch exists. Lomant and Fresco (1975) observed that the change in T_m with change in sodium ion concentration of the buffer, ($\Delta T_m/\Delta \log[Na^+]$), was directly proportional to the square of the percent mismatching in the double-stranded nucleic acid under study, and reported in this ratio was 20.0° for poly A:poly U. Billeter et al. (1966) had found that the T_m of the double-stranded form of MS2 RNA increased by 18.5°C when the sodium concentration was increased 10-fold. For Sindbis circles, this ratio is 19.3°C, which is very close to that found for perfectly base-paired RNAs. For molecules containing 40% mismatch, this ratio would be 8.5°C higher (Lomant and Fresco, 1975) and it is thus likely that little or no base mismatch exists.

Therefore, we favor the model that the regions responsible for circularization of 49S RNA are short (~10-20 nucleotides) and perfectly base-paired. The length estimate from the T_m depression is approximately 10-12 nucleotides if these regions are 50% GC. The length of the double-stranded region could be as short as 6 to 7 nucleotides if it were 100% GC, and as long as 30 to 40 nucleotides if it were 100% AU. (It is a pecularity of poly A:poly U that it melts at only 56° in 0.1 M salt, while poly-r(AU):poly-r(AU) melts at 66°C (Riley, Maling, and Chamberlin, 1966).) Thus long stretches of poly A in one strand and poly U in the other could give the observed T_m. 49S RNA is known to have

poly A at the 3' end. However, we have searched for poly U in this RNA without success (Frey and Strauss, submitted for publication) and estimate that a sequence as short as 10-15 nucleotides would have been detected. In addition, a long poly A:poly U sequence would have been apparent in the nuclease experiment. The ΔH of the circularization reaction gives an estimate of 10-20 nucleotides (depending on the base composition) for the length of the double-stranded region, in agreement with the estimate from the T_m depression.

We have also considered the possibility that the base pairing of these regions is destabilized by the large mass of the RNA molecule which remains single-stranded. However, it is known that the single stranded circles formed by the separated strands of adenovirus DNA or by the single stranded DNA's of adeno-associated virus have approximately the same T_m as the full length double stranded molecules (Garon et al., 1972; Koczot et al., 1973). The terminal complementary sequences are 100-500 nucleotides long (Roberts et al., 1974; Wolfson and Dressler, 1973), and therefore the theoretical T_m depression should be only 1 to 5°C in the absence of other destabilizing factors. It is also known that for double-stranded DNA circles of λ DNA, which are formed by hybridization of short complementary regions at the ends of the molecule, the effect of the circular configuration on the stability of the base-paired regions is slight (Wang and Davidson., 1966a).

It is uncertain what is the relationship between such a short double stranded region of 10-20 nucleotides and the panhandles observed in the electron microscope by Hsu et al. (1973), but the correlation

between secondary structure in RNA molecules and the actual nucleotide sequence which form them has not been well studied. Although the nucleotide sequences which result in circularization are known to be located near the ends of the molecule (Hsu et al, 1973), it is possible that they are several hundred nucleotides removed from one or both ends and that other nucleotide sequences near the ends result in the panhandle appearance. Under the conditions of spreading used to visualize the circles, the molecules retain considerable secondary structure and, in fact, we have often observed molecules with multiple panhandles (Fig. 3).

The renaturation of the linear form of Sindbis virus 49S RNA into the circular form can be observed quite readily by sucrose gradient sedimentation. Our results demonstrate that essentially all of the intact linear 49S RNA molecules are capable of reforming circles. Our data also show that physiological conditions would be favorable for the renaturation of circular 49S RNA. From interpolation of the Arrhenius plot in Figure 8, the rate of reformation of circles in 0.1 M NaCl at 37°C is such that renaturation is 50% complete in 10 minutes. Thus on the time scale of the virus life cycles renaturation under physiological conditions is very fast.

The kinetics of renaturation of only one other circular nucleic acid have been extensively studied, the double stranded DNA of bacteriophage λ (Wang and Davidson, 1966a,b). Although the structure of λ DNA, a fairly rigid molecule with only helical secondary structure, and the structure of Sindbis virus 49S RNA, a molecule with presumably a

tightly folded secondary structure, are very dissimilar, a comparison of the kinetics of renaturation of their circular forms is interesting. The number of base pairs involved in the circularization reaction of the two molecules is very similar. However, the renaturation of the circular form of λ DNA is much less temperature dependent than is the renaturation of the circular form of Sindbis virus 49S RNA. The energy of activation for the circularization of λ DNA is 24 kcal/mole (Wang and Davidson, 1966a) compared to an E_a of 43 kcal/mole for the circularization of Sindbis virus 49S RNA. The high activation energy for Sindbis 49S RNA is presumably required to unfold some secondary structure in the linear molecule, possibly breaking some hydrogen bonds in the process, so that the terminal complementary nucleotide sequences are exposed and circularization can occur.

