THE FUNCTION AND REPLICATION OF SINDBIS VIRUS-SPECIFIC RNA's IN INFECTED CELLS

Thesis by

Daniel Tawil Simmons

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1974

(Submitted April 29, 1974)

To Sue, for the love and support

she has given me

ACKNOWLEDGEMENTS

When I first came to Caltech, knowing practically nothing about virology, I found Jim Strauss' enthusiasm and thoughtful suggestions encouraging in working towards this important goal. To him and to Ellen Strauss, I give my thanks and show my appreciation for the many hours of discussion and for their efforts during the preparation of various manuscripts.

I wish to acknowledge the assistance of Midge Hoover, Edith Lenches, and Sharman Christoph in their invaluable aid throughout these years. The friendship and interest shown to me by John Bell, Charlie Birdwell, Terry Frey, Mark Miller, John Pierce, Rich Uhlmann, and others (especially those in the Sinsheimer group) are deeply appreciated. I am also indebted to the members of my thesis committee, G. Attardi, N. Davidson, R. Russell, J. Strauss, and W. Wood.

Most of all, I wish to thank my wife, Sue, for her help in the preparation of this thesis and for her continuous faith in me and in my work.

ABSTRACT

During infection with Sindbis virus, two species of Sindbis-specific single-stranded RNA are synthesized. One of them, 49S RNA is the RNA of the virus and has a molecular weight of $4.3 \pm 0.3 \times 10^6$ daltons. This molecular weight was estimated by a variety of methods, including polyacrylamide gel electrophoresis, sedimentation after reaction with formaldehyde and analysis of the molecular weight of its double-stranded form. The other species of single-stranded RNA, 26S RNA, was found only in infected cells and has a molecular weight of 1.6×10^6 daltons, determined by sedimentation in dimethylsulfoxide. Hybridization-competition experiments showed that 26S RNA is a specific one-third of the 49S RNA genome.

In infected cells, 26S RNA was primarily associated with ribosomes, and was found to be the predominant species of viral messenger RNA. A small amount (10% by weight) of the messenger RNA in the cells was Sindbis 49S RNA. No other unique and separate species of Sindbis messenger RNA could be detected in infected cells.

The two species of Sindbis single-stranded RNA were translated in lysates of rabbit reticulocytes. Sindbis 26S RNA was translated primarily into the nucleocapsid protein and into a protein shown by others to be the precursor of the two glycoproteins of the virus. These results indicated that Sindbis 26S RNA codes solely for the structural proteins of the virus.

Sindbis 49S RNA was translated *in vitro* into 8 or 9 polypeptides ranging in molecular weight from 60,000 to 180,000 daltons.

None of these polypeptides coincided with any known Sindbis proteins.

The replication of Sindbis-specific RNA was studied by analyzing the forms of double-stranded RNA (or replicative forms) in infected cells. When RNA from infected cells was treated with ribonuclease, three species of Sindbis-specific double-stranded RNA (RF's I, II, and III) were isolated. Their molecular weights were determined to be 8.8×10^6 daltons for RFI, 5.6×10^6 daltons for RFII, and 2.9×10^6 daltons for RFIII.

By hybridization-competition experiments, it was shown that RFI is the double-stranded form of 49S RNA, RFIII, the double-stranded form of 26S RNA, and RFII, the double-stranded form of a species of RNA with molecular weight of 2.8×10^6 daltons and identical to two-thirds of the genome.

The size and structure of Sindbis replicative intermediates (RI's) were studied and found to consist of a double-stranded region the size of RFI and of various lengths of single-stranded tails. Our model for the replication of Sindbis-specific RNA predicts that Sindbis RI's exist in two classes, called RIa and RIb. RIa is the template for the synthesis of 49S RNA and is reduced to RFI when treated with ribonuclease. When RIb is treated with ribonuclease, it is reduced to RF's II and III due to a single-stranded gap in the "plus" strand of the RI (the virus RNA is "plus"-stranded). The portion of RIb corresponding to RFIII is the template for the synthesis of 26S RNA, and the portion corresponding to RFII is the template for synthesis of a species of RNA of 2.8×10^6 daltons, which has not been detected in its

single-stranded form. We hypothesize that there must be two different regions on RIb where chain synthesis is initiated since 26S RNA is synthesized at a much faster rate than the product of RFII.

TABLE OF CONTENTS

INTRODUCTION

Physical Characteristics of Sindbis Virus and Related Animal Viruses.

Sindbis virus is a member of the family of animal viruses called togaviruses. This term applies to the group of viruses which were formerly in the group A or group B arboviruses. In Latin, a toga is a "loose garment worn by citizens appearing in public." The nomenclature accordingly refers to the fact that these viruses contain a lipid membrane envelope. Sindbis virus belongs to the genus alphaviruses (formerly group A arboviruses) which include such viruses as Semliki Forest virus and Western and Eastern Equine Encephalomyelitis. The former group B arboviruses are now called flavoviruses and include dengue virus and Japanese B encephalitis virus among others. The arboviruses (arthropod-borne) were so called because they are carried by and slowly grow in arthropod vectors and they infect susceptible vertebrates.

The togaviruses contain infectious single-stranded RNA as their genetic material (Cheng, 1958; Ada and Anderson, 1959; Sokol et al., 1959) and measure in the case of the alphaviruses approximately 50 to 75 nm in diameter (Horzinek, 1973). The flavovirus virion is somewhat smaller, measuring 40-50 nm in diameter. The following discussion pertains specifically to alphaviruses, although some of the characteristics of the alphaviruses and flavoviruses are quite similar. These virus particles are also composed of three structural proteins, which, in the case of Sindbis, make up 65% of the weight of the virus particle

(Pfefferkorn and Hunter, 1963). One of the structural proteins (the nucleocapsid protein) is in close association with the RNA of the virus and has an approximate molecular weight of 30,000 daltons (Strauss et al., 1969). The other two are glycoproteins and have approximate molecular weights of 50 to 60,000 daltons (Stollar, 1969; Strauss et al., 1970; Uryvayev et al., 1971; Schlesinger et al., 1972). The glycoproteins are about 14% by weight in carbohydrates (Strauss et al., 1970) and are located on the exterior surface of the virus particle (Compans, 1971; Harrison et al., 1971). In addition to these components, alphaviruses contain lipid material consisting of 25% to 30% of the weight of the virus particle (Wachter and Johnson, 1962; Pfefferkorn and Hunter, 1963; Renkonen et al., 1971).

Single-Stranded RNA Species in Cells Infected with some RNA-Containing Animal Viruses.

In cells infected with alphaviruses (Sindbis, Semliki Forest virus), two species of single-stranded virus-specific RNA are synthesized (Sonnabend et al., 1964; Sreevalsan and Lockart, 1966; Friedman et al., 1966). These forms of RNA sediment in sucrose gradients with sedimentation coefficients of 42S-49S and 26S. The larger species of RNA (which will henceforth be called "49S RNA" for consistency with the body of this thesis) is identical or nearly identical with the RNA isolated from purified virions and has a molecular weight of approximately 4.3×10^6 daltons (Dobos and Faulkner, 1970;

Simmons and Strauss, 1972a, and this thesis). Virus-specific 26S RNA has a molecular weight of 1.6 x 10^6 daltons and is a specific one-third of the 49S RNA genome (Simmons and Strauss, 1972a). Both species of alphavirus single-stranded RNA have messenger activity in infected cells although 26S RNA is the predominant (90% by weight) form of viral messenger (Kennedy, 1972; Mowshowitz, 1973; Rosemond and Sreevalson, 1973; D. T. Simmons and J. H. Strauss, submitted for publication, and this thesis).

The replication of alphaviruses seems to proceed differently from the replication of RNA bacteriophages (QB, MS2, R17) or picornaviruses (poliovirus, Encephalomyocarditis virus). The RNA of the virion is the only major viral form of single-stranded RNA synthesized after infection with RNA bacteriophages or picornaviruses (see, for example, reviews by Weissmann et al., 1973; Hindley, 1973; Baltimore, 1969). Soon after these viruses infect the cell, the RNA in the virion functions as messenger for the synthesis of virus-specific proteins (Tobey, 1964; Godson and Sinsheimer, 1967). Progeny RNA molecules are found mainly associated with ribosomes during infection and function, therefore, primarily as messengers (Penman et al., 1963; Godson, 1968).

Other animal RNA viruses appear to replicate in a fashion similar to the replication of alphaviruses on the basis that fragments of RNA smaller than the viral genome are synthesized in infected cells. Thus, cells infected with paramyxoviruses [Newcastle disease virus (NDV), Sendai] for example, produce, in addition to the viral genome,

three or four smaller pieces of RNA which have messenger function and which are complementary to the RNA in the virus (Kingsbury, 1966; Bratt and Robinson, 1967). Similarly, infection with rhabdoviruses [vesicular stomatitis virus (VSV)] produces four or five species of messenger RNA which are smaller than the viral genome and complementary to it (Schaffer et al., 1968; Newman and Brown, 1969; Schincariol and Howatson, 1970; Huang et al., 1970). In these systems, the virion RNA itself does not function as a messenger (Kingsbury, 1973; Morrison et al., 1974).

Other RNA-containing animal viruses must employ different strategies for replication since their genome is in a segmented form. Influenza virus (myxoviruses) RNA is in at least seven distinct fragments when isolated from purified virions (Duesberg, 1968; Pons and Hirst, 1968a). In infected cells, complementary RNA is made in different relative amounts from each fragment of influenza virus RNA and can be isolated from polysomes (Pons, 1972).

The genome of reovirus and of similar viruses consists of 10 double-stranded RNA fragments which, in infected cells, act as independent templates for the synthesis of the 10 corresponding species of single-stranded RNA which function as messengers (Prevec and Graham, 1966; Bellamy and Joklik, 1967; Shatkin and Rada, 1967; Shatkin et al., 1968).

Thus, various forms of messenger RNA, different in size and/or in strandedness from the RNA present in virus particles, may be synthesized in cells infected with various RNA animal viruses. The

reason for this is apparently to control the amounts of polypeptides translated from each species of messenger RNA formed in infected cells. Regulation at the level of RNA synthesis would then directly or indirectly influence the relative amounts of virus-specific proteins made during infection. One level of regulation might be placed on the relative affinity of various RNA species for ribosomes (i.e., not all viral-specific single-stranded RNA molecules may be found in polysomes). A second level of regulation can be executed at the relative efficiency of translation of various messenger RNA species. (The relative amounts of virus-specific polypeptides synthesized may be different than the amounts predicted from the relative amounts of each corresponding species of messenger RNA.) It is understandable that in a highly efficient and successful infection, the protein synthetic machinery of the cell is conserved and utilized optimally to make the correct relative amounts of various virus-specific proteins. Virion-associated structural proteins almost always (with the possible exception of picornaviruses) constitute the major polypeptide products of an infection with an RNA virus. Regulatory proteins and virus-specified enzymes are usually made in smaller relative amounts.

The function of Sindbis virus 26S RNA as a messenger is most probably to code for the structural proteins of the virion (D. T. Simmons and J. H. Strauss, in press, and this thesis; R. Cancedda and M. J. Schlesinger, in press). In infected cells, the structural proteins of the virus or their precursors constitute the large majority of virus-specific proteins (see below). This is easily understood in terms of the predominance of 26S RNA in association with ribosomes.

In vivo, 26S RNA is probably translated into one large polypeptide precursor (molecular weight 130,000 daltons) which contains the amino acid sequences present in all three structural proteins (Strauss et al., 1969; Schlesinger and Schlesinger, 1973). Very rapidly, and possibly while still on the polysomes, the precursor polypeptide chain is cleaved to yield the nucleocapsid protein and a second polypeptide (protein B) which contains the amino acid sequences of the two glycoproteins of the virus (Schlesinger and Schlesinger, 1972; D. T. Simmons and J. H. Strauss, in press). Protein B is cleaved and the resulting polypeptides are glycosylated in a series of steps to yield the two glycoproteins of the virion (Schlesinger and Schlesinger, 1972; Sefton and Burge, 1973).

The translation products of Sindbis messenger 49S RNA in infected cells are not known. In vitro, 49S RNA is translated into 8 or 9 polypeptides ranging from 60,000 to 180,000 daltons in molecular weight (D. T. Simmons and J. H. Strauss, in press, and this thesis). It is not known if these polypeptide products are also synthesized in infected cells.

As in the case of the alphaviruses, proteolytic cleavages of viral polypeptide precursors occur in cells infected with poliovirus (or other picornaviruses) (Summers and Maizel, 1968; Jacobson and Baltimore, 1968; Holland and Kiehn, 1968). The formation of specific proteins occurs at the post-translational stage since animal virus messenger RNA probably lack start/stop signals for translation and since in these cells, there is only one species of viral messenger

RNA. Apparently, these viruses cannot regulate the relative amounts of virus-specific proteins in infected cells, since structural and non-structural proteins are made in approximately equal quantities.

The restriction on start and stop signals for translation of viral messenger RNA is solved in a different fashion by other RNA animal viruses. Thus, the multiple species of viral messenger RNA in cells infected with reovirus are each monocistronic (Loh and Shatkin, 1968; Smith et al., 1969) and proteolytic cleavage is not necessary (although some cleavage does occur). Although all 10 species of messenger RNA are found associated with ribosomes, only six or seven reovirus-specific proteins (the structural proteins) are detectable in infected cells (Watanabe et al., 1968; Zweerink and Joklik, 1970). In the absence of very rapid polypeptide degradation, this implies that, in addition to transcriptional control, there is a control at the level of translation of each reovirus messenger, such that some messengers are translated more efficiently than others.

Several and possibly all of the species of influenza virus messenger RNA (complementary to virion RNA) are monocistronic (Content and Duesberg, 1970). Etchison et al. (1971) have observed cleavage of influenza-specific polypeptides in infected cells, but there has been a report to the contrary (Holland et al., 1972). In these cells, the polypeptides which predominate are the ones found in purified virions (Joss et al., 1969; Taylor et al., 1969). However, the relative amounts of messenger RNA forms would predict different relative amounts of influenza proteins in infected cells. As with the reoviruses, the

translation of influenza virus messenger RNA is probably controlled.

Similarly, the VSV specific messenger RNA species are monocistronic since, *in vitro*, translation of each species of messenger RNA produced a specific polypeptide also found in infected cells (Morrison *et al.*, 1974). Proteolytic cleavage of VSV proteins *in vivo* has not been detected (Wagner *et al.*, 1970).

Finally, the several species of NDV complementary RNA are related in size and abundance to the NDV-specified polypeptides in infected cells and is indicative that each species of paramyxovirus messenger RNA is monocistronic (Kingsbury, 1973; B. S. Collins and M. A. Bratt, in press).

Mechanisms of RNA Replication in Infected Cells.

Bacteriophage and picornavirus RNA replication proceed in a very similar manner. The first step is the synthesis of a complementary "minus" strand from the infecting "plus"-stranded genome (Montagnier and Sanders, 1963; Erikson *et al.*, 1964; Weissmann and Feix, 1966; Hori *et al.*, 1967; Feix *et al.*, 1968). The minus strand, in turn, becomes the template for the synthesis of "plus"-stranded RNA molecules indistinguishable from virion RNA (Weissmann *et al.*, 1968; Girard, 1969). The complex on which the synthesis of "plus" single-stranded RNA occurs is called the replicative intermediate (RI). As extracted from the cell, the RI is composed of a double-stranded RNA "backbone" and of one or more single-stranded RNA chains of varying lengths (Baltimore, 1968). In the infected cell, the replicative intermediate is probably largely in a single-stranded form; however,

when the complex is deproteinized, complementary regions of the RI anneal to form a partially double-stranded structure (Weissmann et al., 1968). Treatment of RI's with RNase under controlled conditions degrades the single-stranded regions and the resistant portion is nearly or fully double-stranded (this structure has been called the replicative form or RF) (Baltimore and Girard, 1966).

In other virus systems where the replication of the RNA has been well characterized, more than one form of replicative intermediate may be involved. For example, each segment of influenza virus RNA replicates independently on a template which is partially double-stranded when isolated from infected cells (Duesberg, 1968; Pons and Hirst, 1968b; Lerner and Hodge, 1969; Content and Duesberg, 1971).

In reovirus-infected cells, the 10 double-stranded viral RNA segments serve as independent templates for the synthesis of 10 corresponding pieces of single-stranded RNA (Watanabe et al., 1967; Prevec et al., 1968). However, several segments of reovirus RNA are transcribed more frequently than others in infected cells (Joklik et al., 1970; Zweerink and Joklik, 1970).

We have proposed the existence of two forms of replicative intermediates in cells infected with Sindbis virus (Simmons and Strauss, 1972b, and this thesis). One RI is believed to serve as the template for the synthesis of virion RNA and the second RI for the synthesis of 26S RNA, the major form of messenger RNA in infected cells.

A similar mechanism which allows for the independent synthesis of messenger RNA on one RI template and of virion RNA on a second RI

template is likely to operate in cells infected with any of the togaviruses, the paramyxoviruses (NDV, Sendai) or the rhabdoviruses (VSV). Thus, cells infected with flavoviruses (group B arboviruses) synthesize two species of single-stranded RNA which apparently correspond to Sindbis 26S and 49S RNA (Stollar et al., 1967; Trent et al., 1969). We expect, therefore, that flavovirus-specific RNA replication involves two forms of replicative intermediates as has been postulated in the case of Sindbis virus.

Similarly, there appear to be two classes of replicative intermediates in cells infected with NDV. One form of RI synthesizes only NDV messenger RNA since, when it is denatured, the various forms of messenger RNA and the virion RNA are released (note that NDV messenger RNA is complementary to the RNA in the virion) (Portner and Kingsbury, 1972). A second species of replicative intermediate has been detected in cells infected with Sendai virus and appears to be the template for synthesis of virion RNA (this second form of RI presumably also exists in cells infected with NDV) (Robinson, 1971; Portner and Kingsbury, 1972).

To date, only one form of replicative intermediate has been detected in cells infected with VSV (Huang et al., 1970; Schaffer et al., 1968). This RI is the template for the synthesis of the four or five species of VSV messenger RNA. A second RI might not be readily detectable since virion RNA is synthesized in relatively small amounts compared to the forms of messenger RNA.

This thesis deals with three main problems of the replication of Sindbis virus. 1) What species of virus-specific single-stranded RNA are found in infected cells and how are they related to the RNA in the virus? The first chapter of this thesis discusses these questions. 2) What is the function of these forms of RNA? This is discussed in Chapters 2 and 3. Finally, how are these RNA molecules replicated? This is described in Chapter 4.

REFERENCES

Ada, G. L. and Anderson, S. G. (1959). Nature 183, 799-800.

Baltimore, D. (1968). J. Mol. Biol. 32, 359-368.

Baltimore, D. (1969). In The Biochemistry of Viruses. (H. B. Levy, Ed.) Marcel Dekker, Inc. pp. 101-176.

Baltimore, D. and Girard, M. (1966). Proc. Nat. Acad. Sci. U.S. 56, 741-748.

Bellamy, A. R. and Joklik, W. K. (1967). J. Mol. Biol. 29, 19-26.

Bratt, M. A. and Robinson, W. S. (1967). J. Mol. Biol. 23, 1-21.

Cheng, P. Y. (1958). Nature 181, 1800.

Compans, R. W. (1971). Nature New Biol. 229, 114-116.

Content, J. and Duesberg, P. H. (1970). J. Virol. 6, 707-716.

Content, J. and Duesberg, P. H. (1971). J. Mol. Biol. 62, 273-285.

Dobos, P. and Faulkner, P. (1970). J. Virol. 6, 145-147.

Duesberg, P. H. (1968). Proc. Nat. Acad. Sci. U.S. 59, 930-937.

Erikson, R. L., Fenwick, M. L., and Franklin, R. M. (1964). J. Mol. Biol. 10, 519-529.

Etchison, J., Doyle, M., Penhoet, E., and Holland, J. (1971). J. Virol. 7, 155-167.

Feix, G., Pollet, R., and Weissmann, C. (1968). Proc. Nat. Acad. Sci. U.S. 59, 145-152.

Friedman, R. M., Levy, B. H., and Carter, W. B. (1966). Proc. Nat. Acad. Sci. U.S. 56, 440-446.

Girard, M. (1969). J. Virol. 3, 376-384.

Godson, G. N. (1968). J. Mol. Biol. 34, 149-163.

Godson, G. N. and Sinsheimer, R. L. (1967). J. Mol. Biol. 23, 495-521.

Harrison, S. C., David, A., Jumblatt, J., and Darnell, J. E. (1971). J. Mol. Biol. 60, 523-528.

Hindley, J. (1973) Prog. Biophys. and Mol. Biol. 26, 269-321.

Holland, J. J., Doyle, M., Perrault, J., Kingsbury, D. T., and Etchison, J. (1972). Biochem. Biophys. Res. Comm. 46, 634-639.

Holland, J. J. and Kiehn, E. D. (1968). Proc. Nat. Acad. Sci. U.S. 60, 1015-1022.

Hori, K., Foyang, L., Banerjee, A. K., and August, J. T. (1967). Proc. Nat. Acad. Sci. U.S. 57, 1790-1797.

Horzinek, M. C. (1973). Prog. Med. Virol. 16, 109-156.

Huang, A. S., Baltimore, D. and Stampfer, M. (1970). Virology 42, 946-957.

Jacobson, M. F. and Baltimore, D. (1968). Proc. Nat. Acad. Sci. U.S. 61, 77-84.

Joklik, W. K., Skehel, J. J., and Zweerink, H. J. (1970). Cold Spring Harbor Symp. Quant. Biol. 35, 791-801.

Joss, A., Gandhi, S. S., Hay, A. J., and Burke, D. C. (1969). J. Virol. 4, 816-822.

Kennedy, S. I. T. (1972). Biochem. Biophys. Res. Comm. 48, 1254-1258.

Kingsbury, D. W. (1966). J. Mol. Biol. 18, 204-214.

Kingsbury, D. W. (1973). J. Virol. 12, 1020-1027.

Lerner, R. A. and Hodge, L. D. (1969). Proc. Nat. Acad. Sci. U.S. 64, 544-551.

Loh, P. C. and Shatkin, A. J. (1968). J. Virol. 2, 1353-1359.

Montagnier, L. and Sanders, F. K. (1963). Nature 199, 664-667.

Morrison, T., Stampfer, M., Baltimore, D., and Lodish, H. F. (1974). J. Virol. 13, 62-72.

Mowshowitz, D. (1973). J. Virol. 11, 535-543.

Newman, J. F. E. and Brown, F. (1969). J. Gen. Virol. 5, 305-313.

Penman, S., Scherrer, K., Becker, Y., and Darnell, J. E. (1963). Proc. Nat. Acad. Sci. U.S. 49, 654-662.

Pfefferkorn, E. R. and Hunter, H. S. (1963). Virology 20, 433-445.

Pons, M. W. (1972). Virology 47, 823-832.

Pons, M. W. and Hirst, G. K. (1968a). Virology 34, 385-388.

Pons, M. W. and Hirst, G. K. (1968b). Virology 35, 182-184.

Portner, A. and Kingsbury, D. W. (1972). Virology 47, 711-725.

Prevec, L. and Graham, A. F. (1966). Science 154, 522-523.

Prevec, L., Watanabe, Y., Gauntt, C. J., and Graham, A. F. (1968). J. Virol. 2, 289-297.

Renkonen, O., Kääriäinen, L., Simons, K., and Gahmberg, C. G. (1971). Virology 46, 318-326.

Robinson, W. S. (1971). Virology 43, 90-100.

Rosemond, H. and Sreevalsan, T. (1973). J. Virol. 11, 399-415.

Schaffer, F. L., Hackett, A. J., and Soergel, M. E. (1968). Biochem. Biophys. Res. Comm. 31, 685-692.

Schincariol, A. L. and Howatson, A. F. (1970). Virology 42, 732-743.

Schlesinger, M. and Schlesinger, S. (1973). J. Virol. 11, 1013-1016.

Schlesinger, S. and Schlesinger, M. (1972). J. Virol. 10, 925-932.

Schlesinger, M. J., Schlesinger, S., and Burge, B. W. (1972). Virology 47, 539-541.

Sefton, B. M. and Burge, B. W. (1973). J. Virol. 12, 1366-1374.

Shatkin, A. J. and Rada, B. (1967). J. Virol. 1, 24-35.

Shatkin, A. J., Sipe, J. D., and Loh, P. (1968). J. Virol. 2, 986-991.

Simmons, D. T. and Strauss, J. H. (1972a). J. Mol. Biol. 71, 599-613.

Simmons, D. T. and Strauss, J. H. (1972b). J. Mol. Biol. 71, 615-631.

Smith, R. E., Zweerink, H. J., and Joklik, W. K. (1969). Virology 39, 791-810.

Sokol, F., Libiková, H., and Zemba, J. (1959). Nature 184, 1581.

Sonnabend, J., Dalgarno, L., Friedman, R. M., and Martin, E. M. (1964). Biochem. Biophys. Res. Comm. 17, 455-460.

Stollar, V. (1969). Virology 39, 426-438.

Stollar, B., Schlesinger, R. W., and Stevens, T. M. (1967). Virology 33, 650-658.

Sreevalsan, T. and Lockart, R. Z. (1966). Proc. Nat. Acad. Sci. U.S. 55, 974-981.

Strauss, J. H., Burge, B. W., and Darnell, J. E. (1969). Virology 37, 367-376.

Strauss, J. H., Burge, B. W., and Darnell, J. E. (1970). J. Mol. Biol. 47, 437-448.

Summers, D. F. and Maizel, J. V. (1968). Proc. Nat. Acad. Sci. U.S. 59, 966-971.

Taylor, J. M., Hampson, A. W., and White, D. O. (1969). Virology 39, 419-425.

Trent, D. W., Swensen, C. C., and Qureshi, A. A. (1969). J. Virol. 3, 385-394.

Tobey, R. A. (1964). Virology 23, 23-29.

Uryvayev, L. V., Derkach, Y. S., Yershov, F. I., and Zhdanov, V. M. (1971). Experientia 27, 348-349.

Wachter, R. F. and Johnson, E. W. (1962). Federation Proc. 21, 461.

Wagner, R. R., Snyder, R. M., and Yamazoki, S. (1970). J. Virol. 5, 548-558.

Watanabe, Y., Millward, S., and Graham, A. F. (1968). J. Mol. Biol. 36, 107-123.

Watanabe, Y., Prevec, L., and Graham, A. F. (1967). Proc. Nat. Acad. Sci. U.S. 58, 1040-1046.

Weissmann, C., Billeter, M. A., Goodman, H. M., Hindley, J., and Weber, H. (1973). Ann. Rev. Biochem. 42, 303-328.

Weissmann, C. and Feix, G. (1966). Proc. Nat. Acad. Sci. U.S. 55, 1264-1268.

Weissman, C., Feix, G., and Slor, H. (1968). Cold Spring Harbor Symp. Quant. Biol. 33, 83-100.

Zweerink, H. J. and Joklik, W. K. (1970). Virology 41, 501-518.

CHAPTER 1

Replication of Sindbis Virus: Relative Size and Genetic Content of 26S and 49S RNA

by

Daniel T. Simmons and James H. Strauss

This chapter is from the
Journal of Molecular Biology
and is reproduced with the
permission of the publisher.

J. Mol. Biol. (1972) **71**, 599–613

Replication of Sindbis Virus

I. Relative Size and Genetic Content of 26 s and 49 s RNA

DANIEL T. SIMMONS AND JAMES H. STRAUSS

Division of Biology, California Institute of Technology
Pasadena, Calif. 91109, U.S.A.

(*Received 14 February 1972, and in revised form 10 July 1972*)

The genome of Sindbis virus, 49 s RNA, is a single, intact polynucleotide chain having a molecular weight of $4{\cdot}3 \pm 0{\cdot}3 \times 10^6$ daltons. This has been determined using a variety of methods including polyacrylamide gel electrophoresis, sedimentation after reaction with formaldehyde, determination of the molecular weights of the double-stranded forms of Sindbis-specific RNA and hybridization competition. The second major species of single-stranded RNA made after infection with Sindbis, 26 s RNA, has been found to have a molecular weight of $1{\cdot}6 \times 10^6$ daltons as determined by sedimentation in dimethylsulfoxide. Hybridization-competition experiments carried out between these two species of RNA, using double-stranded forms of Sindbis RNA isolated from infected cells, showed that 26 s RNA contains only one-third of the base sequences in 49 s RNA and thus represents a unique fraction of the viral genome.

1. Introduction

At least two species of single-stranded RNA are made after infection of animal cell cultures with group A arboviruses (Sonnabend, Dalgarno, Friedman & Martin, 1964; Pfefferkorn, Burge & Coady, 1967; Sreevalsan & Lockart, 1966). In the case of Sindbis virus, these have sedimentation coefficients of 26 s and 49 s. Purified virions contain only 49 s RNA (Pfefferkorn *et al.*, 1967), and subviral core particles contain predominantly, if not exclusively, 49 s RNA (Yin & Lockart, 1968; Friedman & Berezesky, 1967). It has been suggested that 26 s and 49 s RNA have the same genetic information; i.e. that they differ only in configuration (Sreevalsan, Lockart, Dodson & Hartman, 1968), or that 49 s RNA is a dimer or a trimer of 26 s (Dobos & Faulkner, 1969,1970; Dobos, Arif & Faulkner, 1971; Cartwright & Burke, 1970). In these experiments, 49 s RNA was treated with dimethylsulfoxide, urea or heat, and apparently was reduced to the size of 26 s RNA. Sreevalsan *et al.* (1968) found that both species of RNA had the same base ratios, and that the RNA from the 26 s region of sucrose gradients was partially infectious, although the infectivity may have been due to Sindbis replicative intermediates which sediment in the same region (Pfefferkorn *et al.*, 1967; Yin & Lockart, 1968). The possibility that these two molecules were only configurational variants was eliminated by the finding that 49 s RNA migrated more slowly in polyacrylamide gels than did 26 s RNA (Dobos & Faulkner, 1969; Levin & Friedman, 1971). Other evidence suggested that 49 s RNA is not a structural dimer or trimer of 26 s. End-terminal analysis of Sindbis RNA gave a minimal molecular weight estimate of 4×10^6 daltons (E. R. Pfefferkorn, personal communication). The viral RNA has

been denatured by heating with formaldehyde and found to remain in one large piece of comparable size (Mudd & Summers, 1970). Finally, Scheele & Pfefferkorn (1969) showed that puromycin affected the synthesis of 49 s and 26 s RNA differently, implying that these two species were synthesized independently.

In this report, we show that 49 s RNA is a single polynucleotide chain with a molecular weight of $4{\cdot}3 \pm 0{\cdot}3 \times 10^6$, whereas 26 s RNA has a molecular weight of $1{\cdot}6 \times 10^6$. Hybridization competition experiments showed that 26 s RNA contains only one-third the base sequence information in 49 s, as expected on the basis of their respective molecular weights, and therefore represents a unique fragment of the genome.

2. Materials and Methods

(a) *Materials*

Actinomycin D was purchased from Calbiochem. [5-^{3}H]Uridine (spec. act. 23 Ci/m-mole) and [^{32}P]phosphoric acid (carrier free) were obtained from Schwarz/Mann. [2-^{14}C]Uridine (spec. act. 55 mCi/m-mole) was from New England Nuclear. Sodium dodecyl sulfate, TEMED† and dimethylsulfoxide (Me_2SO) (spectroquality) were all purchased from Matheson, Coleman and Bell. The sodium dodecyl sulfate was recrystallized once from 95% ethanol; TEMED was either used fresh or was vacuum redistilled at 15 mm mercury and stored under nitrogen. British Drug Houses Ltd supplied the 4-aminosalicylate. Tri-isopropylnaphthalenesulfonate, diethyl oxydiformate, acrylamide ("for electrophoresis") and *N,N'*-methylenebisacrylamide (bis-acrylamide) were all from Eastman Organic Chemicals. The acrylamide was further purified by recrystallizing it once from chloroform. Mallinckrodt supplied the liquefied phenol and meta-cresol, which were redistilled and stored frozen at −20°C, as well as the 8-hydroxyquinoline, the formaldehyde solution and the ammonium persulfate. Agarose (pure powder) was obtained from Aldrich Chemical Co., and a 2% solution was filtered hot before use. Vinyl tubing (electrophoresis tubing) was purchased from Cadillac Plastic Co. and was soaked in a 1 : 4 dilution of sulfuric acid/$Na_2Cr_2O_7$ cleaning solution before use. Dimethylsulfoxide-d_6 was purchased from BioRad Laboratories. Pancreatic ribonuclease was obtained from Sigma and deoxyribonuclease was "RNase-free" from Worthington Biochemical.

(b) *Preparation of RNA*

(i) *Infection and labeling of Sindbis-specific RNA*

Six hr after changing the medium over the cells, confluent monolayers of chick embryo fibroblasts (Pfefferkorn & Hunter, 1963) or of hamster cells (BHK21), in 75 cm^2 T-flasks or in 800 cm^2 roller bottles, were infected with Sindbis virus (HR strain; Burge & Pfefferkorn, 1966) at a multiplicity of 10 plaque-forming units per cell. The medium used was Eagle's minimal essential medium (Eagle, 1959), containing Earle's salts (GIBCO) 5% dialyzed calf serum and 1 μg actinomycin D/ml. After a 1-hr adsorption period, sufficient amounts of the above medium were added to completely bathe the cells (6 ml./75 cm^2). Radioactive label was added at various times after infection depending upon the experiment. Usually [5-^{3}H]uridine was used at a concentration of 20 μCi/ml., [2-^{14}C]-uridine at 2 μCi/ml. and $H_3{}^{32}PO_4$ at 0·1 to 1 mCi/ml. (the last in medium containing one-tenth the normal phosphate concentration).

(ii) *Lysis and extraction of the RNA*

At the end of the appropriate labeling period, the medium overlay was removed and the cells were rapidly chilled to 0°C by dipping the T-flask (or the roller bottle) into an ice-water slurry. The cells were then washed 3 consecutive times with cold 0·01 M-Tris, 0·0015 M-$MgCl_2$, 0·01 M-KCl, pH 7·4, and treated in one of three ways.

Method A. The cells were lysed with Tris/NaCl/EDTA buffer (0·01 M-Tris, 0·06 M-NaCl, 0·001 M-EDTA, pH 7·2) containing 1% sodium dodecyl sulfate and 3% diethyl oxy-

† Abbreviation used: TEMED, *N,N,N',N'*-tetramethylethylenediamine.

diformate. In all cases, the volumes of lysis mixture were 1·5 ml./T-flask and 10 ml./roller bottle. This lysis buffer had to be added at room temperature, but it was allowed to cool at the bottom of the culture vessel before coming in contact with the cell monolayer. The lysate was then extracted at 0°C by the phenol/chloroform technique of Penman (1966).

Method B. The cells were lysed at 0°C with the lysis mixture of Parish & Kirby (1966) (Tris/NaCl/EDTA buffer containing 1% w/v tri-isopropylnaphthalenesulfonate, 6% w/v 4-aminosalicylate, 1% w/v NaCl and 6% v/v phenol/meta-cresol/8-hydroxyquinoline (900 : 100 : 1)). Extraction of the lysate was carried out in the cold with one volume of the phenol/cresol/8-hydroxyquinoline mixture 2 consecutive times followed by a chloroform/1% isoamyl alcohol extraction.

Method C. The cells were lysed at 0°C with Tris/NaCl/EDTA buffer containing 1% sodium dodecyl sulfate, and the lysate was extracted by the Penman method at 55°C.

The extracted aqueous solutions were made 0·2 M in potassium acetate (pH 6·0) and the RNA was precipitated at −20°C overnight following the addition of 2·5 vol. of 95% ethanol. The RNA precipitate was collected by centrifugation at 10,000 *g* for 10 min at 0°C, washed once with 95% ethanol and then dissolved in a small vol. (usually 0·4 to 0·5 ml./T-flask) of the appropriate buffer. The RNA solutions were stored at −55°C in a Revco freezer.

(iii) *Preparation of ^3H- or ^{32}P-labeled single-stranded RNA for hybridization*

One or more T-flasks of chick embryo fibroblasts was infected with Sindbis and mass-labeled from 1 to 8 hr post-infection with either [5-^{3}H]uridine or $H_3{}^{32}PO_4$. The RNA was prepared by method A and dissolved in 0·4 ml. of Tris/NaCl/EDTA buffer containing 0·2% sodium dodecyl sulfate. To purify the RNA from residual protein and DNA, it was sedimented at 40,000 rev./min for 4 hr at 23°C in a linear 15 to 30% sucrose gradient made in the same buffer. The fractions containing 49 s and 26 s RNA were pooled together, and the RNA was precipitated with ethanol and resuspended in 0·2 ml. of 0·01 M-Tris, 0·005 M-sodium acetate, 0·0005 M-EDTA (pH 7·8). Preparative electrophoresis was carried out to separate the two RNA species and is described below.

(iv) *Preparation of double-stranded RNA for hybridization*

An infected roller bottle of hamster cells (BHK21, about 10^8 cells) was labeled with [5-^{3}H]uridine from 1 to 8 hr after infection. The RNA was prepared by method C and resuspended in 4 ml. of 0·01 M-Tris, 0·1 M-NaCl, 0·01 M-$MgCl_2$, 0·001 M-EDTA (pH 7·2), containing 10 μg pancreatic RNase/ml. and 50 μg DNase/ml., and incubated at 37°C for 30 min. The solution was made 1% in sodium dodecyl sulfate, and extracted twice with phenol and once with ether at room temperature. The RNA was precipitated and dissolved in 0·5 ml. of 0·001 M-EDTA, 0·05 M-NaCl, pH 7·0. It was then passed through a Sephadex G150 column (25 cm × 1 cm) in the same buffer. Double-stranded RNA was totally excluded from the column while all other RNA fragments were retained on it. Fractions containing the excluded volume were pooled; the RNA was precipitated and resuspended in 0·001 M-EDTA, pH 7·0.

(v) *Preparation of hamster ribosomal RNA*

Actively growing hamster cells were labeled for 6 hr with 10 μCi [5-^{3}H]uridine/ml. The label was chased with fresh complete medium containing 1 μg actinomycin D/ml. for 2 hr, and the RNA was prepared by method B.

(vi) *Preparation of other RNA's*

Escherichia coli ribosomal RNA was prepared by the method of Godson & Sinsheimer (1967).

(c) *Polyacrylamide–agarose gel electrophoresis*

The technique used was a modification of that of Peacock & Dingman (1968*a*). Here we describe the preparation of 10 ml. of gel solution containing 1·85% acrylamide, 0·0925% bis-acrylamide, and 0·4% agarose. Appropriate changes were made for gels of other concentrations.

Two ml. of gel buffer (0·2 M-Tris, 0·1 M-sodium acetate, 0·01 M-EDTA (pH 7·8)) and 1·65 ml. of water were mixed and kept at 45°C. Two ml. of a 2% solution of agarose were melted by heating to 90°C, cooled to 60°C and then added to the diluted gel buffer at 45°C. This mixture was transferred to a 37°C warm room which contained all solutions, pipettes and tubes used in making the gel. 2·5 ml. of a 0·14% solution of ammonium persulfate were immediately added to the agarose gel buffer mixture and the tube mixed well. Finally 1·85 ml. of a solution containing 10% acrylamide, 0·5% bis-acrylamide and 6 μl. of TEMED were added. The gel solution was poured in vinyl tubing or occasionally in glass tubing (15 or 10 cm long, 3/8 or 1/4 in. inside diameter) with Parafilm sealing the bottom. The gels were left at 37°C for 30 min to allow the acrylamide to polymerize. They were then transferred to room temperature, and the agarose permitted to solidify for at least 2 hr before using the gel. A piece of nylon stocking was attached with a rubber band to the top of the gel tube. The Parafilm was removed, the gel tube was inverted and the gel was allowed to slide down. This gave a flat surface for sample application. The 1/4 in. gels were pre-run for 1 hr at 5 mA/gel and the 3/8 in. gels were pre-run for 1·5 hr at 8 mA/gel with the reservoir buffer consisting of 0·04 M-Tris, 0·02 M-sodium acetate, 0·002 M-EDTA (pH 7·8) and also containing 0·2% sodium dodecyl sulfate if the gel was to be used for single-stranded RNA. The RNA sample in a 1 to 4 dilution of this reservoir buffer (but containing 0·2% sodium dodecyl sulfate) was made 8% in sucrose, and 5 to 10 μl. of 0·05% bromophenol blue was added. The sample was layered onto the top of the gel and subjected to electrophoresis at the same amperage and with the same reservoir buffer used in the pre-run.

Analytical gels were fractionated with a gel slicer made from razor blades set 1·1 mm apart, and the slices were transferred to scintillation vials. The RNA in the slices was hydrolyzed with 0·5 ml. of concentrated NH_4OH at 40°C for 6 hr or more. The ammonia was allowed to evaporate before adding Bray's scintillation fluid (Bray, 1960).

Preparative gel electrophoresis was carried out in a 10 cm long and 3/8 in. diameter gel within an apparatus made in our shop. The RNA was eluted from the bottom of the gel and transported in anode buffer to a fraction collector. The fractions from each peak were pooled, the RNA precipitated with ethanol and dissolved in 1 ml. of 0·001 M-EDTA, pH 7·0. Alternatively, large amounts of RNA were prepared by using the gels described earlier. The RNA was eluted from the gel slices with 1 ml. of 0·001 M-EDTA, 0·2% sodium dodecyl sulfate over a period of 12 hr at room temperature. Unfortunately, this also liberated small particles of gel which prevented the RNA from being concentrated more than 10-fold. Nevertheless, ^{32}P-labeled RNA was prepared in this manner and used for hybridization after removing the sodium dodecyl sulfate by dialysis.

(d) *Dimethylsulfoxide gradients*

The deuterated Me_2SO gradients of Sedat, Lyon & Sinsheimer (1969) were used. A 10-μl. sample of RNA solution in Tris/NaCl/EDTA buffer, 0·2% sodium dodecyl sulfate was mixed with 100 μl. of Me_2SO and 10 μl. of dimethylformamide and then layered on the top of the gradient. Sedimentation was for 12 hr at 50,000 rev./min in a Spinco SW50.1 rotor at 25°C. Two or three-drop fractions were collected on filter paper discs, the RNA precipitated with trichloroacetic acid and each fraction counted for radioactivity in toluene–fluor.

(e) *Formaldehyde–sucrose gradients*

The method used was that of Boedtker (1968) adapted for sucrose gradients. The gradients were linear 15 to 30% sucrose in 1 M-formaldehyde, 0·1 M-phosphate, 0·001 M-EDTA and 0·2% sodium dodecyl sulfate, adjusted to pH 7·1. The RNA was dissolved in, or dialyzed into, the above buffer. The RNA solution was then heated to 63°C for 10 min and applied to the top of the above gradient. Sedimentation was at 40,000 rev./min for 10 hr at 23°C in a Spinco SW41 rotor. The gradient was fractionated and assayed for radioactivity as described in section (d) above.

(f) *Hybridization*

The method of Weissman (1965) was used with some modifications. The appropriate amounts of double-stranded RNA and of ^{3}H- and ^{32}P-labeled single-stranded RNA were

allowed to evaporate on a sheet of aluminium foil. The RNA was taken up in 30 to 40 μl. of water, adjusted to 0·4 M-NaCl and introduced quantitatively into a capillary tube which was then sealed at both ends. The capillary tubes were heated to 120°C for 3 min in an ethylene glycol bath to denature the RNA and then incubated at 80°C for 12 hr to permit annealing. Following the annealing period, both ends of each tube were broken and the sample mixed in with 0·5 ml. of 0·01 M-Tris, 0·1 M-NaCl, 0·01 M-$MgCl_2$, 0·001 M-EDTA (pH 7·2) containing 10 μg pancreatic RNase/ml. The enzyme treatment was for 1 hr at 37°C. The hybridized RNA was then acid-precipitated and counted for radioactivity as before.

The 26 s RNA preparation used as competing RNA was found to be contaminated with 49 s RNA fragments. Since we were not able to determine directly the fraction of the ^{32}P-labeled 49 s RNA which was effectively competed by the competing 26 s RNA, we derived a function (equation (2)) which gave us this value by back extrapolation to zero competing RNA. We also derived a relation (equation (1)) which operates when the competing RNA competes effectively with all of the ^{32}P-labeled RNA.

Let: a = picomoles of complementary strand in double-stranded RNA,
b = picomoles of ^{32}P-labeled RNA,
c = picomoles of ^{3}H-labeled competing RNA,
α = the molar fraction of contamination of the competing 26 s RNA preparation with 49 s fragments,
β = the fraction of the base sequences in the molecule of ^{32}P-labeled RNA which is also present in the competing RNA, and
Z = percentage of maximum ^{32}P hybridized,

or $$\frac{Z}{100} = \frac{\%\text{ of input which hybridizes in the presence of competing RNA}}{\%\text{ of input which hybridizes in the absence of competing RNA}}.$$

Note that plus strands of RNA (RNA with the polarity of the virion RNA) are contributed by the double-stranded RNA added to the annealing mixture as well as by the ^{32}P-labeled (single-stranded) RNA and by the competing RNA. Thus there are more plus strands in the mixture than minus strands (RNA complementary to the virion RNA). If the efficiency of annealing is high, so that all of the minus strands end up in hybrids, the fraction of plus strands that hybridizes is given by the ratio of minus strands to plus strands.

Then, if $\beta = 1$,

$$\frac{Z}{100} = \frac{a/(a+b+c)}{a/(a+b)},$$

or $$Z = -Zc/(a+b)+100. \quad (1)$$

A plot of Z *versus* Zc gives a straight line of slope $= -1/(a+b)$ and a Zc intercept $= 100\ (a+b)$.

If $\beta \neq 1$, as is the case when 26 s RNA is used to compete with ^{32}P-labeled 49 s RNA, then

$$\frac{Z}{100} = \frac{a\beta/(a+b+c)+a(1-\beta)/(a+b+ac)}{a/(a+b)}.$$

If α is small and $c \gg a+b$, then

$$\frac{Z}{100} \cong (a+b)(1-\beta)/(a+b+ac),$$

or $$Z = -\alpha Zc/(a+b)+100(1-\beta). \quad (2)$$

A plot of Z *versus* Zc gives a straight line of slope $= -\alpha/(a+b)$ and a Z intercept $= 100\ (1-\beta)$.

3. Results

(a) *Polyacrylamide–agarose gel electrophoresis of Sindbis-specific RNA*

Acrylamide gel electrophoresis has been used to estimate the molecular weight of single-stranded RNA (Mills, Peterson & Spiegelman, 1967; Peacock & Dingman,

1968*b*). These authors found empirically that the distance traveled by an RNA species varies approximately as the logarithm of its molecular weight. Configurational differences between different RNA molecules appeared to have a negligible effect on the rates of migration.

Dobos & Faulkner (1969) and Levin & Friedman (1971) have subjected Sindbis 49 s and 26 s RNA to acrylamide gel electrophoresis and estimated their molecular weights to be around 4×10^6 and $1{\cdot}7\times10^6$ daltons, respectively. These results were verified when we analyzed Sindbis-specific RNA by gel electrophoresis in the presence of purified *E. coli* rRNA as markers (Fig. 1). Using the molecular weights for the *E. coli* rRNA of 0·56 and $1{\cdot}07\times10^6$ daltons (Stanley & Bock, 1965), the molecular weights for 49 s and 26 s RNA extrapolate to 4×10^6 and $1{\cdot}6\times10^6$ daltons, respectively.

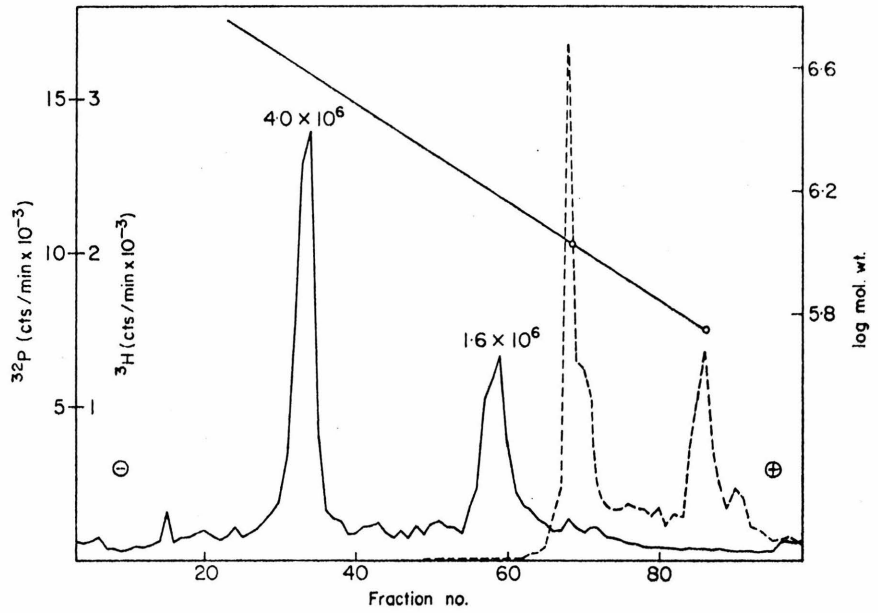


FIG. 1. Acrylamide gel electrophoresis of the RNA made in Sindbis-infected cells. $1{\cdot}5\times10^7$ chick embryo cells were infected at a multiplicity of 10 and labeled with 150 μCi/ml. $H_3{}^{32}PO_4$ from 1 to 8 hr after infection in the presence of 1 μg actinomycin D/ml. The RNA was purified by method A of Materials and Methods and dissolved in 0·5 ml. of 0·01 M-Tris, 0·01 M-sodium acetate, 0·0005 M-EDTA (pH 7·8). 1% of this RNA was mixed with ^{3}H-labeled *E. coli* rRNA, bromophenol blue and sucrose, and the total volume brought to 50 μl. Electrophoresis was for 4 hr at 5 mA on a 15 cm by 0·25 in. gel containing 1·85% acrylamide, 0·0925% bis-acrylamide and 0·4% agarose. The gel was sliced and counted for radioactivity. The bromophenol blue band was found near fraction 125. (——) ^{32}P-labeled Sindbis-specific RNA; (- - - -) ^{3}H-labeled *E. coli* rRNA; —○—○—, log mol. wt.