It is also of interest to compare the ratio of the probability densities, j, for λ DNA and Sindbis RNA. For a random coil, j is the probability density for one end of the coil to lie in the vicinity of the other. By combining the Jacobson-Stockmayer (1950) expression

$$j = \left(\frac{3}{2\pi Lb}\right)^{\frac{3}{2}} \tag{4}$$

and the expression for the radius of gyration (R_G) of a random coil

$$R_G^2 = \frac{Lb}{6} \tag{5}$$

we arrive at the relationship

$$\frac{j_1}{j_2} = \left(\frac{R_{G_2}}{R_{G_1}}\right)^3 = \left(\frac{L_2}{L_1}\right)^{\frac{3}{2}} \qquad (6)$$

In these expressions, L is the contour length of the molecule and b is the length of the Kuhn statistical segment. We can approximate R_G for λ DNA and Sindbis virus RNA by using the expression

$$R_G = \frac{M(1-\bar{\nu}_2\rho_o)}{6\pi\eta N\xi_f S^o} \qquad (7)$$

Using sedimentation coefficients (S^o) of 33.6 S for λ DNA and 49S for Sindbis virus RNA, molecular weights (M) of 32×10^6 for λ DNA and 4.3×10^6 for Sindbis virus RNA, partial specific volumes ($\bar{\nu}_2$) of 0.53 for DNA and 0.58 for RNA (Boedtker, 1960), approximating the density of the solvent (ρ_o) as 1 g/cc, the viscosity (η) as 10^{-2} poise, and using a value of 0.665 for ξ_f (a constant which relates the R_G of a random coil to the radius of a corresponding sphere), we calculate R_G of λ DNA to be 6.0×10^{-5} cm and the R_G of Sindbis virus RNA to be 4.9×10^{-6} cm. The ratio of the probability densities is thus 1700. This means that, everything but the radius of gyration being equal, Sindbis virus RNA should recircularize 1700 times faster than λ DNA. However, in 0.1 M NaCl at 50°C Sindbis virus RNA recircularizes only 50 times faster than λ DNA (compare Fig. 8 with the data of Wang and Davidson (1966a)). This discrepancy in the observed and theoretical difference in the rates of recircularization for the two nucleic acids is probably due to the larger E_a for the circularization of Sindbis virus RNA.

The results presented here on Sindbis virus RNA should serve as a useful reference point for studies on the circularization of other viral RNAs. As an example, the molecular weights of the three circular virion RNA species of Uukuniemi virus are 1.9×10^6, 0.9×10^6 and 0.4×10^6 daltons for the large, medium and small RNA species respectively (Hewlett, Pettersson and Baltimore, 1977). From equation (6), if all other aspects of the circularization of Sindbis virus 49S RNA and Uukuniemi virus RNAs are similar, the large, medium and small species of Uukuniemi virus RNA should renature into their circular forms 3.5, 10, and 35 times more rapidly than Sindbis virus 49S RNA, respectively. It is obvious from this that denaturation of the small Uukuniemi RNA will be difficult to observe. From Figure 8, the half time for reformation of 49S RNA circles in 0.1 M NaCl at 49°C is about 1 minute, and some renaturation occurs during rapid cooling of mixtures heated above the T_m. For the small Uukuniemi RNA the half time for reformation of circles would be on the order of 2 seconds under these conditions and renaturation would probably be completed during cooling of a heated mixture. It is possible that denaturation can be studied in lower ionic strengths, however, where recircularization is slower.

The function of the circularity of alphavirus 49S RNA in the replication of the virus is unclear. It does appear, however, that the base sequences responsible for circularization have evolved so that, on the one hand, 49S RNA molecules will rapidly circularize under physiological conditions (in the absence of factors to keep the ends apart)

and, on the other hand, the T_m is sufficiently low that the free energy required to linearize the molecule could readily be supplied by proteins (the viral replicase, translation initiation factors, possible binding proteins) which interact with the RNA. The circular structure of the alphavirus 49S RNA could be functional in translation, replication or encapsidation of 49S RNA. It is unlikely that the circular structure has a role in translation since there is no evidence that any eukaryotic messenger RNA, including Sindbis virus 26S RNA, is circular. The circular structure could well be involved in RNA transcription, however, possibly acting as a replicase binding site. We note that the minus strand of alphavirus RNA and that alphavirus defective interfering RNAs (which contain the 5' and 3' terminal sequences of the 49S RNA (Guild, Flores and Stollar, 1976; Kennedy, 1976)) also contain the same sequences and will be able to circularize. In addition it appears that only species of viral RNA which are capable of forming circles are encapsidated, and thus the circular structure could be functional in encapsidation, perhaps through interactions of these sequences with the capsid protein.

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