(b) *Dimethylsulfoxide velocity sedimentation*

When 49 s RNA was prepared from infected hamster cells lysed with sodium dodecyl sulfate alone, the RNA received 1·5 cleavages per molecule, on the average, as determined by sedimentation under denaturing conditions (unpublished observations). Yet, under non-denaturing conditions, in sucrose gradients or in polyacrylamide gels, this same RNA appeared over 90% intact. The same phenomenon was observed qualitatively with 26 s RNA. Thus, both 49 s and 26 s RNA seemed to "hide" their

nicks very well. For the purposes of studying Sindbis-specific RNA under denaturing conditions it was, therefore, necessary to use a lysis method which would introduce as few nicks as possible. The tri-isopropylnaphthalenesulfonate method of Parish & Kirby (1966) was found to introduce no more than one cleavage for every four molecules of 49 s RNA, and was the lysis method used in these experiments.

Strauss, Kelly & Sinsheimer (1968) showed that Me_2SO completely denatures single-stranded and double-stranded RNA, and that, in this solvent, the sedimentation coefficient of RNA varies as the 0·31 power of the molecular weight. To establish the molecular weight dependence on sedimentation coefficient in our Me_2SO gradients, which also contained deuterated Me_2SO, we sedimented hamster rRNA and *E. coli* rRNA in the same gradient (Fig. 2). When the logarithm of the molecular weight of

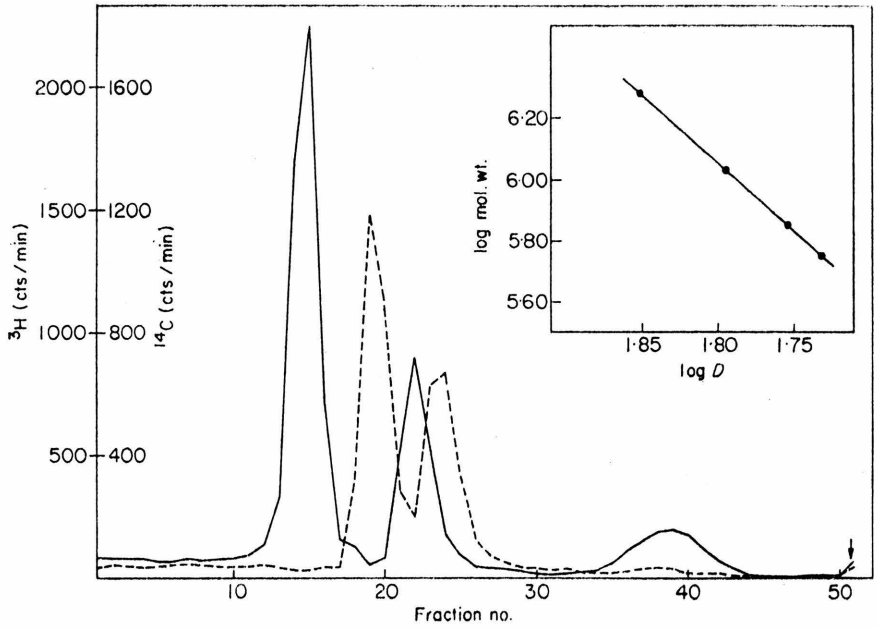


FIG. 2. Calibration of Me_2SO gradients. Five μl. of hamster cell rRNA in Tris/NaCl/EDTA buffer containing 0·2% sodium dodecyl sulfate was mixed with 3 μl. of ^{14}C-labeled *E. coli* rRNA, 100 μl. of Me_2SO and 10 μl. of dimethylformamide and sedimented in a Me_2SO gradient for 12 hr at 50,000 rev./min. The top of the gradient is at fraction 51.

(——) ^{3}H-labeled hamster cell rRNA; (----) ^{14}C-labeled *E. coli* rRNA.

Insert. Plot of the logarithm of the molecular weight *versus* logarithm of the distance traveled (log *D*). Molecular weights used were 0·56 and $1·07 \times 10^6$ daltons for *E. coli* rRNA (Stanley & Bock, 1965) and 0·71 and $1·90 \times 10^6$ daltons for hamster cell rRNA (McConkey & Hopkins, 1969).

the RNA was plotted against the logarithm of the distance traveled, all four points fell on a straight line (see insert of Fig. 2). This result indicates that these Me_2SO gradients can be used to determine molecular weights in this range.

When Sindbis-specific RNA was sedimented in a Me_2SO gradient, only a single peak was observed (Fig. 3). From its position in the gradient (relative to *E. coli* rRNA), this RNA has a molecular weight of $1·58 \pm 0·02 \times 10^6$ daltons (average of four determina-

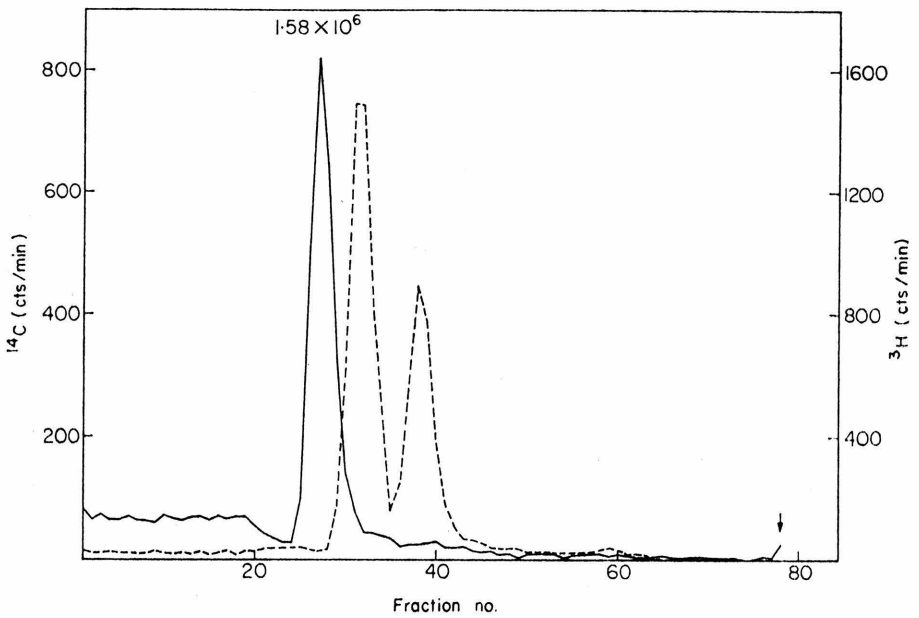


FIG. 3. Me_2SO gradient of Sindbis-specific RNA. Hamster cells were labeled with [^{14}C]uridine at 1 μCi/ml. from 4 to 7 hr after infection. The RNA was purified by method B with the addition that the RNA was also twice precipitated from 2 M-LiCl. ^{3}H-labeled *E. coli* rRNA was also added in the overlay. The top of this gradient is at fraction 77·5.

(——) Sindbis-specific [^{14}C]RNA; (– – – –) *E. coli* ^{3}H-labeled rRNA.

tions), which is in good agreement with the molecular weight expected for 26 s RNA. It was possible that 49 s RNA was being broken down to a size which cosedimented with 26 s RNA, but we suspected that the 49 s RNA had pelleted because 30% of the radioactivity in this gradient was found at the bottom of the centrifuge tube. To determine directly where 49 s RNA sediments in Me_2SO, it was first purified from other Sindbis-specific RNA species by sucrose–velocity sedimentation, and then examined on a Me_2SO gradient. Approximately 50% of the RNA was found in a pellet (mol. wt $> 3\times10^7$), and the rest was evenly distributed throughout the lower fifth of the gradient. No single peak of radioactivity was ever observed when 49 s RNA was sedimented in Me_2SO. Furthermore, no significant amount of radioactivity was ever found at the position corresponding to a molecular weight of $1{\cdot}6\times10^6$ daltons. The viral RNA was not being reduced in size to pieces smaller than $2{\cdot}5\times10^6$ daltons, but instead aggregated in this solvent to a rapidly sedimenting form. We conclude, therefore, that the species of RNA sedimenting at $1{\cdot}58\times10^6$ daltons is 26 s RNA.

We have tried several methods in an attempt to prevent or reduce the aggregation of 49 s RNA in Me_2SO. These include hot phenol re-extractions, repeated treatment of the RNA with 0·01 M-EDTA, 5% sodium dodecyl sulfate/phenol extractions, passage of the RNA through ion-exchange columns, reacting the RNA first with formaldehyde before sedimenting in Me_2SO, sedimentation in Me_2SO gradients containing 0·5 M-LiCl, sedimentation in Me_2SO gradients lacking deuterated Me_2SO and isolation of the 49 s RNA from purified virions. All these methods failed to prevent 49 s RNA from aggregating. However, the RNA did not aggregate when it was

degraded into smaller pieces. We can offer no explanation for the apparent insolubility of the intact molecule in Me_2SO.

(c) *Formaldehyde–sucrose velocity sedimentation*

Since 49 s RNA did not sediment as a band in Me_2SO, we decided to denature the RNA and sediment it in the presence of formaldehyde. This experiment should distinguish between different structural forms of the RNA (i.e. intact *versus* dimer or trimer), and should give an approximation of its molecular weight. Boedtker (1968) found that reliable molecular weights could be obtained from sedimentation coefficients in formaldehyde for many species of single-stranded RNA.

When the same RNA used in the Me_2SO gradient of Figure 3 was heated in the presence of formaldehyde and sedimented in a sucrose gradient containing formaldehyde, two peaks of Sindbis RNA were clearly visible (Fig. 4). Relative to the *E. coli*

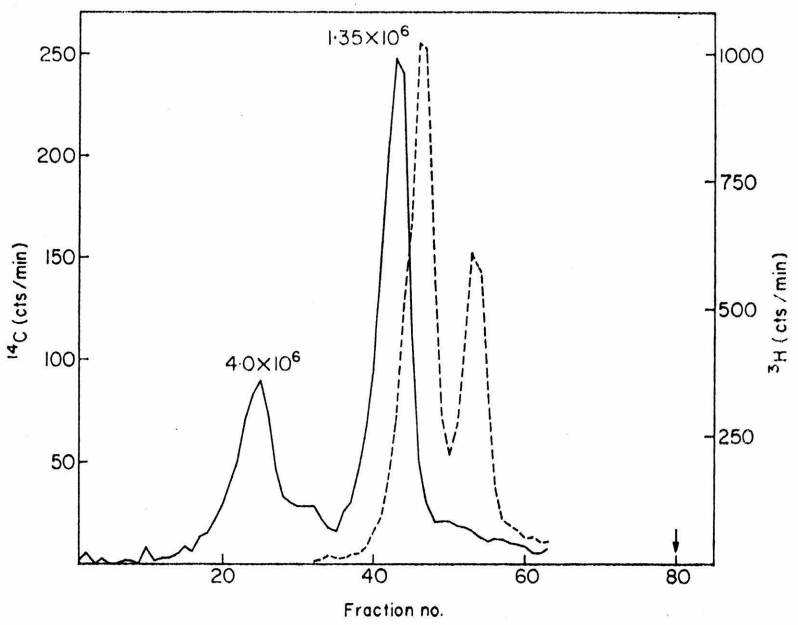


FIG. 4. Formaldehyde–sucrose velocity gradient of Sindbis-specific RNA. The RNA used in Fig. 3 was dialyzed for 3 hr against 1 M-formaldehyde in 0·1 M-phosphate, 0·001 M-EDTA, 0·2% sodium dodecyl sulfate (pH 7·1) and analyzed on a formaldehyde-containing sucrose gradient as described in Materials and Methods.

(——) Sindbis-specific [^{14}C] RNA; (– – – –) *E. coli* ^{3}H-labeled rRNA.

rRNA, these have molecular weights of $4{\cdot}0 \pm 0{\cdot}2 \times 10^6$ daltons and $1{\cdot}35 \pm 0{\cdot}02 \times 10^6$ daltons (average of two determinations). To show that the RNA with a molecular weight of 4×10^6 corresponded to Sindbis 49 s RNA, we isolated 49 s RNA from a sucrose gradient and resedimented it after reaction with formaldehyde (Fig. 5). The single peak in the formaldehyde gradient corresponds to a molecular weight of $4{\cdot}1 \times 10^6$ daltons. Hence, 49 s RNA remains as a single intact polynucleotide chain after formaldehyde denaturation.

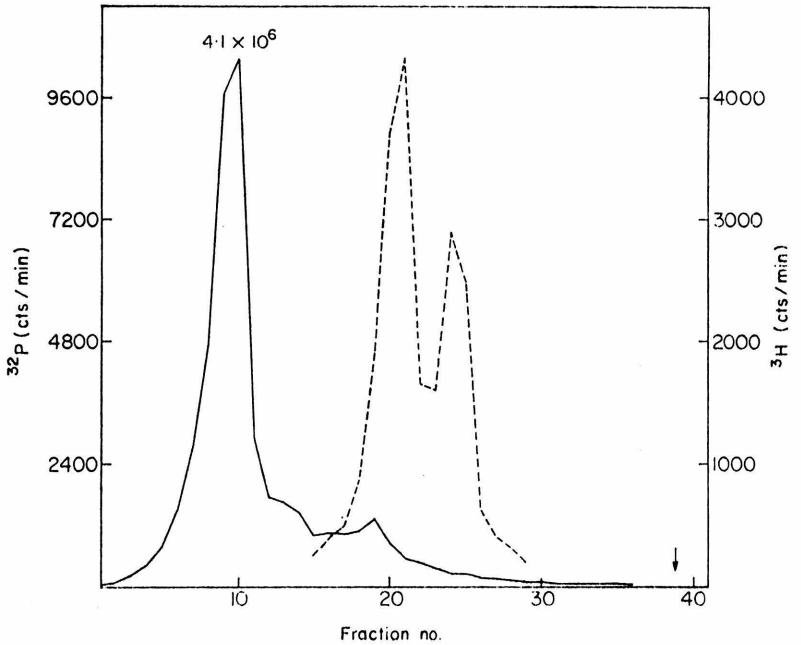


FIG. 5. Formaldehyde–sucrose velocity gradient of 49 s Sindbis RNA. Chick embryo cells were infected and labeled with 400 μCi $H_3{}^{32}PO_4$/ml. from 1 to 9 hr after infection. The cells were lysed by method A of Materials and Methods. The RNA in 0·4 ml. of Tris/NaCl/EDTA buffer containing 0·2% sodium dodecyl sulfate was sedimented in a 15 to 30% sucrose gradient in the same buffer in order to isolate the 49 s RNA. Five μl. of the peak fraction of 49 s RNA were mixed with 20 μl. of ^{3}H-labeled *E. coli* rRNA and with 200 μl. of the 1 M-formaldehyde buffer. This mixture was heated and sedimented in a formaldehyde–sucrose gradient as before.
(——) 49 s Sindbis [^{14}C]RNA; (– – – –) *E. coli* ^{3}H-labeled rRNA.

(d) *Hybridization competition*

The relation between the base sequences of 26 s and 49 s RNA was determined by hybridization of each of these two species of RNA with Sindbis-specific double-stranded RNA in the presence of increasing amounts of the other RNA. The double-stranded RNA was obtained from Sindbis-infected cells after treating the extracts with RNase and DNase. This preparation contained a mixture of RNA's (Simmons & Strauss, 1972) equivalent to the double-stranded form of 49 s RNA. In addition, two preparations of RNA, one labeled with ^{32}P at a very high specific activity (approximately one ^{32}P atom per 4×10^6 daltons of RNA), the other with [^{3}H]uridine for use as competing RNA, were made. (^{3}H was used to simplify the isolation procedure and to determine relative RNA concentrations.) From both of these samples, the 49 s and 26 s RNA were separated by preparative gel electrophoresis. To give an indication of the resolution obtained, the gel pattern of the ^{3}H-labeled RNA is shown in Figure 6.

In a preliminary experiment, it was found that 49 s and 26 s RNA do not hybridize to themselves or to each other (Table 1). These RNA's have the same polarity, therefore, and these RNA preparations contain no detectable minus strands.

Hybridizations with double-stranded RNA added as a source of minus strand were carried out such that each annealing tube contained the same amount of double-

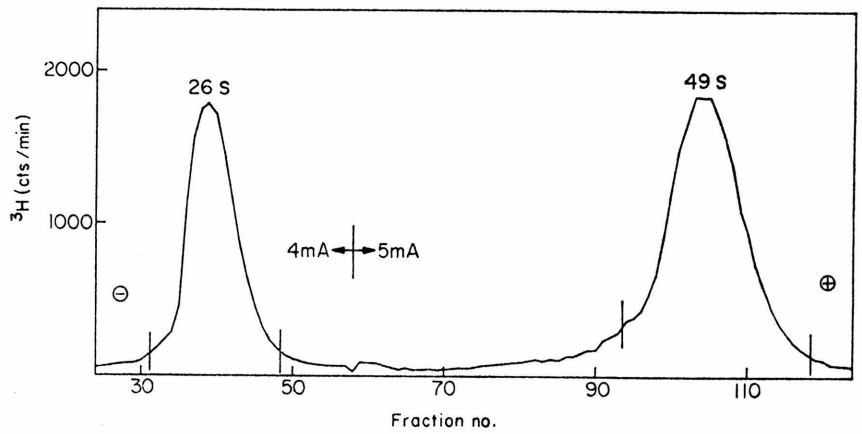


FIG. 6. Preparative acrylamide gel electrophoresis of Sindbis 26 s and 49 s RNA. Preparation and electrophoresis of the RNA are described in Materials and Methods sections (b) and (c). Starting 11 hr after electrophoresis at 4 mA, 10-min fractions were collected. The amperage was increased to 5 mA at fraction 58. Peak fractions between marks were pooled and each species of RNA concentrated by ethanol precipitation.

TABLE 1

Absence of intra- and intermolecular annealing by 26 s and 49 s RNA

	Input (cts/min)	Cts/min after treatment with RNase	
		Hybridized	Not hybridized
(1) ^{3}H-labeled 26 s RNA alone	20,000	324	260
(2) ^{3}H-labeled 49 s RNA alone	10,000	168	61
(3) ^{3}H-labeled 49 s RNA	10,000	69	
and ^{32}P-labeled 26 s RNA	1500	36	

26 s and 49 s Sindbis RNA were purified by preparative gel electrophoresis. The RNA was denatured and allowed to hybridize as described in Materials and Methods. The concentrations of RNA in the annealing mixture were 1·3 μg/ml. and 0·7 μg/ml. for ^{3}H-labeled 26 s RNA and 49 s RNA, respectively, and 20 ng/ml. for the ^{32}P-labeled 26 s RNA.

stranded RNA (15 ng) and the same amount of either ^{32}P-labeled 49 s or ^{32}P-labeled 26 s RNA (1 ng). Varying amounts (0 to 400 ng) of competing RNA (26 or 49 s) were added to each mixture. Following the 12-hour annealing period, the RNA was treated with RNase, and the amounts of hybridized ^{32}P-labeled RNA were determined for each reaction mixture. The concentration of double-stranded RNA in each tube was sufficient for complete (~ 90%) reannealing, under the conditions used, in the absence of other RNA. After addition of ^{32}P-labeled RNA (26 s or 49 s), the ratio of minus strands to plus strands is such that 75% of the input ^{32}P hybridizes in the absence of competing RNA.

Figure 7 shows the percentage of this maximum ^{32}P hybridized as a function of the amount of competing RNA added, for each of four different combinations of RNA. When 49 s RNA was used in competition with ^{32}P-labeled 49 s RNA or when 26 s RNA competed with ^{32}P-labeled 26 s RNA, the levels of ^{32}P-labeled hybrids remaining

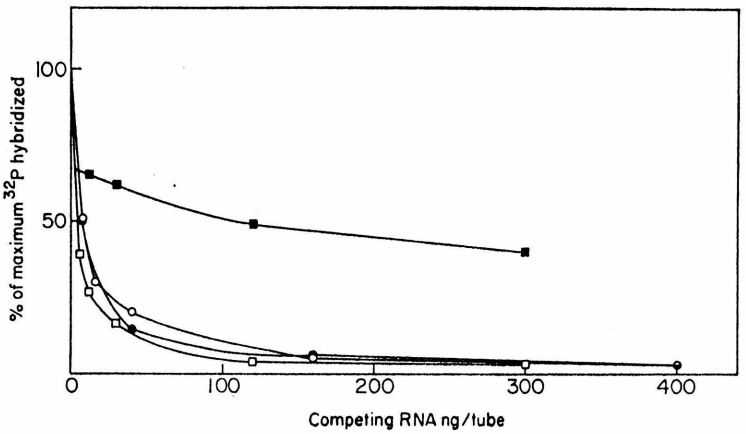


FIG. 7. Hybridization competition between 26 s and 49 s RNA for Sindbis-specific double-stranded RNA. Approximately 15 ng of Sindbis-specific double-stranded RNA was mixed with 1 ng of ^{32}P-labeled, gel purified 26 s or 49 s RNA. Various amounts of competing RNA (labeled with ^{3}H), either 26 s or 49 s, were also added. The RNA solutions, in 0·001 M-EDTA, were taken to dryness and brought up in 33 μl. of 0·4 M-NaCl. The annealing capillary tubes were heated to 120°C for 3 min followed by incubation at 80°C for 12 hr. Each RNA sample was digested with RNase (10 μg/ml.) for 1 hr at 37°C. The RNA was precipitated with trichloroacetic acid and counted for radioactivity. The amount of ^{32}P hybridized, expressed as a percentage of the ^{32}P hybridized in the absence of competing RNA, is plotted as a function of the amount of competing RNA used. Concentrations of RNA were estimated by absorbance at 260 nm (single-stranded RNA) or by electron microscopy (double-stranded RNA).

—●—●—, 49 s ^{32}P-labeled RNA competing 49 s ^{3}H-labeled RNA; —■—■—, 49 s ^{32}P-labeled RNA competing 26 s ^{3}H-labeled RNA; —○—○—, 26 s ^{32}P-labeled RNA competing 49 s ^{3}H-labeled RNA; —□—□—, 26 s ^{32}P-labeled RNA competing 26 s ^{3}H-labeled RNA.

dropped rapidly to very low levels, as expected. The same situation was observed when 49 s RNA was used in competition with ^{32}P-labeled 26 s RNA. Therefore, we conclude that all base sequences in 26 s RNA are found in 49 s RNA. However, where 26 s competed with ^{32}P-labeled 49 s RNA, only a portion of the 49 s RNA was rapidly competed. Competition is incomplete, even at a 40-fold excess of competing RNA to complementary strand in the double-stranded RNA. Thus, there are some base sequences present in 49 s RNA which are not present in 26 s RNA.

Since the competing 26 s RNA preparation was obviously contaminated with 49 s RNA fragments (as evidenced by the continuing decline in ^{32}P-labeled 49 s RNA hybridizing in the presence of increasing amounts of 26 s RNA), it was necessary to determine more accurately the fraction of the 49 s RNA molecule identical to 26 s RNA. The percentage of maximum ^{32}P hybridized (Z) was plotted against the percentage of maximum ^{32}P hybridized multiplied by the picomoles of competing RNA (Zc) (Fig. 8). This function should result in a straight line (see Materials and Methods for derivation). For the three curves involving complete and rapid competition, equation (1) applies, and the reciprocal of the slope of the line as well as the Zc intercept divided by 100 should correspond to the picomoles of plus strand in the reaction mixture in the absence of competing RNA. The points from the competition curve where 26 s is the ^{32}P-labeled RNA and 49 s is the competing RNA are shown on an expanded scale in the insert. The scatter of the points is due to the difficulties in obtaining precise numbers when only a small fraction of the ^{32}P hybridizes and the

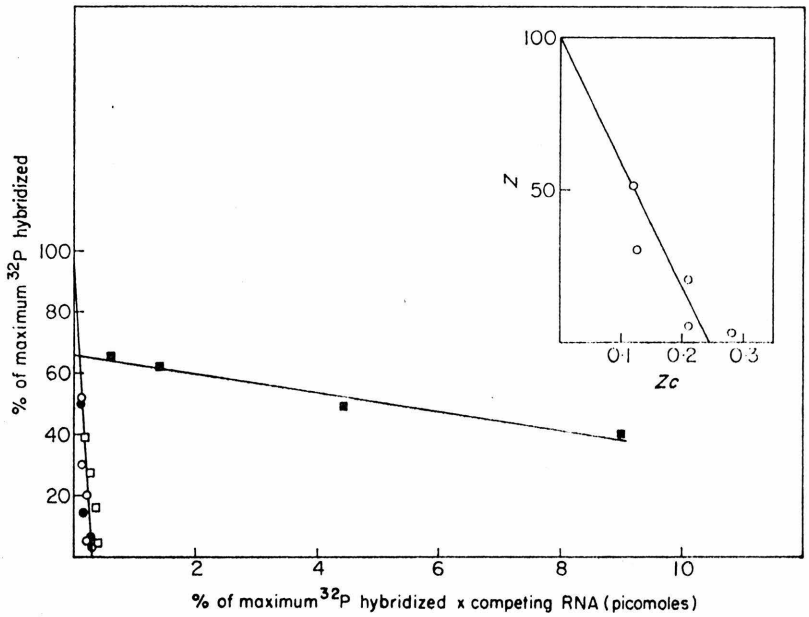


FIG. 8. Plot of the percentage of maximum ^{32}P hybridized (Z) *versus* the picomoles of competing RNA (c) times Z. The data from all four competition curves are included. The meaning of the symbols is the same as in Fig. 7.

Insert. Z *versus* Zc plot for the competition curve between ^{32}P-labeled 26 s RNA and ^{3}H-labeled 49 s RNA.

amount of radioactivity is therefore small. Nevertheless, the Zc intercept divided by 100 gives a value of $2{\cdot}4\times10^{-3}$ picomoles of plus strand per tube (in the absence of competing RNA), in good agreement with the actual amounts used ($2{\cdot}1\times10^{-3}$ picomoles).

When 26 s RNA was used in competition with ^{32}P-labeled 49 s, on the other hand, equation (2) applies. The Z intercept gives a value of 66% for $1-\beta$, or 34% for β, the term which denotes the fraction of the 49 s RNA molecule that is identical to 26 s RNA. This indicates that 26 s RNA contains only 34% of the base sequences present in 49 s RNA. The ratio of the slopes of the two lines of Figure 8 gives a value of 2 to 3% for the percentage mass contamination of the 26 s competing RNA with fragments of 49 s RNA ($\alpha \simeq 0{\cdot}8\%$ molar contamination). Therefore, 26 s RNA must be a unique fragment of the genome.

4. Discussion

The molecular weight of Sindbis 26 s RNA is approximately $1{\cdot}6\times10^{6}$ daltons as determined by sedimentation in Me_2SO and by acrylamide gel electrophoresis. Sedimentation after reaction with formaldehyde gives the somewhat lower value of $1{\cdot}35\times10^{6}$ daltons. However, sedimentation coefficients in formaldehyde may not give exact molecular weights, presumably because denaturation of RNA may be incom-

plete. Thus the large species of eukaryotic rRNA behaves anomalously (Strauss, 1967; Fenwick, 1968). Although 28 s rRNA has a molecular weight of $1{\cdot}9\times10^6$ daltons (McConkey & Hopkins, 1969), its sedimentation coefficient in our formaldehyde system corresponds to a molecular weight of 3×10^6 daltons. On the other hand, Me_2SO is a very effective denaturing agent for RNA (Strauss *et al.*, 1968), and sedimentation coefficients in this solvent are expected to give more reliable estimates of molecular weight.

We have obtained three experimental values for the molecular weight of Sindbis 49 s RNA. These are:

(1) $4{\cdot}0\times10^6$ daltons. This value was obtained by electrophoresis in polyacrylamide–agarose gels and by sedimentation after reaction with formaldehyde. However, molecular weight estimates based on gel electrophoresis are not always accurate because the RNA is not denatured. (For example, this method gives a value of $1{\cdot}6\times10^6$ as the molecular weight of the larger species of hamster rRNA; whereas sedimentation in Me_2SO gives the more accurate figure of $1{\cdot}9\times10^6$.) The molecular weight estimate in formaldehyde is subject to the limitations discussed above.

(2) $4{\cdot}6\times10^6$ daltons. Several lines of evidence indicate that 49 s RNA is almost three times the size of 26 s RNA. These include hybridization-competition experiments, the ratio of molecular weights obtained from sedimentation in formaldehyde, and the ratio of the molecular weights of double-stranded forms of 49 s and 26 s RNA (Simmons & Strauss, 1972). The value of $4{\cdot}6\times10^6$ daltons is based upon a molecular weight of $1{\cdot}6\times10^6$ daltons for 26 s RNA.

(3) $4{\cdot}4\times10^6$ daltons. This value is based on the molecular weight of the double-stranded form of 49 s RNA ($8{\cdot}8\times10^6$ daltons; Simmons & Strauss, 1972).

Hybridization-competition data showed that 49 s RNA must be a polynucleotide chain containing three times as many base sequences as does 26 s RNA. The absence of any significant amount of hybridization between 26 s and 49 s RNA also indicates that these two molecules have the same polarity, and therefore that 26 s RNA must be a fraction of the genome. Furthermore, 49 s RNA must be a single covalently linked molecule since it resists dissociation when denatured with formaldehyde or Me_2SO.

We believe that our results differ from those obtained previously by other authors (see Introduction) primarily because of the difficulties in obtaining unnicked RNA from cultured cells or from purified virions. Nicked RNA maintains its integrity quite well in the absence of denaturing agents, but falls apart when it is denatured by heat, Me_2SO or formaldehyde.

Recently, Arif & Faulkner (1972) have also concluded that 49 s RNA is a single polynucleotide chain. In contrast to our results, they found that their 49 s RNA preparations sedimented as a discrete band in Me_2SO. Since the molecular weight they obtained in Me_2SO was only slightly larger than that of 28 s rRNA, however, their sedimentation pattern may have resulted from slight degradation of their 49 s RNA.

We are pleased to acknowledge the expert technical assistance of Edith M. Lenches and Mildred Quisenberry Hoover. We thank Drs Ellen G. Strauss and John S. Pierce for their helpful editorial advice for the construction of this manuscript. This investigation was supported in part by the National Science Foundation grant no. GB13508 and by the U.S. Public Health Service grant no. GM06965. One of us (D. T. S.) was supported by the National Institutes of Health training grant no. 5-T1-GM86.

REFERENCES

Arif, B. M. & Faulkner, P. (1972). *J. Virol.* **9**, 102.
Boedtker, H. (1968). *J. Mol. Biol.* **35**, 61.
Bray, G. A. (1960). *Analyt. Biochem.* **1**, 279.
Burge, B. W. & Pfefferkorn, E. R. (1966). *Virology*, **30**, 204.
Cartwright, K. L. & Burke, D. C. (1970). *J. Gen. Virol.* **6**, 231.
Dobos, P. & Faulkner, P. (1969). *J. Virol.* **4**, 429.
Dobos, P. & Faulkner, P. (1970). *J. Virol.* **6**, 145.
Dobos, P., Arif, B. M. & Faulkner, P. (1971). *J. Gen. Virol.* **10**, 103.
Eagle, H. (1959). *Science*, **130**, 432.
Fenwick, M. L. (1968). *Biochem. J.* **107**, 851.
Friedman, R. M. & Berezesky, I. K. (1967). *J. Virol.* **1**, 374.
Godson, G. N. & Sinsheimer, R. L. (1967). *Biochim. biophys. Acta*, **149**, 476.
Levin, J. G. & Friedman, R. M. (1971). *J. Virol.* **7**, 504.
McConkey, E. H. & Hopkins, J. W. (1969). *J. Mol. Biol.* **39**, 545.
Mills, D. R., Peterson, R. L. & Spiegelman, S. (1967). *Proc. Nat. Acad. Sci., Wash.* **58**, 217.
Mudd, J. A. & Summers, D. F. (1970). *Virology*, **42**, 328.
Parish, J. H. & Kirby, K. S. (1966). *Biochim. biophys. Acta*, **129**, 554.
Peacock, A. C. & Dingman, C. W. (1968*a*). *Biochemistry*, **6**, 1818.
Peacock, A. C. & Dingman, C. W. (1968*b*). *Biochemistry*, **7**, 668.
Penman, S. (1966). *J. Mol. Biol.* **17**, 117.
Pfefferkorn, E. R., Burge, B. W. & Coady, H. M. (1967). *Virology*, **33**, 239.
Pfefferkorn, E. R. & Hunter, H. S. (1963). *Virology*, **20**, 433.
Scheele, C. M. & Pfefferkorn, E. R. (1969). *J. Virol.* **4**, 117.
Sedat, J., Lyon, A. & Sinsheimer, R. L. (1969). *J. Mol. Biol.* **44**, 415.
Simmons, D. T. & Strauss, J. H. (1972). *J. Mol. Biol.* **71**, 615.
Sonnabend, J. A., Dalgarno, L., Friedman, B. M. & Martin, E. M. (1964). *Biochem. Biophys. Res. Comm.* **17**, 455.
Sreevalsan, T. & Lockart, R. Z., Jr. (1966). *Proc. Nat. Acad. Sci., Wash.* **55**, 974.
Sreevalsan, T., Lockart, R. Z., Jr., Dodson, M. L., Jr. & Hartman, K. A. (1968). *J. Virol.* **2**, 558.
Stanley, W. M. & Bock, R. M. (1965). *Biochemistry*, **4**, 1302.
Strauss, J. H. (1967). Ph.D. Thesis, California Institute of Technology, Pasadena, California.
Strauss, J. H., Kelly, R. B. & Sinsheimer, R. L. (1968). *Biopolymers*, **6**, 793.
Weissman, C. (1965). *Proc. Nat. Acad. Sci., Wash.* **54**, 202.
Yin, F. H. & Lockart, R. Z., Jr. (1968). *J. Virol.* **2**, 728.

CHAPTER 2

Replication of Sindbis Virus: Polyribosomes and Messenger RNA in Infected Cells

by

Daniel T. Simmons and James H. Strauss

This chapter has been prepared
for publication in
The Journal of Virology

SUMMARY

Cells infected with wild-type Sindbis virus contain at least two forms of messenger RNA, 26S RNA and 49S RNA. Sindbis 26S RNA (mol. wt. 1.6 x 10^6 daltons) constitutes 90% by weight of the mRNA in infected cells and is thought to specify the structural proteins of the virus. Sindbis 49S RNA, the viral genome (mol. wt. 4.3 x 10^6 daltons), constitutes approximately 10% of the mRNA in infected cells and is thought to supply the remaining viral functions.

In cells infected with ts2, a temperature-sensitive mutant of Sindbis virus, the messenger forms are 26S RNA and a second species of RNA with a sedimentation coefficient of 33S and an apparent molecular weight of 2.3 x 10^6 daltons. Hybridization-competition experiments showed that 90% of the base sequences in 33S RNA from these cells are also present in 26S RNA. Sindbis 33S RNA was also isolated from cells infected with wild type virus. After reaction with formaldehyde, this species of 33S RNA appeared to be completely converted to 26S RNA. These results indicate that 33S RNA isolated from cells infected with either wild-type Sindbis or ts2 is not a unique and separate form of Sindbis RNA.

INTRODUCTION

In cells infected with group A arboviruses (alphaviruses), at least two species of virus-specific single-stranded RNA are manufactured (14, 15, 4). In the case of Sindbis virus, these two species of RNA have sedimentation coefficients of 49S and 26S. Sindbis 49S RNA is the viral genome and has a molecular weight of $4.3 \pm 0.3 \times 10^6$ daltons (3, 12). Sindbis 26S RNA has a molecular weight of 1.6×10^6 daltons and consists of one-third of the viral genome (12).

It has been determined that 26S RNA functions as a messenger in the infected cell (5, 7, 11). Since 26S RNA has only one-third of the coding capacity of the genome, it is expected that the other two-thirds of the viral functions are specified by other species of Sindbis-specific messenger RNA. Kennedy (5) and Mowshowitz (7) described a second species of Sindbis messenger RNA having a sedimentation coefficient of 33S and a molecular weight of 2.2 to 2.4×10^6 daltons. Rosemond and Sreevalson (11) described several species of messenger RNA smaller than 26S RNA in infected cells. In an earlier publication (13), we obtained indirect evidence for the existence of a species of single-stranded RNA with a molecular weight of 2.8×10^6 daltons which contains two-thirds of the base sequence information present in 49S RNA but none of the information present in 26S RNA. We hypothesized that this species of RNA functions as a messenger in infected cells.

In this paper, we support the evidence that 26S RNA is the predominant messenger in cells infected with Sindbis virus and we show that 49S RNA, the viral genome, serves as the second form of

messenger in infected cells. We also present evidence that Sindbis 33S RNA is a structural variant of 26S RNA and not a separate species of Sindbis-specific RNA. No other unique species of RNA was detected in preparations of messenger RNA.

MATERIALS AND METHODS

Except as described below, the materials and methods used in these experiments have been previously described (12). These include the methods for sucrose gradient- and formaldehyde sucrose gradient-velocity sedimentation, analytical and preparative acrylamide gel electrophoresis and hybridization-competition.

Isolation of Sindbis-specific polysomes. Chick embryo fibroblasts were infected with Sindbis virus as previously described (12). The cells were labeled with 5-^{3}H-uridine (specific activity 22.4 Ci/mmole) for various periods of time and/or with ^{14}C-amino acids (Schwarz hydrolysate, specific activity 50 mCi/mmole) for 2 min. In some cases, the cells were treated with 50 μg/ml of cycloheximide (Nutritional Biochemicals Corporation) for 7 min prior to harvesting the infected cells.

The flask of cells was rapidly cooled to 0 C in an ice-water slurry and the cells washed three times in ice cold 0.01 M Tris, 0.01 M KCl and 0.0015 M $MgCl_2$ (pH 7.4). The cell monolayer was scraped off the surface of the flask with a rubber policeman into 0.5 ml of the same buffer. After 10 min at 0 C to allow the cells to swell, the cells were homogenized with 10 to 15 strokes of a glass Dounce homogenizer. Nuclei and unbroken cells were pelleted at 1000 g

for 8 min at 0 C. The supernatant was made 0.5% with respect to deoxycholate (Matheson, Coleman & Bell) and sedimented at 10,000 g for 10 min to remove cell fragments and undissolved membranes. The clarified supernatant containing the polysomes as well as other cytoplasmic material was sedimented in a linear 15 to 40% sucrose gradient in the above buffer for 2 hr 15 min (unless otherwise stated) at 40,000 rev/min and 1.5 C in the Spinco SW40 or SW41 rotor.

If the gradient was to be analyzed for radioactivity, it was first pumped through a flow-cell in a Gilford recording spectrophotometer to determine the position of the monosome band. The material in each fraction was precipitated with trichloroacetic acid and counted in toluene-fluor scintillation fluid.

If the labeled RNA in the gradient containing Sindbis-specific polysomes was to be analyzed by velocity sedimentation, the gradient was first fractionated directly into 2 ml beakers containing 20 µl of of 10% SDS and the RNA from selected fractions was subjected to sucrose gradient-velocity sedimentation (13) for 4.5 hr at 40,000 rev/min in the Spinco SW41 rotor.

Preparation of messenger RNA from infected cells. To prepare messenger RNA, polysomes were sedimented through a sucrose gradient such that those consisting of more than 10 ribosomes were pelleted in the centrifuge tube. The gradient above the pellet was carefully removed by aspiration and the pellet was resuspended in 0.2 ml of 0.01 M Tris, 0.01 M KCl and 0.0015 M $MgCl_2$ (pH 7.4). The solution was made 0.01 M in EDTA to release the messenger RNA from ribosomes and 100 µg/ml in dextran sulfate (Sigma Chemical Co.) to inhibit RNase.

To separate the released mRNA from nucleocapsids present in the polysomal pellet, the material was sedimented in a sucrose gradient as described above for the sedimentation of polysomes with the exception that the time of centrifugation was increased to 3 hr. The gradient was collected in beakers containing 20 μl of 10% SDS and an aliquot of each fraction was counted for radioactivity. The RNA in the pooled fractions was made 0.2 M in K acetate (pH 6.0) and precipitated with 2.5 volumes of ethanol overnight at -20 C. The RNA was resuspended in 0.01 M Tris, 0.06 M NaCl, 0.001 M EDTA and 1% SDS (pH 7.2), extracted twice with phenol-chloroform (8) at 0 C and twice more at room temperature. The RNA was then subjected to electrophoresis in an acrylamide-agarose gel as previously described (12).

Preparation of Sindbis 33S RNA. Sindbis 33S RNA was prepared from chick cells infected with either wild-type Sindbis virus or ts2.

When the source of the 33S RNA was cells infected with wild-type Sindbis (HR), total cellular RNA was prepared as previously described (12, Method A, but without diethyl oxydiformate). The RNA was subjected to sedimentation in a 15-30% sucrose gradient in 0.01 M Tris, 0.06 M NaCl, 0.001 M EDTA, 0.2% SDS (pH 7.2) for 4.5 hr at 40,000 rev/min and 23 C in the Spinco SW41 rotor, and the fractions corresponding to Sindbis 26S RNA and 33S RNA were pooled together. The RNA was precipitated with ethanol and the resuspended RNA was subjected to preparative gel electrophoresis (12). The fractions containing 33S RNA were combined and the RNA was concentrated by ethanol precipitation.

When the source of the 33S RNA was cells infected with ts2 at 39 C, polysomes were prepared and subjected to sedimentation as described above. The fractions containing polysomes with 4 to 10 ribosomes were made 1% with respect to SDS and the samples were extracted with phenol-chloroform (8). The species of 33S RNA was purified by electrophoresis in a preparative acrylamide gel (12).

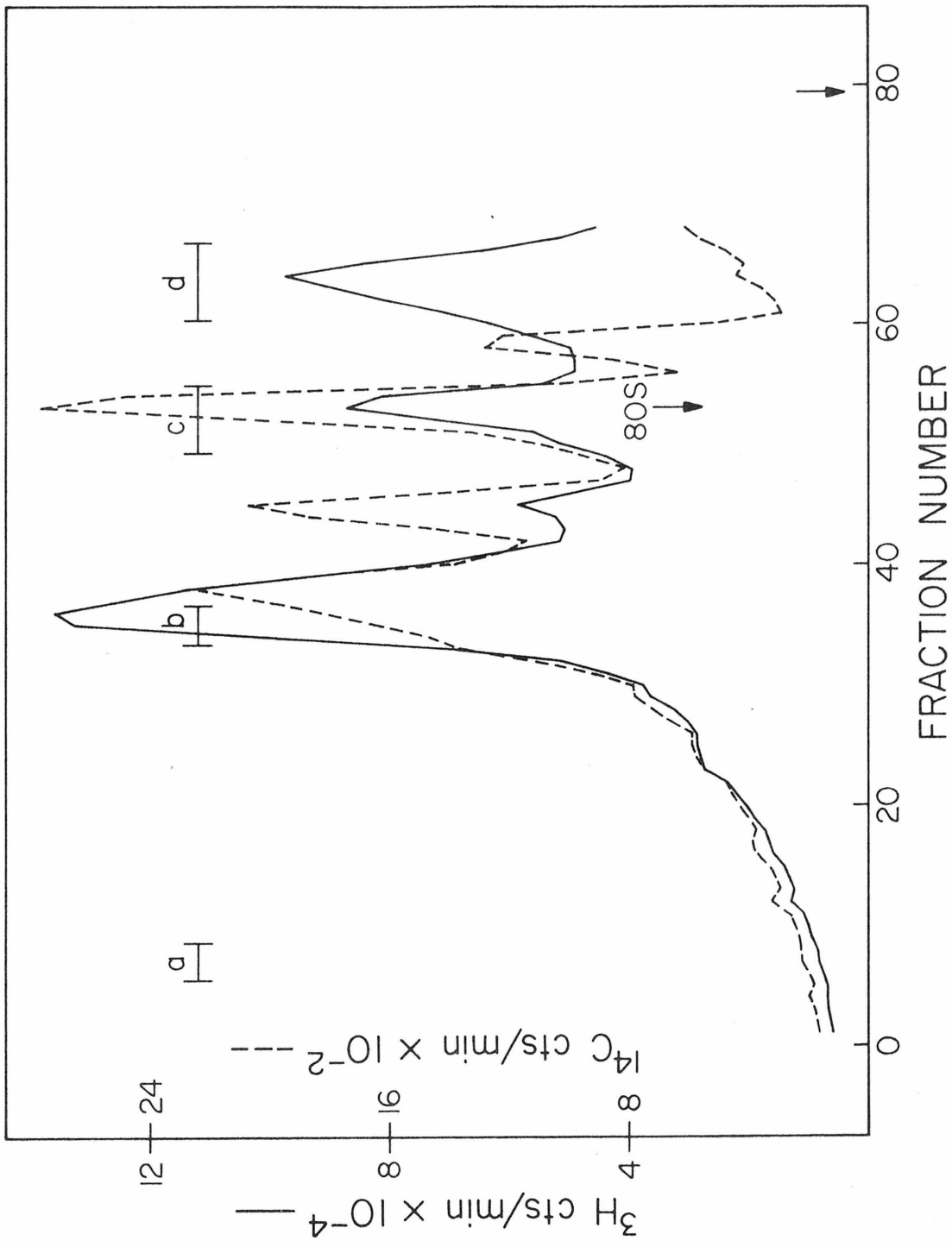
Preparation of viral nucleocapsids. Nucleocapsids were isolated from detergent-treated Sindbis virus which had been purified as previously described (9). The virus solution was diluted 3-fold in 0.01 M Tris, 0.01 M KCl and 0.005 M EDTA (pH 7.4) and Triton X-100 (Sigma Chemical Co.) was added to a final concentration of 0.5%. The nucleocapsids were sedimented in a 15 to 40% sucrose gradient in the same buffer for 3 hr at 40,000 rev/min and 1.5 C in the Spinco SW40 rotor. The fractions containing the nucleocapsids were pooled and stored frozen.

RESULTS

Polysomes of infected cells. Polysomes prepared from cells infected with Sindbis virus served as the source of the messenger RNA in these experiments. It was therefore important to analyze the size of these polysomes and to determine if they were involved in protein synthesis.

When cells infected with wild-type (HR strain) Sindbis virus were briefly labeled for 2 min with ^{14}C-labeled amino acids, the sedimentable radioactivity was associated primarily with monosomes, disomes, and trisomes (Fig. 1) indicating that these structures were

FIG. 1. Polysomes from cells infected with wild-type Sindbis virus. 1.5 x 10^7 chick embryo cells were infected with wild-type (HR) Sindbis virus and labeled with 20 μCi/ml of 5-^{3}H-uridine from 0.5 to 4.5 hr after infection and with 100 μCi/ml of ^{14}C-amino acids (Schwarz hydrolysate) for 2 min prior to cell harvesting. The polysomes were prepared and sedimented as described in Materials and Methods. The bars labeled a, b, c, or d indicated in the figure refer to certain fractions which were pooled for analysis of the labeled RNA (see Fig. 4). The arrow on the right in this and all other figures represents the top of the gradient. Symbols: (———) ^{3}H-uridine label, (- - - - -) ^{14}C-amino acid label.

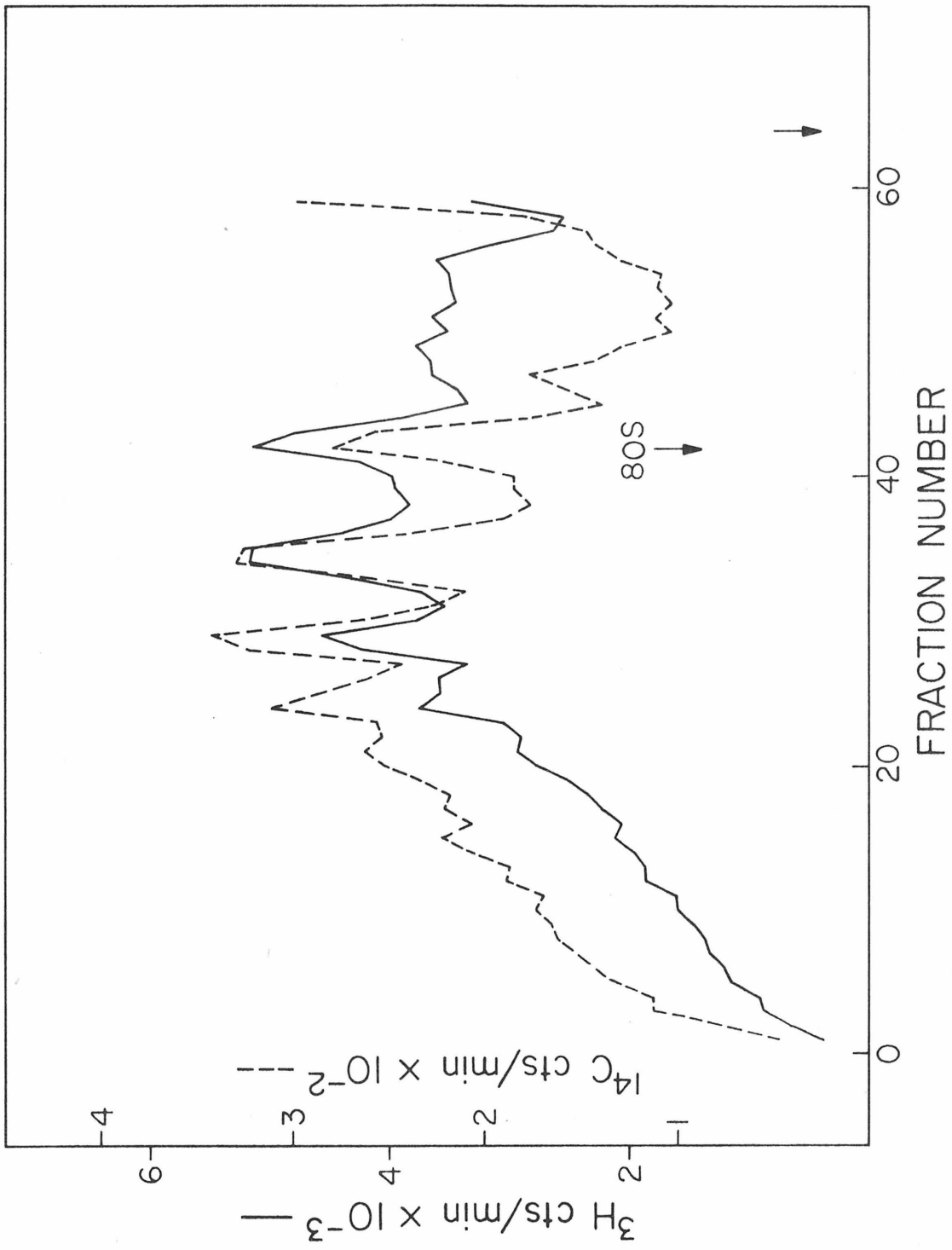

^{3}H cts/min x 10^{-4} ——
^{14}C cts/min x 10^{-2} ---
12
8
4
24
16
8
0
20
40
60
80
FRACTION NUMBER
a
b
c
d
80S

involved in protein synthesis. Smaller amounts of amino acid label were incorporated into larger polysomes. The polysomes were also labeled with ^{3}H-uridine which was added at 30 min after infection in the presence of actinomycin D (in order to label only virus-specific RNA). In addition, the uridine label was incorporated into viral nucleocapsids which sediment at 140S. In these gradients, most of the nucleocapsids sedimented slightly faster than trisomes and at the position shown by bar b in Fig. 1. Little amino acid label was associated with nucleocapsids after only 2 min of labeling.

In cells infected with certain mutants of Sindbis virus (ts2, ts5, ts13, and ts106), the synthesis of viral nucleocapsids is temperature sensitive (1, 2; E. Strauss, personal communication). When polysomes were prepared from cells infected with ts2 at the restrictive temperature, uridine label was found in polysomes as expected, but little or no uridine had been incorporated into nucleocapsids (Fig. 2) (nucleocapsids, if present, would be found near fraction 27 in this gradient). These polysomes were also labeled with amino acids in a 2 min pulse showing that all classes of ts2 polysomes were involved in protein synthesis.

Since polysome preparations from cells infected with ts2 were free from large amounts of nucleocapsids, we were able to calculate the relative amounts of ^{14}C-amino acids to ^{3}H-uridine incorporated into each class of polysomes. Thus, in Fig. 2, the ratio of ^{14}C-amino acid to ^{3}H-uridine label increases as the polysomes get larger. This is expected on the basis that larger polysomes synthesize a greater total length of polypeptide chains per unit length of messenger RNA than do

FIG. 2. Polysomes from cells infected with ts2. The experimental procedures were as described in the legend to Fig. 1 with the exception that the cells were infected with ts2 at 39 C. Symbols: (———) ^{3}H-uridine label, (-----) ^{14}C-amino acid label.


^{3}H cts/min x 10^{-3} ——
^{14}C cts/min x 10^{-2} ---
FRACTION NUMBER
80S
0
20
40
60
2
4
6
1
2
3
4

smaller polysomes. In Fig. 1; the ^{14}C to ^{3}H ratio in polysomes cannot be calculated due to the presence of large amounts of nucleocapsids in the gradient (see below). However, these results suggest that polysomes from cells infected with wild-type Sindbis virus are smaller than polysomes from cells infected with ts2.

Furthermore, to show that polysomes were not degraded during the isolation procedure, we prepared polysomes from uninfected cells after a brief labeling period with amino acids. Most of the sedimentable radioactivity was associated with large polysomes consisting of 5 or more ribosomes while relatively little label was in monosomes or disomes (Fig. 3). Moreover, as shown below, the RNA isolated from polysomes of infected cells was usually undegraded. These results indicate that, under the conditions of infection, polysomes from cells infected with either wild-type Sindbis or ts2 were smaller than those from uninfected cells.

Characterization of the RNA from gradients of Sindbis-specific polysomes. Polysomes labeled with ^{3}H-uridine were prepared from cells infected with wild-type (HR) Sindbis virus and subjected to velocity sedimentation in a sucrose gradient. In this gradient, the profile of ^{3}H radioactivity was similar to the one shown in Fig. 1. Four different samples of this gradient were pooled as shown by the bars labeled a, b, c, or d in Fig. 1, and the structures in these samples were disrupted with SDS. The RNA in each sample was sedimented in a linear sucrose gradient in the presence of E. coli rRNA (Fig. 4). Sample (a) which apparently consisted of large polysomes and sample (b) which consisted of trisomes, tetrasomes and viral nucleocapsids

FIG. 3. Polysomes from uninfected cells. A culture of uninfected chick embryo cells was labeled with 100 μCi/ml of ^{3}H-amino acids (Schwarz hydrolysate) for 2 min prior to cell harvesting. The polysomes were subjected to velocity sedimentation as described in the legend to Fig. 1 with the exception that the time of centrifugation was reduced to 2 hr.

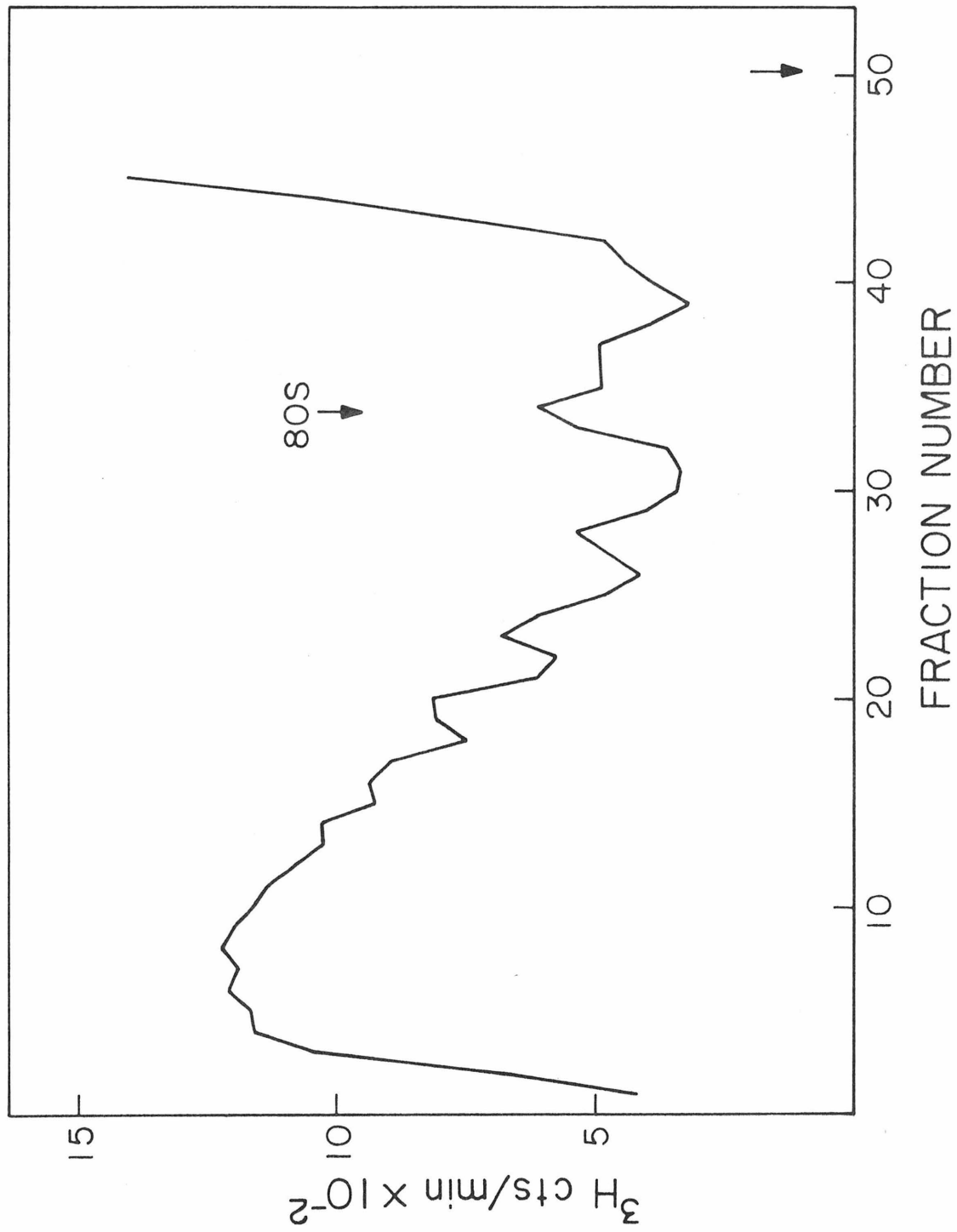
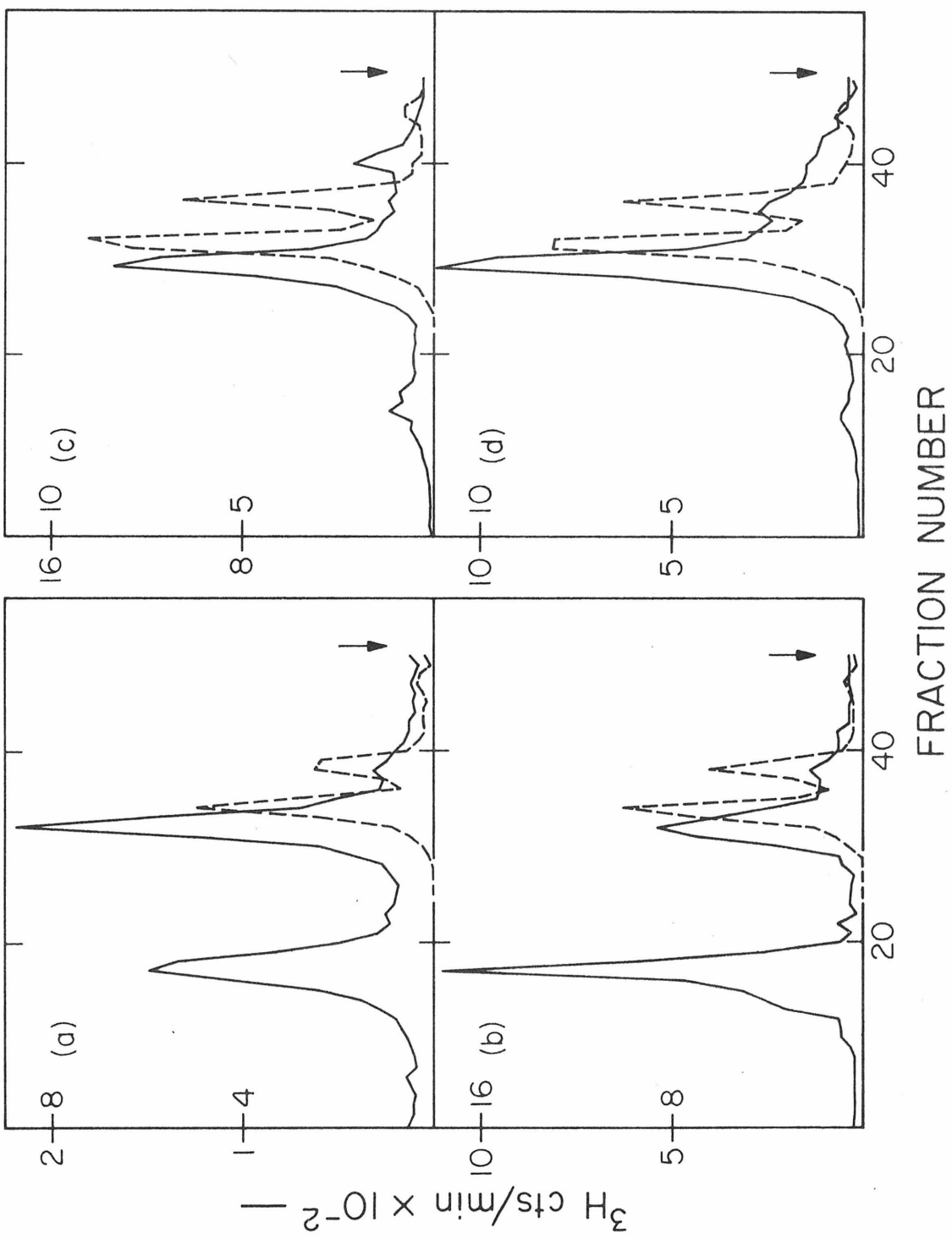

80S
15
10
5
^{3}H cts/min x 10^{-2}
10
20
30
40
50
FRACTION NUMBER

FIG. 4. Characterization of the RNA from gradients of Sindbis-specific polysomes. Chick cells infected with wild-type Sindbis virus were labeled with 20 μCi/ml of 5-^{3}H-uridine from 0.5 to 4.5 hr post-infection. The polysomes were prepared and sedimented, and the gradient was collected for analysis of the RNA as described in Materials and Methods. The RNA in each of the 4 samples, corresponding to the regions of the gradient shown by bars a, b, c, or d in Fig. 1, was subjected to sedimentation in a 15-30% sucrose gradient at 40,000 rev/min and 23 C for 4.5 hr in the Spinco SW41 rotor using ^{14}C-labeled E. coli rRNA as markers. Symbols: Fig. 4(a) to (d) (———) ^{3}H-labeled Sindbis-specific RNA in samples (a), (b), (c), and (d) respectively, (-----) ^{14}C-labeled E. coli rRNA.


(a)
(b)
(c)
(d)
^{3}H cts/min x 10^{-2} —
^{14}C cts/min x 10^{-2} ---
FRACTION NUMBER
20
40

contained both Sindbis 26S RNA and 49S RNA [Fig. 4(a) and (b)]. On the other hand, samples (c) and (d) which consisted of monosomes and slower sedimenting structures, respectively, contained primarily 26S RNA [Fig. 4(c) and (d)]. The interpretation of these results is complicated by the presence of large amounts of nucleocapsids which sedimented faster than 140S throughout the bottom half of the gradient containing polysomes. (For example, it was shown that after EDTA treatment and resedimentation of the material in sample (a) of Fig. 1, 35% of the ^{3}H-label was in viral nucleocapsids.) This observation and the data shown in Fig. 4 probably indicate that the large majority of the 49S RNA in these gradients of Sindbis-specific polysomes was in the form of nucleocapsids whereas most of the 26S RNA was associated with ribosomes.

The RNA in each of the samples (a) through (d) was treated with RNase to detect the presence of labeled double-stranded RNA. Only sample (d) contained RNase-resistant RNA, and this was shown to consist of the three forms of Sindbis RF's (13) (results not shown). The conclusion was that only sample (d) contained the Sindbis replicative intermediates.

Messenger RNA in cells infected with wild-type Sindbis virus. In order to isolate messenger RNA, polysomes from cells infected with wild-type Sindbis virus were sedimented such that those consisting of more than 10 ribosomes were pelleted in the centrifuge tube. For this experiment, the cells were treated with cycloheximide to increase the average size of the polysomes and to reduce the relative amounts of nucleocapsids in the pelleted material. The polysomes in the pellet

were dissociated with EDTA and the components sedimented in a sucrose gradient to separate released RNA (i.e., mRNA) from nucleocapsids (Fig. 5). The polysomal pellet contained relatively little uridine label in nucleocapsids as shown by the small amounts of ^{3}H radioactivity sedimenting with the nucleocapsid marker. Fig. 5 shows as well that the RNA released by EDTA was associated with structures (presumably RNA-protein complexes) sedimenting at approximately 35S to 40S (sample I) and at 60S to 70S (sample II). After deproteinization, the RNA in each of samples I and II was subjected to acrylamide gel electrophoresis (Fig. 6). Sample I consisted primarily of 26S RNA [Fig. 6(a)] whereas 49S RNA was the predominant species of RNA in sample II [Fig. 6(b)]. Other species of Sindbis mRNA could possibly be present in sample II since another band (or bands) could be hidden under the leading side of the 49S RNA peak in Fig. 6(b). Approximately 10% of the label in the mRNA fraction (samples I and II) was in 49S RNA. These results indicate that 26S RNA is the predominant species of messenger RNA in infected cells but that 49S RNA has messenger activity as well.

Messenger RNA in cells infected with ts2. Since the virus-specific polypeptides synthesized in cells infected with ts2 are different in size from the polypeptides synthesized in cells infected with wild-type Sindbis virus (16), we characterized the species of messenger RNA in ts2-infected cells. The messenger RNA forms were subjected to electrophoresis in an acrylamide gel using purified Sindbis 26S RNA as marker (Fig. 7). They consisted primarily of 26S RNA and of small amounts of another species of RNA with an apparent

FIG. 5. Disruption of polysomes with EDTA. 1.5×10^7 chick embryo cells were infected with wild-type (HR) Sindbis virus and labeled with 20 μCi/ml of 5-^{3}H-uridine from 3 to 5 hr after infection. Seven min prior to cell harvesting, the medium was made 50 μg/ml with respect to cycloheximide to increase the average size of the polysomes. The polysomes were sedimented at 40,000 rev/min for 2 hr 15 min and 1.5 C in a Spinco SW40 rotor and the polysomes in the pellet were treated with EDTA. The material was subjected to velocity sedimentation as described in Materials and Methods. ^{14}C-labeled purified Sindbis nucleocapsids were added as a marker. Bars labeled I and II refer to certain fractions of the gradient which were pooled for analysis of the RNA by acrylamide gel electrophoresis (see Fig. 6). Symbols: (———) ^{3}H-uridine label, (-----) ^{14}C-labeled Sindbis nucleocapsids.

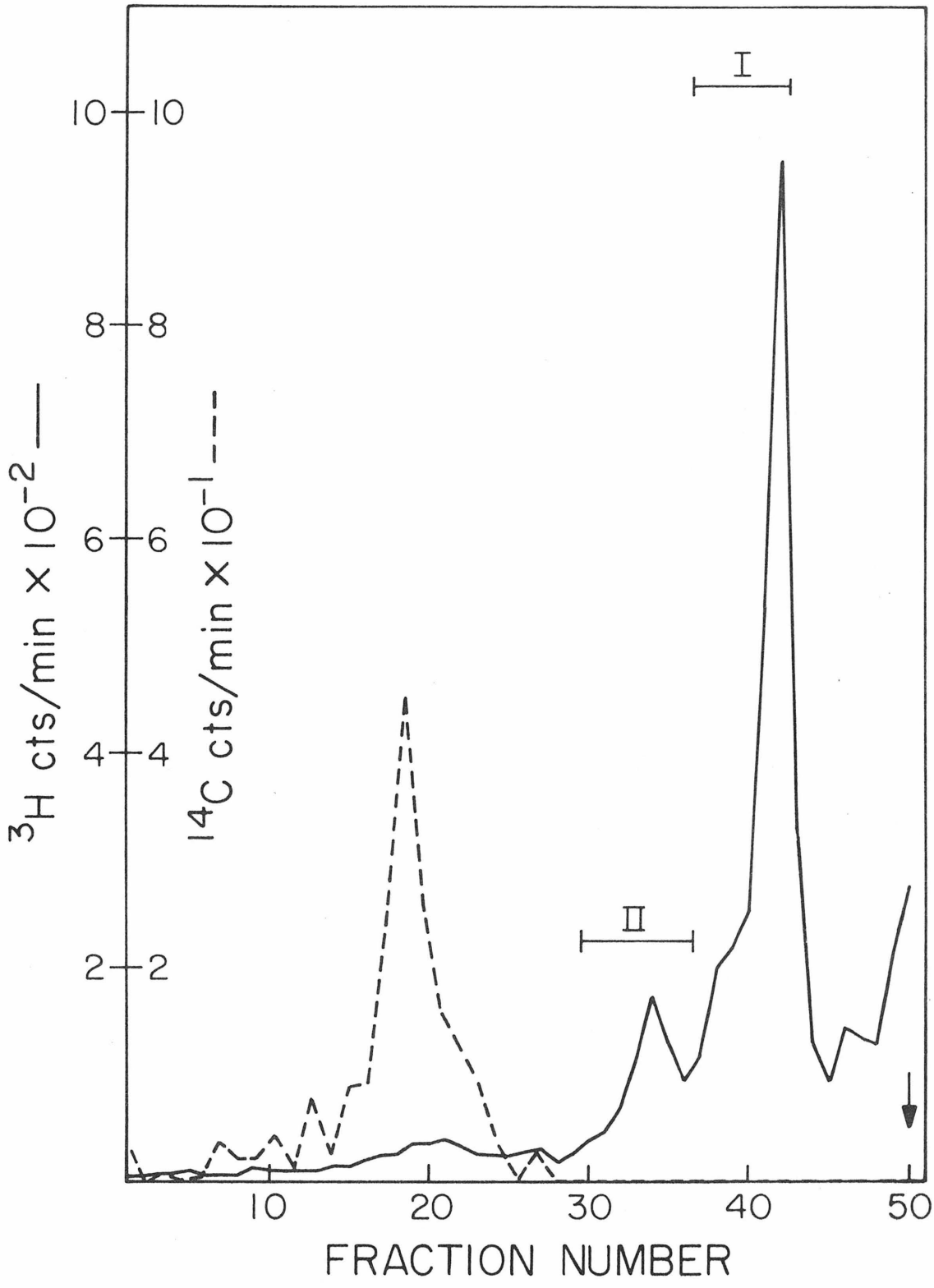

I
II
10
8
6
4
2
10
8
6
4
2
^{3}H cts/min X 10^{-2} ——
^{14}C cts/min X 10^{-1} ---
10
20
30
40
50
FRACTION NUMBER

FIG. 6. Messenger RNA from cells infected with wild-type Sindbis virus. The RNA in each of samples I and II shown in Fig. 5 was subjected to acrylamide gel electrophoresis as described in Materials and Methods. ^{14}C-labeled E. coli rRNA were added as markers and the direction of electrophoresis was from left to right. Symbols: Fig. 6(a) and (b) (——) ^{3}H-labeled Sindbis mRNA in sample I and in sample II respectively, (- - - -) ^{14}C-labeled E. coli rRNA.

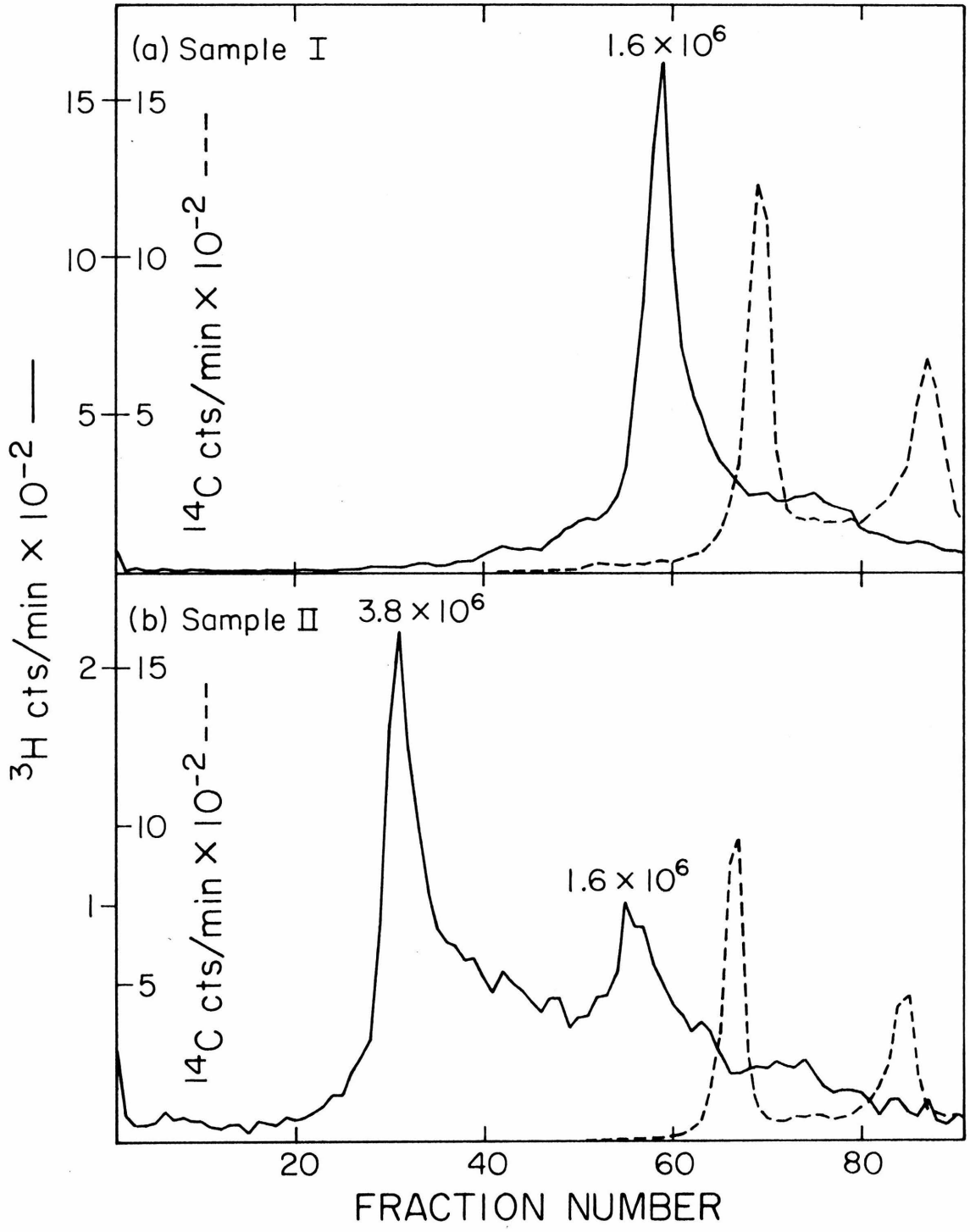
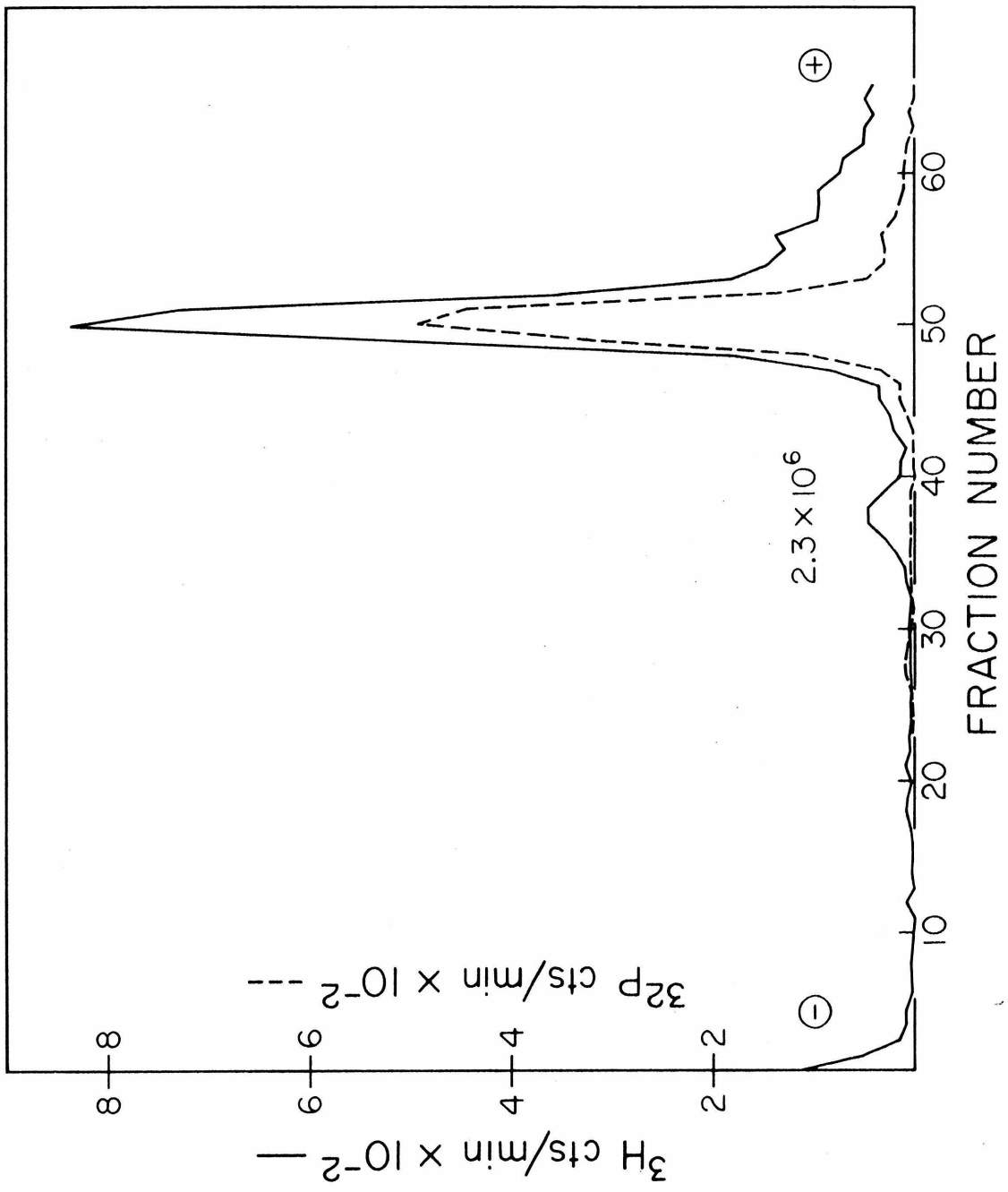

(a) Sample I
1.6 × 10^6
15
10
5
15
10
5
^{14}C cts/min × 10^{-2} ---
^{3}H cts/min × 10^{-2} —
(b) Sample II
3.8 × 10^6
1.6 × 10^6
2
1
15
10
5
^{14}C cts/min × 10^{-2} ---
20
40
60
80
FRACTION NUMBER

FIG. 7. Messenger RNA from cells infected with ts2. Chick cells infected with ts2 at 39 C were labeled with 20 μCi/ml of 5-^{3}H-uridine from 0.5 to 3.25 hr postinfection. The messenger RNA fraction was isolated as described in Materials and Methods and the RNA was subjected to acrylamide gel electrophoresis using purified ^{32}P-labeled Sindbis 26S RNA as marker. Symbols: (———) ^{3}H-labeled ts2 mRNA, (-----) ^{32}P-labeled purified Sindbis 26S RNA.

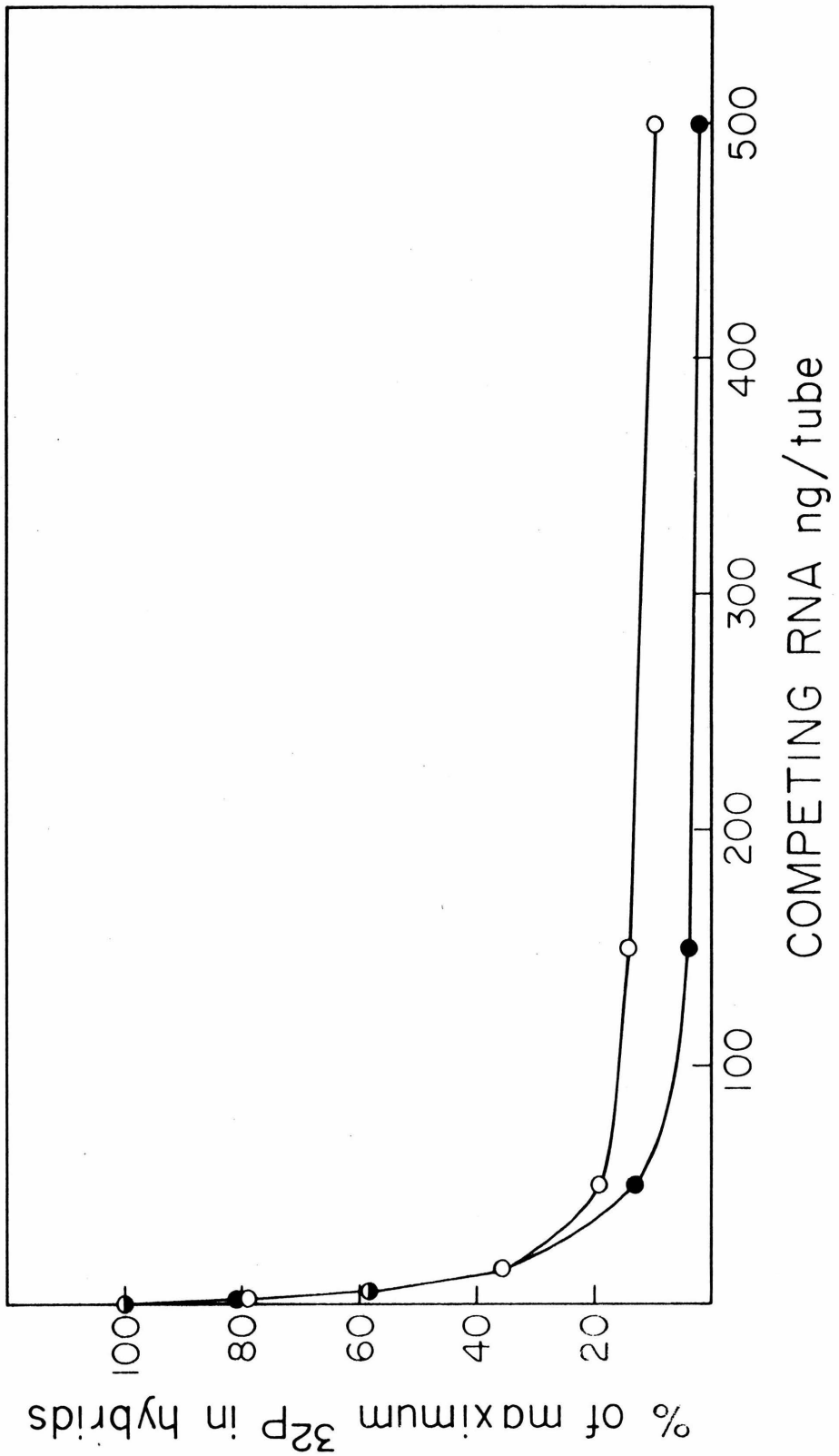

^{3}H cts/min x 10^{-2} ——
^{32}P cts/min x 10^{-2} ---
2
4
6
8
10
20
30
40
50
60
FRACTION NUMBER
2.3×10^6
⊖
⊕

molecular weight of 2.3×10^6 daltons. This second species of RNA has been detected previously in cells infected with Semliki Forest virus or with Sindbis virus (6) and in mRNA preparations from these infected cells (4, 7). Unlike mRNA from cells infected with wild-type Sindbis, little 49S RNA was detected in messenger RNA from cells infected with ts2.

Characterization of Sindbis 33S RNA. The species of RNA with a molecular weight of 2.3×10^6 daltons (33S RNA) was isolated from polysomes of cells infected with ts2 at the restrictive temperature and purified by preparative gel electrophoresis. A hybridization-competition experiment using 33S RNA was performed to determine whether or not this form of Sindbis-specific RNA was separate and distinct from Sindbis 26S RNA. When 33S RNA was hybridized to Sindbis double-stranded RNA, unlabeled 26S RNA was able to effectively compete with it for the sites on the complementary strand of the double-stranded RNA (Fig. 8). Only about 10% of the base sequences in 33S RNA were not in competition with excess unlabeled 26S RNA. The curve for the control experiment where 26S RNA was hybridized to Sindbis double-stranded RNA in the presence of increasing amounts of unlabeled 26S RNA is also shown in Fig. 8. These results indicate that at least 90% of the base sequences in 33S RNA from cells infected with ts2 are also present in 26S RNA.

If, as the hybridization-competition results indicate, 33S RNA is a structural variant of 26S RNA, denaturation of this form of RNA should convert it to 26S RNA. For the following experiment, 33S RNA was isolated from total cellular RNA after infection with wild-type

FIG. 8. Hybridization-competition experiments with 33S RNA. 10^8 chick embryo cells were infected with ts2 at 39 C and labeled with 250 μCi/ml of $H_3{}^{32}PO_4$ from 0.5 to 4.5 hr postinfection. The 33S RNA was isolated from polysomes as described in Materials and Methods. The annealing mixtures contained 20 ng of double-stranded RNA (12), 2 ng of ^{32}P-labeled 26S or 33S RNA and various amounts of unlabeled purified 26S RNA. The RNA was hybridized as previously described (12). Symbols: (O——O) Hybridization of ^{32}P-labeled 33S RNA, (●——●) Hybridization of ^{32}P-labeled 26S RNA.


% of maximum 32P in hybrids
100
80
60
40
20
100
200
300
400
500
COMPETING RNA ng/tube

Sindbis virus and was purified by electrophoresis in a preparative acrylamide gel. The native form of the RNA was subjected to sucrose gradient-velocity sedimentation with E. coli rRNA as markers (Fig. 9). In agreement with its size estimated by gel electrophoresis, the RNA had a sedimentation coefficient of 33.5S. However, when the purified RNA was denatured by heating in formaldehyde, it sedimented with an apparent molecular weight of only 1.4×10^6 daltons (Fig. 10). After reaction with formaldehyde, Sindbis 26S RNA sedimented with this same apparent molecular weight (12). It appears, therefore, that when 33S RNA from cells infected with wild-type Sindbis virus was denatured with formaldehyde, it was converted to 26S RNA.

DISCUSSION

Polysomes isolated from cells infected with either wild-type Sindbis virus or ts2 appear to be smaller, under the conditions of infection, than polysomes of uninfected cells. This is in contrast with the results obtained on polysomes of cells infected with poliovirus (10).

Sindbis 26S RNA is the predominant messenger in cells infected with either wild-type Sindbis or ts2. In wild-type infections, Sindbis 49S RNA serves as a messenger as well but to a lesser extent than 26S RNA. The majority of the 49S RNA in gradients containing Sindbis-specific polysomes was found in nucleocapsids whereas the majority of the 26S RNA was probably associated with ribosomes.

In vitro and, by inference, in infected cells, 26S RNA codes for the structural proteins of the virus (D. T. Simmons and

FIG. 9. Sedimentation of 33S RNA in sucrose gradients. Chick cells were infected with wild-type (HR) Sindbis virus and labeled with 5-^{3}H-uridine from 0.5 to 4.5 hr postinfection. The 33S RNA was isolated from total cellular RNA as described in Materials and Methods and subjected to velocity sedimentation as described in the legend to Fig. 4. Symbols: (———) ^{3}H-labeled 33S RNA, (- - - - -) ^{14}C-labeled E. coli rRNA.

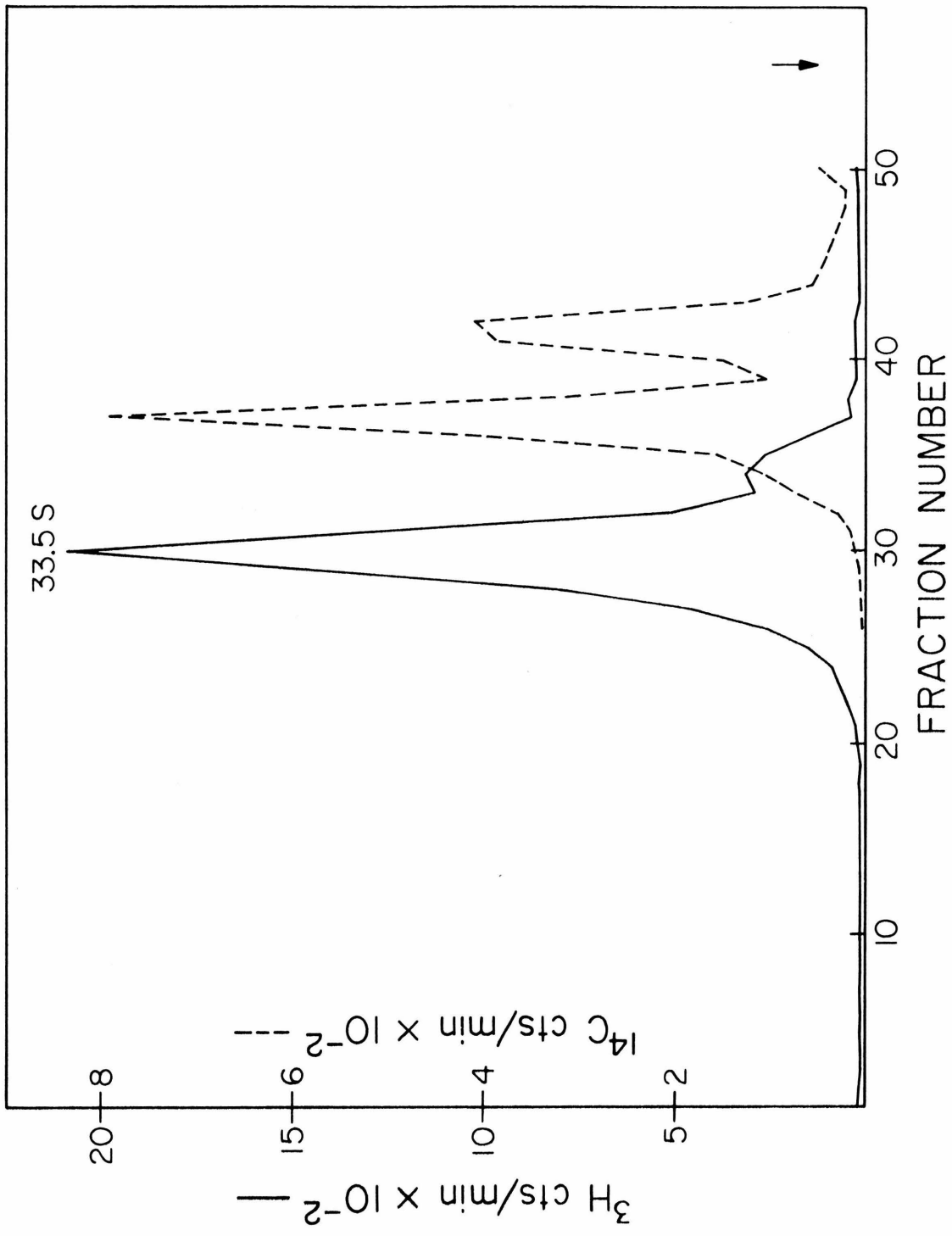
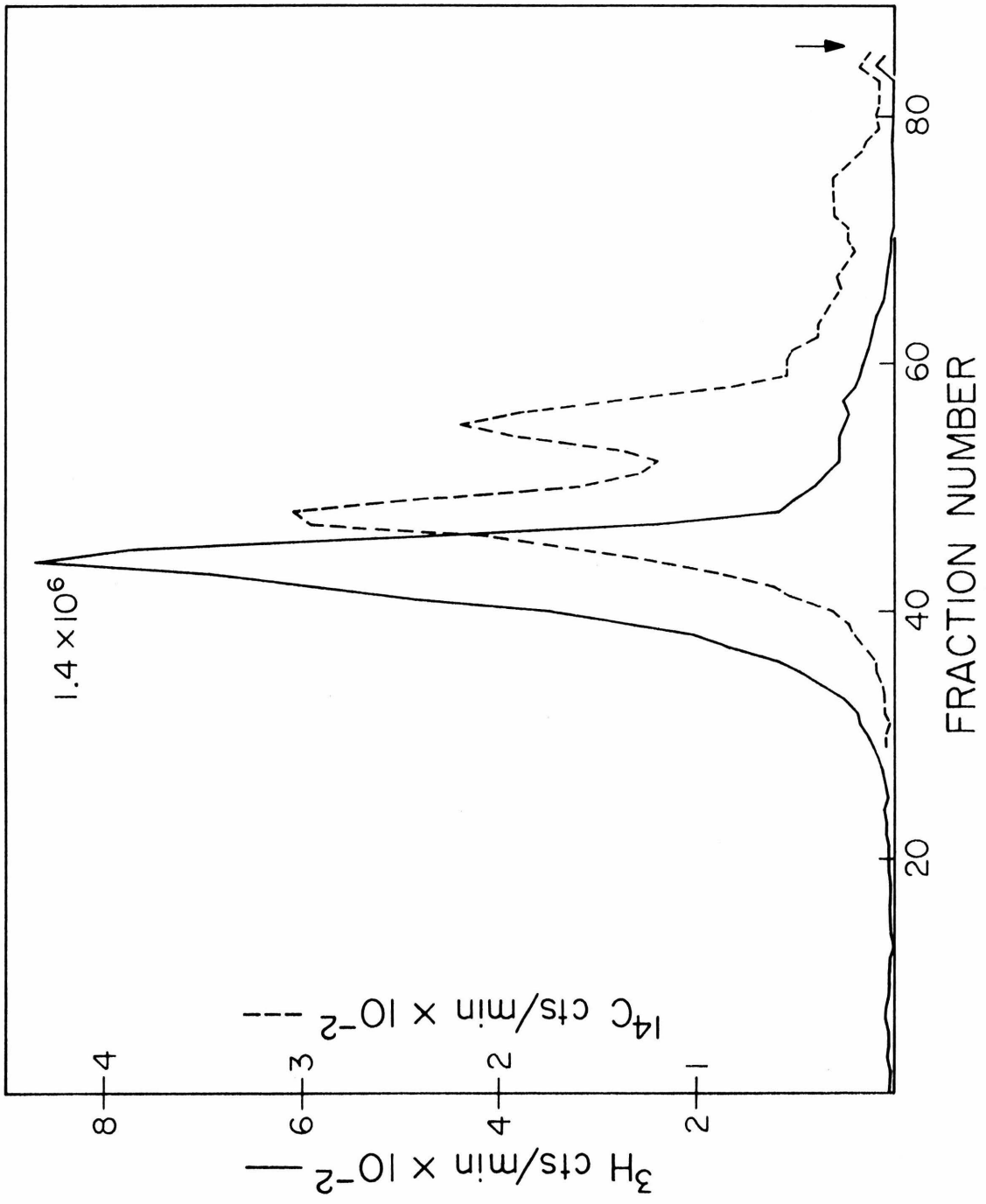

33.5 S
FRACTION NUMBER
10
20
30
40
50
^{3}H cts/min x 10^{-2} ——
^{14}C cts/min x 10^{-2} ---
5
10
15
20
2
4
6
8

FIG. 10. Sedimentation of 33S RNA after reaction with formaldehyde. 33S RNA was prepared as described in the legend to Fig. 8, denatured by heating in the presence of formaldehyde (12) and sedimented in a formaldehyde-sucrose gradient for 10.5 hr at 40,000 rev/min and 23 C in the Spinco SW40 rotor. Formaldehyde-treated E. coli rRNA were used as markers. Symbols: (———) ^{3}H-labeled formaldehyde-treated 33S RNA, (-----) ^{14}C-labeled formaldehyde-treated E. coli rRNA.


1.4 x 10^6
3H cts/min x 10^-2 ——
14C cts/min x 10^-2 ---
2
4
6
8
1
2
3
4
20
40
60
80
FRACTION NUMBER

J. H. Strauss, in press; R. Cancedda and M. J. Schlesinger, in press). In infected cells, the remaining viral functions seem to be coded for by 49S RNA. The proteins specified by the 49S RNA genome are not known but they presumably include one or two RNA replicases and regulatory proteins needed in small quantities. In lysates of rabbit reticulocytes, 49S RNA specifies 8 or 9 polypeptides ranging in molecular weight from 60,000 to 180,000 daltons (D. T. Simmons and J. H. Strauss, in press). It is not known if these polypeptides are synthesized in infected cells. Our results indicate that 33S RNA isolated from infected cells (5, 6, 7) is not a unique species of Sindbis RNA. When 33S RNA isolated from cells infected with wild-type Sindbis virus was denatured with formaldehyde it appeared to be completely converted to 26S RNA. Hybridization-competition experiments indicate that 33S RNA from cells infected with ts2 shares 90% of its base sequences with 26S RNA. We hypothesize that 33S RNA from either source is either a structural variant of 26S RNA or a complex of 26S RNA in association with a nonribonucleic acid component.

In a previous publication (13), we presented a model for the replication of Sindbis-specific RNA which showed the synthesis of a hypothetical unique species of single-stranded RNA (molecular weight 2.8×10^6 daltons) equivalent to two-thirds of the genome. A rigorous search for this species of RNA has failed. We have abandoned the possibility that 33S RNA is the molecule in question. However, in that publication (13), we presented strong evidence that such a molecule is synthesized on Sindbis replicative intermediates. Therefore, if this

species of RNA is released from the replicative complex, it is most likely very rapidly degraded and does not accumulate.

ACKNOWLEDGEMENTS

We wish to acknowledge the expert technical assistance of Edith M. Lenches and Sharman E. S. Christoph in preparing the cultures of chick embryo fibroblasts. We also wish to thank Dr. Ellen G. Strauss for helpful suggestions in writing this manuscript. This investigation was supported in part by the National Science Foundation Grant No. GB 31763X and by the U. S. Public Health Service Grant No. GM 06965. One of us (DTS) was supported by the National Institutes of Health Training Grant No. 5-T1-GM-86.

LITERATURE CITED

1. Burge, B. W., and E. R. Pfefferkorn. 1966. Isolation and characterization of conditional-lethal mutants of Sindbis virus. Virology 30:204-213.

2. Burge, B. W., and E. R. Pfefferkorn. 1968. Functional defects of temperature-sensitive mutants of Sindbis virus. J. Mol. Biol. 35:193-205.

3. Dobos, P., and P. Faulkner. 1970. Molecular weight of Sindbis virus ribonucleic acid as measured by polyacrylamide gel electrophoresis. J. Virol. 6:145-147.

4. Friedman, R. M., B. H. Levy, and W. B. Carter. 1966. Replication of Semliki Forest virus: Three forms of viral RNA produced during infection. Proc. Nat. Acad. Sci. U.S.A. 56:440-446.

5. Kennedy, S. I. T. 1972. Isolation and characterization of the virus-specified RNA species found on membrane-bound polyribosomes of chick embryo cells infected with Semliki Forest virus. Biochem. Biophys. Res. Commun. 48:1254-1258.

6. Levin, J. G., and R. M. Friedman. 1971. Analysis of arbovirus ribonucleic acid forms by polyacrylamide gel electrophoresis. J. Virol. 7:504-514.

7. Mowshowitz, D. 1973. Identification of polysomal RNA in BHK cells infected by Sindbis virus. J. Virol. 11:535-543.

8. Penman, S. 1966. RNA metabolism in the HeLa cell nucleus. J. Mol. Biol. 17:117-130.

9. Pierce, J. P., E. G. Strauss, and J. H. Strauss. 1974. Effect of ionic strength on the binding of Sindbis virus to chick cells. J. Virol. in press.

10. Rich, A., S. Penman, Y. Becker, J. E. Darnell, and C. Hall. 1963. Polyribosomes: Size in normal and polio-infected HeLa cells. Science 142:1658-1663.

11. Rosemond, H., and T. Sreevalsan. 1973. Viral RNA's associated with ribosomes in Sindbis virus-infected HeLa cells. J. Virol. 11:399-415.

12. Simmons, D. T., and J. H. Strauss. 1972. Replication of Sindbis virus. I. Relative size and genetic content of 26S and 49S RNA. J. Mol. Biol. 71:599-613.

13. Simmons, D. T., and J. H. Strauss. 1972. Replication of Sindbis virus. II. Multiple forms of double-stranded RNA isolated from infected cells. J. Mol. Biol. 71:615-631.

14. Sonnabend, J., L. Dalgarno, R. M. Friedman, and E. M. Martin. 1964. A possible replicative form of Semliki Forest virus RNA. Biochem. Biophys. Res. Commun. 17:455-460.

15. Sreevalsan, T., and R. Z. Lockart, Jr. 1966. Heterogeneous RNA's occurring during the replication of Western Equine Encephalomyelitis virus. Proc. Nat. Acad. Sci. U.S.A. 55:974-981.

16. Strauss, J. H., B. W. Burge, and J. E. Darnell. 1969. Sindbis virus infection of chick and hamster cells: Synthesis of virus-specific proteins. Virology 37:367-376.

CHAPTER 3

Translation of Sindbis Virus 26S RNA and 49S RNA in Lysates of Rabbit Reticulocytes

by

Daniel T. Simmons and James H. Strauss

This chapter has been accepted for publication in the Journal of Molecular Biology

Summary

Sindbis virus-specific polypeptides were synthesized in lysates of rabbit reticulocytes in response to added 26S or 49S RNA. Sindbis 26S RNA was translated into as many as three polypeptides which comigrate in acrylamide gels with proteins found in infected cells.

Wild-type 26S RNA was translated primarily into two polypeptides which appear to be the Sindbis nucleocapsid protein (molecular weight 30,000 daltons) and the precursor of the two glycoproteins of the virion (molecular weight 100,000 daltons). A larger polypeptide (molecular weight 130,000 daltons) was synthesized in response to ts2 26S RNA a species of RNA which was isolated from cells infected with the ts2 mutant of Sindbis virus. This large polypeptide is apparently the protein which accumulates in cells infected with the mutant virus and which is thought to be a precursor of all three viral structural proteins.

These results support the hypothesis that 26S RNA is the messenger for the three structural proteins of the virion and that the RNA codes for one large polypeptide precursor. The precursor may then be cleaved at a specific site to yield the nucleocapsid protein and a second polypeptide which, in infected cells, is cleaved in a series of steps to yield the two glycoproteins of the virion.

Sindbis 49S RNA was translated into 8 or 9 polypeptides ranging from 60,000 to 180,000 daltons in molecular weight. The viral structural proteins, as such, were not synthesized in response to the added 49S RNA.

1. Introduction

Sindbis virion RNA, 49S RNA, is a single polynucleotide chain with molecular weight of $4.3 \pm 0.3 \times 10^6$ daltons (Dobos & Faulkner, 1970; Simmons & Strauss, 1972a). In cells infected with Sindbis virus a second species of virus-specific single-stranded RNA is also synthesized. This species of RNA, 26S RNA, has a molecular weight of 1.6×10^6 daltons and represents a specific region of the viral genome (Simmons & Strauss, 1972a). It has been determined that 26S RNA functions as a messenger in the infected cell (Kennedy, 1972; Rosemond & Sreevalsan, 1973; Mowshowitz, 1973; Simmons & Strauss, submitted for publication). Simmons & Strauss (1972b) suggested that 26S RNA codes for the structural proteins of the virion which consist of a nucleocapsid protein and two envelope glycoproteins. We have shown that, in addition to 26S RNA, Sindbis 49S RNA has messenger activity during the virus life cycle, although it constitutes only a fraction (10% by weight) of the messenger RNA in the infected cell (D. T. Simmons & J. H. Strauss, submitted for publication). Nothing is known of the products of translation of 49S RNA during infection.

We have used lysates of rabbit reticulocytes to translate Sindbis 26S and 49S RNA *in vitro*. Successful translation of several species of exogenous messenger RNA is possible in reticulocyte lysates. These include the messengers for hemoglobins (Schapira *et al.*, 1968; Lockard & Lingrel, 1969), immunoglobulins (Stavnezer & Huang, 1971), reovirus (McDowell et al., 1972), vesicular stomatitis virus (Morrison *et al.*, 1974), *Dictyostelium discoideum* (Lodish, H. F., personal communications), Sendai virus (Kingsbury, 1973) and duck feather

keratin (Partington et al., 1973). Several species of animal virus messenger RNA have been translated with lesser efficiency in lysates of cells other than reticulocytes. Polio and encephalomyocarditis messenger RNA were translated by cell free extracts of Krebs ascites tumor cells (Kerr et al., 1966; Mathews & Korner, 1970; Smith et al., 1970). The species of reovirus messenger RNA have been translated in S-30 fractions of L-cells (Graziadei & Lengyel, 1972), and polyoma proteins have been synthesized in extracts of E. coli (Crawford & Gesteland, 1973).

In this study, we describe the translation of Sindbis 26S and 49S RNA in lysates of rabbit reticulocytes, we characterize the products obtained and compare them with some of the proteins made in infected cells.

2. Materials and Methods

Except as given below, the materials and methods used in these experiments were stated previously (Simmons & Strauss, 1972a).

(a) Preparation of RNA

i) Preparation of 26S RNA

Chick embryo fibroblasts were infected with either the HR strain of Sindbis virus or a temperature sensitive mutant of the virus, ts2 (Burge & Pfefferkorn, 1966) as previously described (Simmons & Strauss, 1972a). The infected cells were labeled with 10 μCi/ml. of [5-^{3}H] uridine (specific activity 24 Ci/mmole) from 0.5 hr to 6.5 hr post infection at 37^{o}C for the wild-type (HR strain) infection or at 39^{o}C

(the non-permissive temperature) for the ts2 infection. The cells were collected and homogenized in a glass Dounce homogenizer (Simmons & Strauss, submitted for publication). Nuclei and unbroken cells were pelleted at 1000 g for 10 min at 0°C. The supernatant was made 0.5% with respect to sodium deoxycholate (Matheson, Coleman & Bell) and 1.0% with respect to sodium dodecyl sulfate and extracted with phenol-chloroform at 0°C (Penman, 1966). The RNA was precipitated with 0.1 volume of 2 M potassium acetate (pH 6.0) and 2.5 volumes of 95% ethanol overnight at -20°C. The resuspended RNA was sedimented at 27,000 rev./min in a linear 15-30% sucrose gradient (Simmons & Strauss, 1972b) in a Spinco SW27 rotor for 8 hr at 23°C. The 26S RNA in the gradient was pooled, precipitated with ethanol and resuspended in 0.15 ml. of 0.01 M-Tris, 0.005 M-Na acetate, 0.0005 M-EDTA and 0.2% sodium dodecyl sulfate (pH 7.8). Preparative gel electrophoresis was carried out as previously described (Simmons & Strauss, 1972a). The purified 26S RNA was dissolved in 0.005 M-Tris (pH 7.4) to a concentration of approximately 20 µg/ml. and was intact as determined by sucrose gradient-velocity sedimentation.

ii) Preparation of 49S RNA.

Purified Sindbis virus was the source of the 49S RNA used in these experiments. Chick fibroblasts were inoculated with virus at a multiplicity of 1/100 and labeled with 4 µCi/ml. of [5-^{3}H] uridine starting at 5 hr post infection. At 20 hr post infection, the culture fluid was harvested and the virus precipitated from the medium with 8% polyethylene glycol (Pierce et al., 1974), and subjected to sucrose

gradient-velocity sedimentation and to equilibrium density banding (Strauss et al., 1969). The banded virus was diluted 2-fold in 0.05 M-Tris, 0.25 M-NaCl and 0.001 M-EDTA (pH 7.2) and pelleted in a Spinco SW50.1 rotor at 45,000 rev./min for 3 hr at 4°C. The virus pellet was resuspended in 1.5 ml. of 0.01 M-Tris, 0.06 M-NaCl, 0.001 M-EDTA and 0.2% sodium dodecyl sulfate (pH 7.2) and extracted by the Penman method at 0°C. The RNA was precipitated with ethanol and resuspended in 0.005 M-Tris (pH 7.4) to a final concentration of 30 µg/ml.

(b) Preparation of lysates of rabbit reticulocytes

One female New Zealand white rabbit (5.2 lbs) was made anemic by daily injections of phenylhydrazine-hydrochloride (Matheson, Coleman & Bell) as described elsewhere (Adamson et al., 1968). The rabbit was bled on the eighth day (Lingrel, 1972) and the blood rapidly cooled to 0°C. Reticulocytes constituted 75% to 80% of the blood cells as determined by staining with New Methylene Blue (Matheson, Coleman & Bell) (Brecher, 1949). The cells were pelleted by centrifugation at 500 g for 10 min in the cold and washed according to Lingrel & Borsook (1963). The packed cells were lysed with one volume of an ice cold solution of 5×10^{-5} M-hemin (Nutritional Biochemicals Corporation) in 0.001 M-Tris and 0.001 M-KCl (pH 7.0) (Adamson et al., 1968). After lysis, cell debris and mitochondria were removed by centrifugation at 12,000 g for 10 min. The supernatant (0.3 ml. aliquots) was frozen and stored in liquid nitrogen.

(c) Composition of the in vitro reaction mixtures

The reaction mixtures contained 62 µl. of reticulocyte lysate

and 20 µl. 0.005 M-Tris (pH 7.4), with or without dissolved messenger RNA, in a total volume of 100 µl. In addition, the cell free system contained, as final concentrations, 0.001 M-ATP, 0.00025 M-GTP, 0.015 M-creatine phosphate, 60 µg/ml. creatine phosphate kinase (all from Calbiochem) (Lingrel, 1972), 0.025 M-Tris, 0.1 M-KCl, 0.0025 M-Mg acetate and 0.005 M-2-mercaptoethanol (pH 7.4). Furthermore, 18 amino acids (Sigma Chemical Co.) excluding methionine were added at concentrations given in Borsook et al. (1957) and either [methyl-^{3}H] L-methionine at a specific activity of 7.7 Ci/mmole or [^{35}S] L-methionine at 144 Ci/mmole (both from Amersham-Searle) was added to a final concentration of 10 to 20 µCi/ml.

Certain precautions were taken to destroy possible traces of ribonuclease activity in the reaction mixtures. The solutions of ATP, GTP, creatine phosphate, creatine phosphate kinase and all non-radioactive amino acids with the exceptions of histidine, arginine and lysine were extracted with 1 mg/ml. bentonite in 0.01 M-K acetate (pH 7.0) (Fraenkel-Conrat et al., 1961). The basic amino acids were heated at 100^{o}C in 1 N-HCl for 30 min. Finally, all salt solutions were autoclaved.

(d) Acrylamide gel electrophoresis

The reaction mixtures were incubated at 27^{o}C or 37^{o}C for 2 to 2.5 hr. Pancreatic RNase was then added to 4 µg/ml. and the resulting mixture incubated at 37^{o}C for 15 min. Two volumes of 10% sodium dodecyl sulfate and 1/20 volume of 2-mercaptoethanol were added. The solutions were heated for 1.5 min at 100^{o}C and dialyzed overnight

against 0.025 M-Tris, 0.1% sodium dodecyl sulfate and 1.0% 2-mercaptoethanol (pH 6.8) at room temperature. To concentrate the samples, the proteins were precipitated by the addition of 8 to 10 volumes of acetone and pelleted by centrifugation at 10,000 g for 10 min at -20°C. The pellets were dried and resuspended in two times the volume of the original reaction mixtures in 0.005 M-Na_2HPO_4 (adjusted to pH 7.1), 3% sodium dodecyl sulfate, 5% v/v 2-mercaptoethanol and 10% v/v glycerol. The samples were heated at 100°C for 30 sec prior to electrophoresis in gels containing 9% acrylamide in the resolving gel and 3% acrylamide in the stacking gel (Laemmli, 1970). Electrophoresis was at constant current for 20 to 25 mA-hr in cylindrical 6 mm by 13 cm gels. The resolving gels were sliced (Mickle gel slicer) into 1 mm slices and counted for radioactivity in toluene-fluor scintillation fluid containing 10% v/v NCS solubilizer (Nuclear Chicago Corporation) and 1% H_2O.

(e) Labeling of Sindbis proteins in infected cells

Hamster cells or chick fibroblasts were infected with Sindbis virus, either the HR strain or ts106 as previously described (Simmons & Strauss, 1972a). The cells were labeled with 20 μCi/ml. of [methyl-^{3}H] L-methionine (specific activity 7.7 Ci/mmole) from 6 hr to 9 hr post infection and washed as in part (a). Cells were lysed by the addition of 1 ml. per T-flask of 0.01 M-Tris, 0.06 M-NaCl, 0.001 M-EDTA, 1% sodium dodecyl sulfate and 5% v/v 2-mercaptoethanol (pH 7.2). Prior to gel electrophoresis, the samples were heated at 100°C for 1 min and dialyzed as in part (d).

3. Results

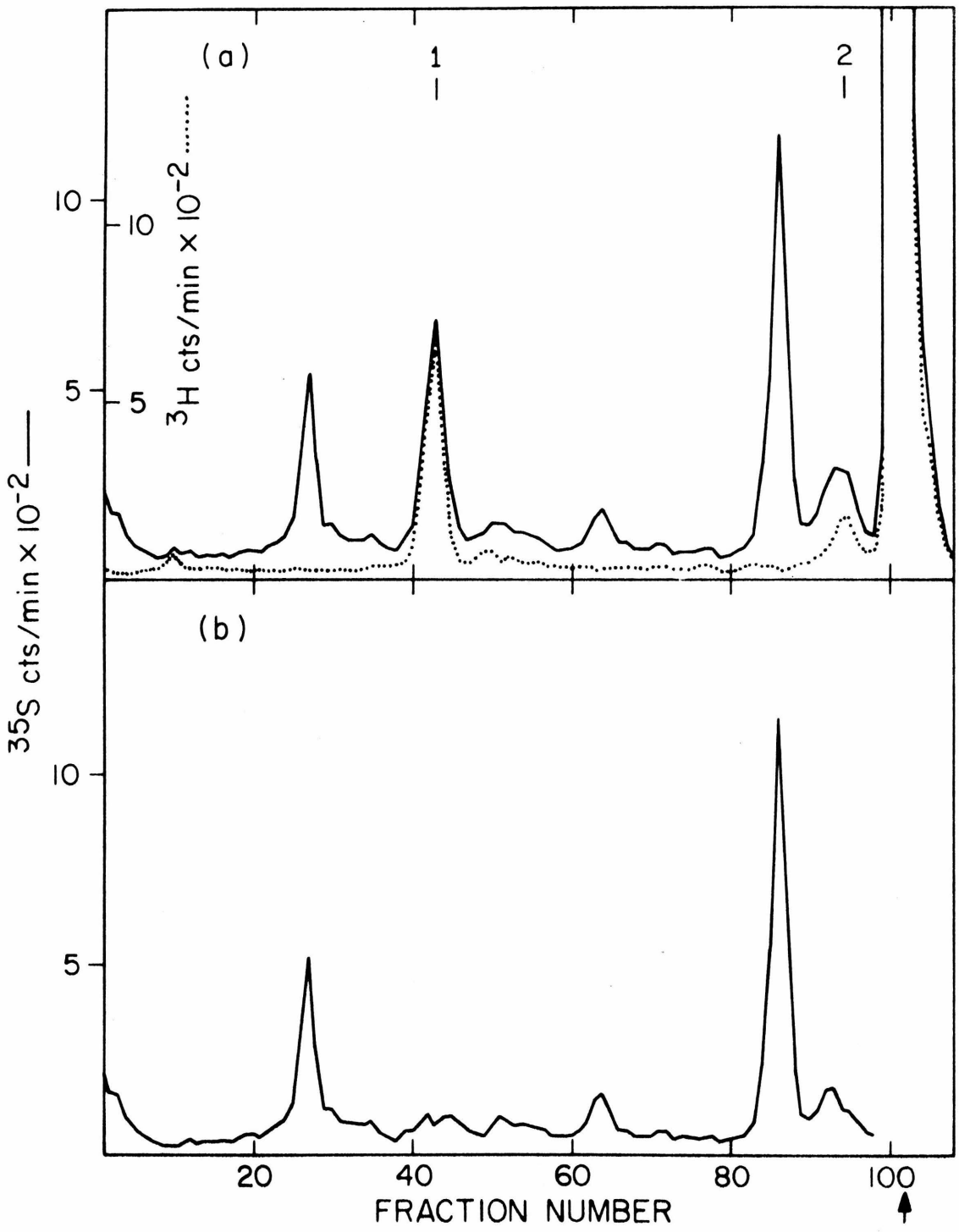
(a) Translation of wild-type 26S RNA

In cells infected with Sindbis virus, 26S RNA serves as the predominant messenger (Kennedy, 1972; Rosemond & Sreevalsan, 1973; Mowshowitz, 1973; Simmons & Strauss, submitted for publication) and has been postulated to be the messenger for the three structural proteins of the virion. To test its messenger activity *in vitro*, 26S RNA was isolated from cells infected with the HR strain of the virus, purified by acrylamide gel electrophoresis and incubated in a lysate of rabbit reticulocytes. Sindbis-specific polypeptides were detected when the polypeptides synthesized in this system were subjected to electrophoresis in acrylamide gels (Fig. 1.). Two separate reaction mixtures were analyzed by electrophoresis in the same gel. One was incubated in the presence of 26S RNA and [^{35}S] L-methionine, and the other was in the presence of [methyl-^{3}H] L-methionine but without added RNA (Fig. 1a). The pattern of labeled rabbit proteins was essentially simple. Hemoglobin migrated with the dye front and accounted for approximately 90% of the ^{3}H-methionine labeled material in the gel. Most of the remaining ^{3}H-label was associated with a protein with molecular weight of 64,000 daltons (protein 1) and one of 20,000 daltons (protein 2) (Lodish & Desalu, 1973). Figure 1b shows that two major polypeptides were synthesized in response to added Sindbis 26S RNA, which together account for approximately 7% of the incorporated methionine label. This pattern was obtained by subtracting, in each gel slice, the ^{35}S radioactivity present in rabbit-specific proteins

FIG. 1. Protein synthesis directed by wild-type 26S RNA at 27°C *in vitro*. 26S RNA was isolated from chick cells infected with wild-type (HR strain) Sindbis virus and purified by preparative gel electrophoresis. The RNA was mixed with a lysate or rabbit reticulocytes and incubated at 27°C in the presence of [^{35}S] L-methionine. A second lysate was incubated in the presence of [methyl-^{3}H] L-methionine without added RNA. Both reaction mixtures were subjected to electrophoresis in the same acrylamide gel (see Materials and Methods). In this and all other figures, the arrow represents the position of the bromophenol blue tracking dye after electrophoresis. The designations shown in the figures for various rabbit or Sindbis-specific proteins are described in the Results section.

a) (———) polypeptides labeled in the presence of 26S RNA; (·····) polypeptides labeled in the absence of added RNA.

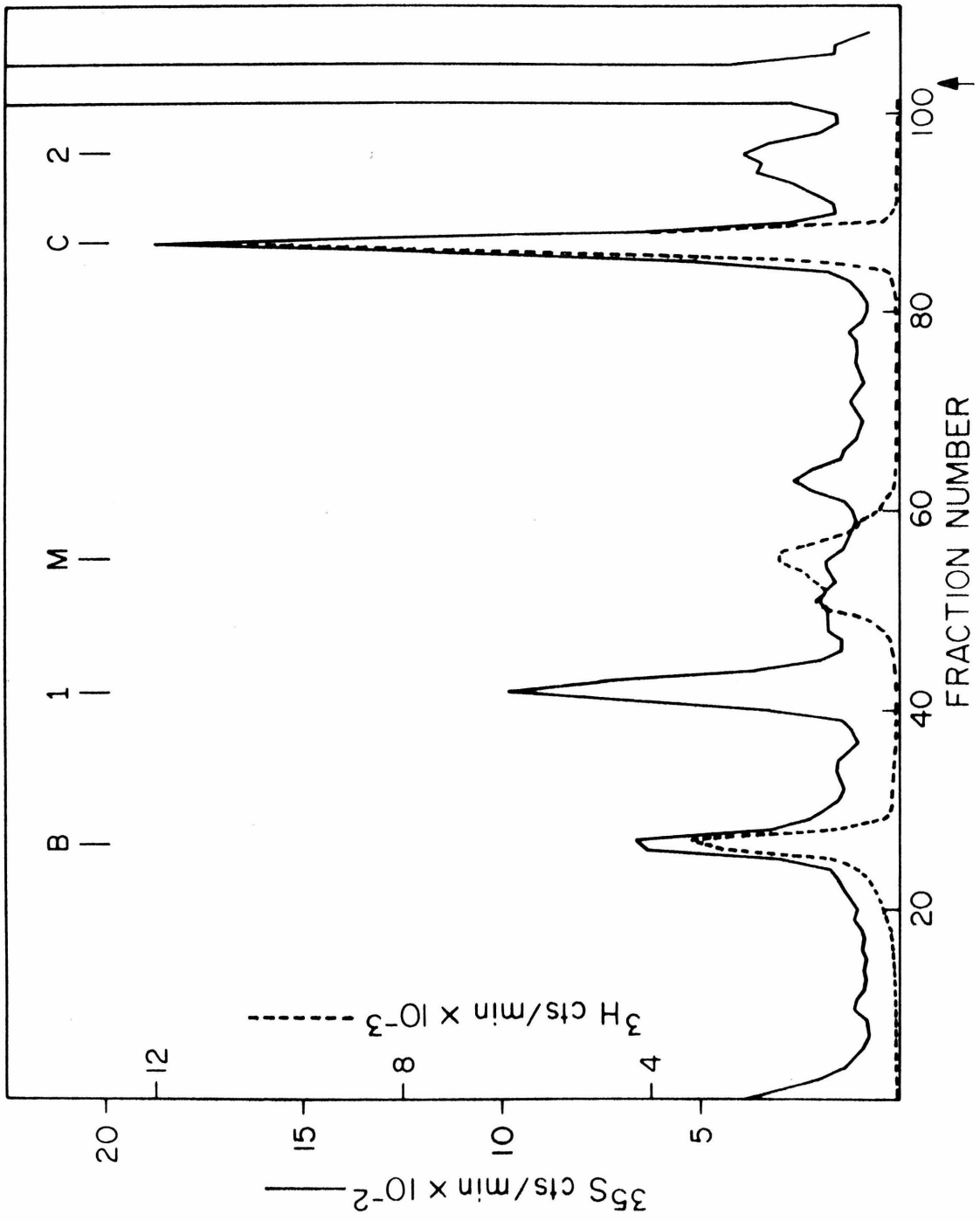
b) Polypeptides made in response to 26S RNA. The ^{35}S radioactivity present in rabbit proteins was subtracted from the total ^{35}S radioactivity in each gel slice.


(a)
1
2
^{3}H cts/min x 10^{-2}
10
5
(b)
^{35}S cts/min x 10^{-2}
10
5
20
40
60
80
100
FRACTION NUMBER

from the total ^{35}S radioactivity. The ^{35}S label in rabbit proteins in each slice was calculated by multiplying the ^{3}H label by a constant number such that the ^{3}H label in hemoglobin was equal to the ^{35}S label in hemoglobin. These calculations used the assumption that the presence of Sindbis messenger RNA in the reaction mixture had a uniform effect on the synthesis of all rabbit proteins. While this assumption may not be completely true, it is useful and allows for a more convenient presentation of the data.

To identify the Sindbis-specific polypeptides made in vitro, the reaction mixture was subjected to electrophoresis in the presence of marker proteins labeled in hamster cells infected with Sindbis virus (Fig. 2). The larger of the two major polypeptides synthesized in vitro migrated with the same mobility as a Sindbis-specific protein having a molecular weight of 100,000 daltons (Strauss et al., 1969) which we refer to as protein B. There is evidence that protein B contains the amino acid sequences present in the two envelope glycoproteins (proteins M) of the virus (Schlesinger & Schlesinger, 1973) and thus serves as precursor to these glycoproteins. The smaller of the two major polypeptides specified by 26S RNA migrated with the same mobility as the nucleocapsid protein of the virus (protein C) with molecular weight of 30,000 daltons (Strauss et al., 1969). Since the two major polypeptides synthesized in vitro comigrated with proteins of infected cells, we assume that the in vivo and in vitro polypeptides are identical or nearly identical, although proof of this assumption must await protein sequence studies.

FIG. 2. Identification of two major Sindbis-specific proteins made *in vitro*. Sindbis 26S RNA was translated in a lysate of reticulocytes as described in the legend to Figure 1. The reaction mixture was subjected to gel electrophoresis in the presence of proteins labeled with [methyl-^{3}H] L-methionine from 6 hr to 9 hr post infection in hamster cells infected with Sindbis virus. (———) polypeptides labeled *in vitro* in the presence of 26S RNA; (- - - -) polypeptides labeled in infected hamster cells.


B
1
M
C
2
20
40
60
80
100
FRACTION NUMBER
^{35}S cts/min x 10^{-2}
5
10
15
20
^{3}H cts/min x 10^{-3}
4
8
12

To provide further evidence that protein B synthesized in vitro was Sindbis-specific, the reaction mixture was incubated with antibodies (IgG fraction) directed against Sindbis virus (antibodies were prepared by C. Birdwell in our laboratory). In the resulting immunoprecipitate, the polypeptide which comigrated with protein B was enriched approximately 30-fold with respect to the total incorporated radioactivity as determined by acrylamide gel electrophoresis. In contrast, the nucleocapsid protein, an internal protein of the virion, was precipitated very poorly by anti-Sindbis antibodies.

In addition to protein B and the nucleocapsid protein, two other polypeptides with molecular weights of 40,000 and 22,000 daltons appeared to be made in response to added 26S RNA (Fig. 1b). Because of the uncertainties in subtracting the label in rabbit specific proteins, it is difficult to be sure these polypeptides are virus specific. They do not seem to correspond to proteins found in infected cells. In other experiments with phenyl methyl sulfonyl fluoride (1.5 mM) (Sigma Chemical Co.), an inhibitor of trypsin and chymotrypsin, these smaller polypeptides were made in lesser amounts relative to protein B (results not shown). Thus, they may represent cleavage products of the larger B protein or of other polypeptides. Alternatively, they could represent products arising from improper termination of the polypeptide chain (Cancedda & Schlesinger, 1974).

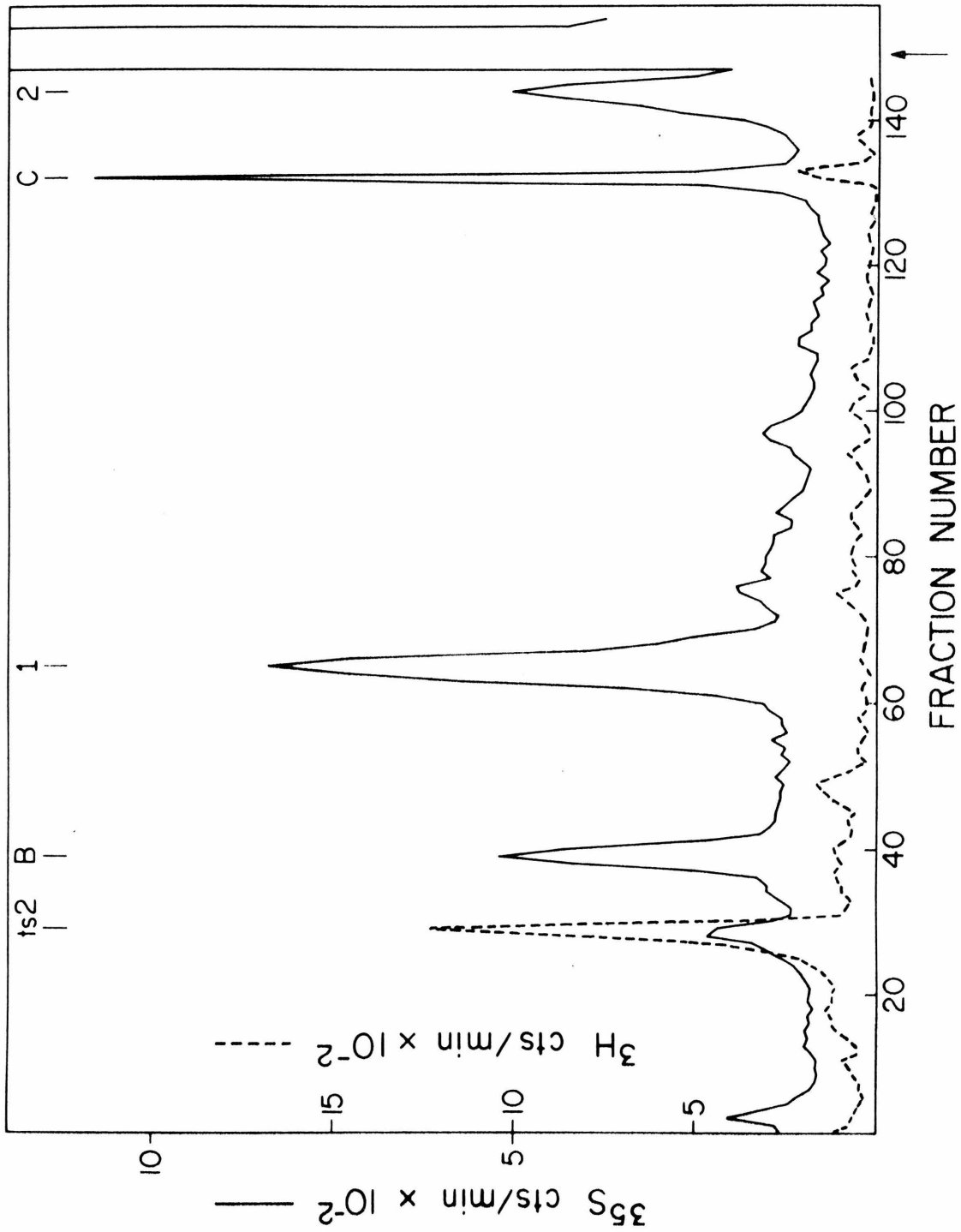
These observations are consistent with the hypothesis that 26S RNA codes solely for the structural proteins of the virus or for their precursors.

(b) Translation of ts2 26S RNA

In cells infected with certain mutants of Sindbis virus (ts2, ts5, ts13, and ts106), the synthesis of viral nucleocapsids is temperature sensitive (Burge & Pfefferkorn, 1966, 1968; Strauss, E., personal communications). At the restrictive temperature, these cells fail to synthesize normal amounts of structural proteins and accumulate instead a large polypeptide with molecular weight of approximately 130,000 daltons (Strauss et al., 1969; Waite, 1973). This polypeptide, which we designate as the ts2 protein, generates tryptic peptides in common with those of all three viral structural proteins (Schlesinger & Schlesinger, 1973), and is thought therefore to represent a precursor of these three proteins. We therefore investigated the possibility that the ts2 protein is a product of the translation of ts2 26S RNA in vitro.

Ts2 26S RNA was isolated from cells infected with the mutant virus at the restrictive temperature. The purified RNA was translated in a lysate of rabbit reticulocytes, as before. When the reaction mixture was incubated at 27°C, a permissive temperature, polypeptides which comigrated with the nucleocapsid protein (C) and the B protein were synthesized as predicted (Fig. 3). In addition, small amounts of the ts2 protein appeared to be made. Notice that in the gels shown in Figures 3 to 5, the markers used are Sindbis-specific proteins labeled in cells infected with ts106 at the restrictive temperature. The ts106 mutation does not completely shut off synthesis of the structural proteins.

FIG. 3. Protein synthesis directed by ts2 26S RNA at 27°C. Ts2 26S RNA was isolated from chick cells infected at 30°C with Sindbis mutant ts2 and translated at 27°C in a lysate of rabbit reticulocytes in the presence of [^{35}S] L-methionine. The reaction mixture was subjected to gel electrophoresis in the presence of proteins labeled with [methyl-^{3}H] L-methionine from 6 hr to 9 hr post infection in chick cells infected at 39°C with ts106. (——) polypeptides labeled in vitro at 27°C in the presence of ts2 26S RNA; (-----) polypeptides labeled in cells infected at 39°C with ts106.


35S cts/min x 10-2
3H cts/min x 10-2
FRACTION NUMBER
ts2
B
1
C
2
5
10
15
20
40
60
80
100
120
140

When ts2 26S RNA was translated at 37°C, a restrictive temperature, the presumptive ts2 protein was the only Sindbis-specific polypeptide detected (Fig. 4). The material in peaks 1 and 2 consist primarily of rabbit-specific proteins. Little or no nucleocapsid protein or B protein were made at the restrictive temperature. Thus, these observations correspond with the results obtained previously on the synthesis of Sindbis-specific proteins in cells infected with these mutants. Furthermore, in pulse-chase experiments with either reticulocyte lysates or infected cells, the label in the ts2 protein did not disappear when the temperature was shifted from restrictive to permissive (Scheele & Pfefferkorn, 1970, and our observation).

Figure 5 shows that when wild-type 26S RNA was translated in a lysate of rabbit reticulocytes at 37°C rather than 27°C, the polypeptides which comigrated with the nucleocapsid protein and the B protein were both made as predicted. A large polypeptide with molecular weight of 130,000 daltons did not accumulate as was the case when the messenger was ts2 26S RNA. The relative amount of B protein to nucleocapsid protein in the reaction mixture was greater at 37°C than at 27°C, however. At 37°C, the molar ratios of the two proteins was approximately 1 to 1, as the case would be if they were derived from a common precursor. It is therefore possible that the RNA chains were not completely translated at the lower temperature. If this is true, then the cistron for the nucleocapsid protein is on the 5'-terminus of the 26S RNA molecule in agreement with Schlesinger & Schlesinger (1972).

FIG. 4. Protein synthesis directed by ts2 26S RNA at 37°C. The experiment was performed as described in the figure legend to Figure 3 with the exception that the reaction mixture was incubated at 37°C instead of 27°C. (——) polypeptides labeled in vitro at 37°C in the presence of ts2 26S RNA; (- - - -) polypeptides labeled in cells infected at 39°C with ts106.

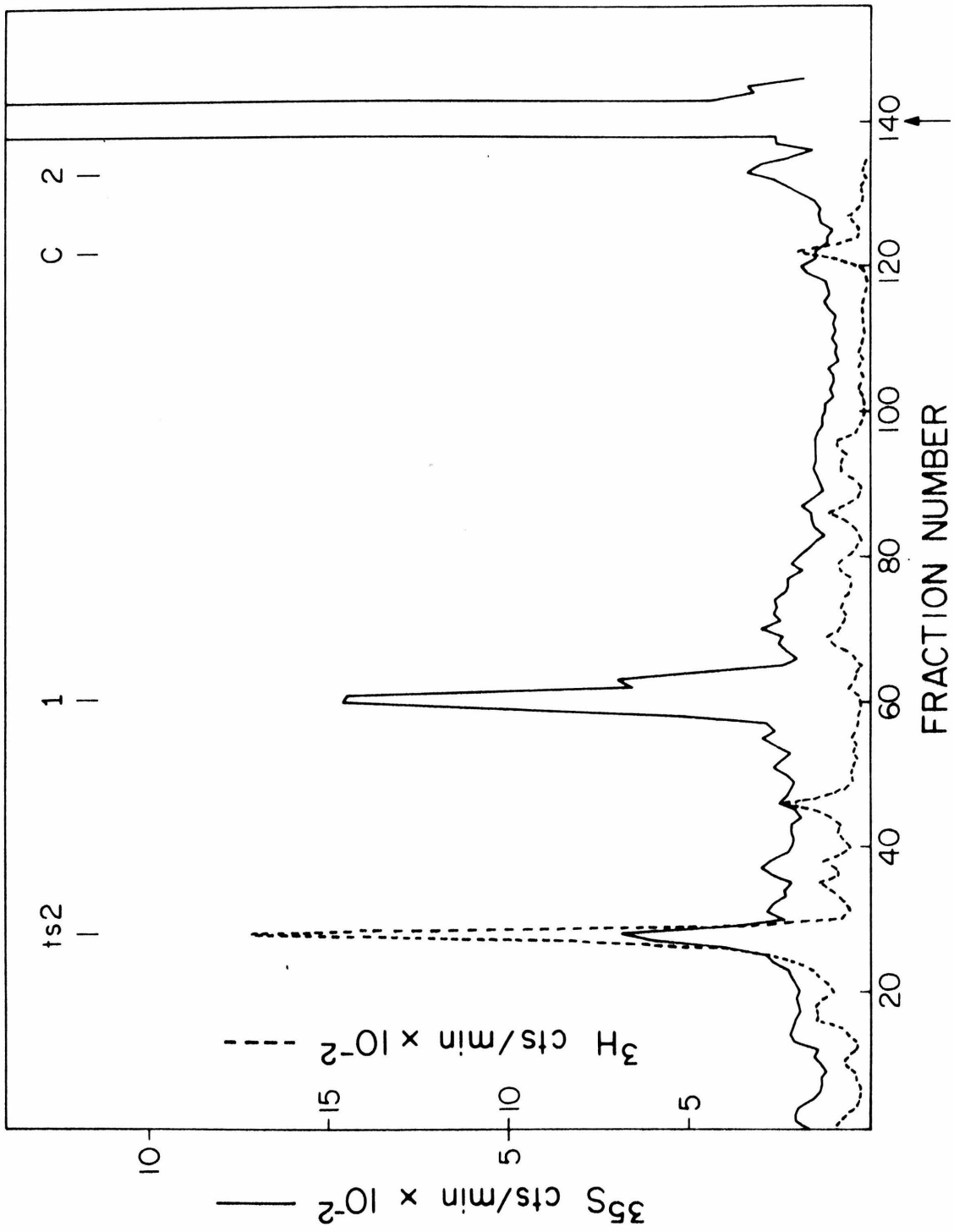
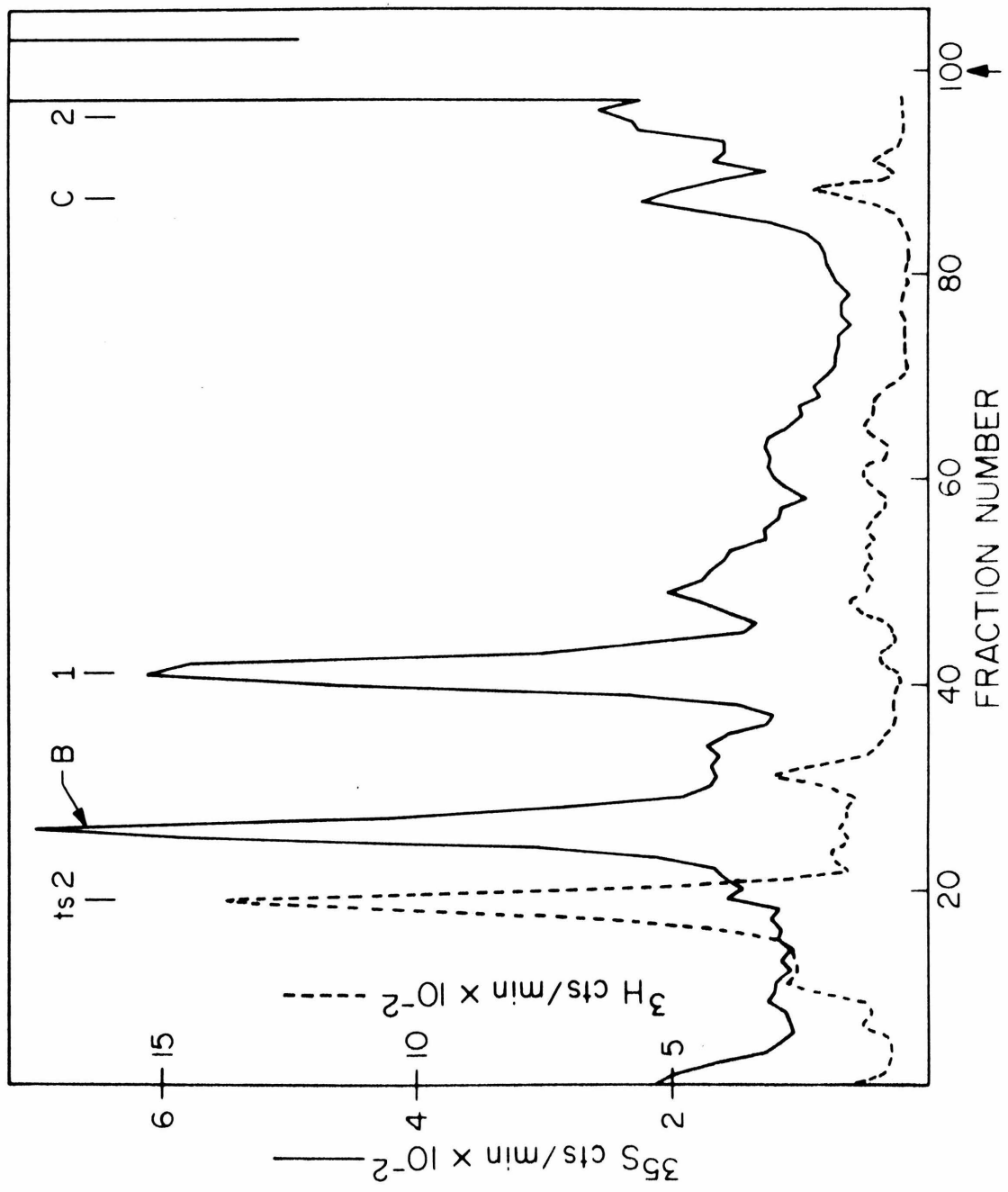

35S cts/min x 10-2
3H cts/min x 10-2
FRACTION NUMBER
ts2
1
C
2

FIG. 5. Protein synthesis directed by wild-type 26S RNA at 37°C. The experiment was performed as described in the figure legend to Figure 3 with the exception that the reaction mixture was incubated at 37°C with wild-type 26S RNA. (———) polypeptides labeled in vitro at 37°C in the presence of wild-type 26S RNA; (- - - -) polypeptides labeled in cells infected at 39°C with ts106.


ts2
B
1
C
2
20
40
60
80
100
FRACTION NUMBER
^{3}H cts/min x 10^{-2}
^{35}S cts/min x 10^{-2}
2
4
6
5
10
15

(c) Translation of Sindbis virion RNA

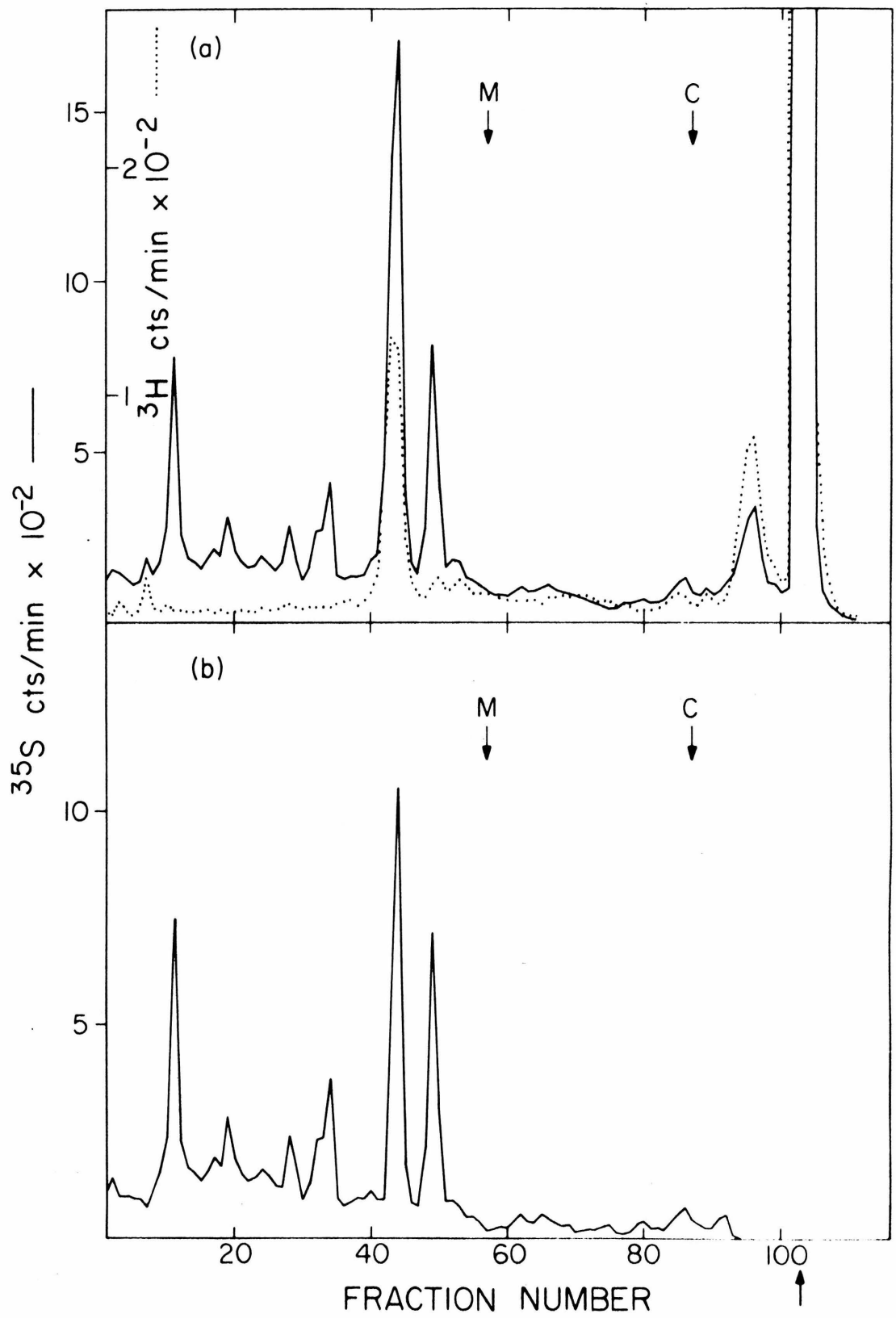
Nothing is known of the products of the translation of 49S RNA in infected cells. It seems likely from the foregoing results that Sindbis-specific proteins detected in infected cells are translation products of 26S RNA. The proteins specified by 49S RNA have probably been undetected because of the high background of host protein synthesis occurring early in infection, and because 49S RNA accounts for only a fraction (10% by weight) of the messenger RNA in infected cells (D. T. Simmons & J. H. Strauss, submitted for publication).

Sindbis 49S RNA was isolated from purified virus and incubated at 27°C in the presence of a lysate of rabbit reticulocytes. The polypeptides labeled in the presence of the RNA were compared to those made in its absence as described above, and at least 8 or 9 virus-specific polypeptides were detected (Fig. 6). The positions in the gel of the nucleocapsid protein (C) and glycoproteins (M) were determined from a separate gel. The virus-specific polypeptides specified by 49S RNA varied from 60,000 to 180,000 daltons in molecular weight. Neither protein B nor the structural proteins were made in response to Sindbis 49S RNA. Nevertheless, it is possible that some of the polypeptides made *in vitro* contained sequences in common with the structural proteins of the virion. However, none of these polypeptides correspond with any Sindbis-specific proteins labeled in infected cells. Together, these products exceed the total coding capacity of the genome and thus some of the larger polypeptides are precursors to others and/or they accumulate as a result of polypeptide chain termination at various

FIG. 6. Protein synthesis directed by Sindbis virion RNA. Sindbis virion RNA (49S RNA) was incubated at 27°C with [^{35}S] L-methionine in the presence of a lysate of rabbit reticulocytes. A second lysate was incubated in the presence of [methyl-^{3}H] L-methionine without added RNA. Both reaction mixtures were subjected to electrophoresis in the same gel.

a) (———) polypeptides labeled in the presence of 49S RNA; (·····) polypeptides labeled in the absence of added RNA.

b) Polypeptides made in response to 49S RNA (see legend to Fig. 1).


(a)
M
C
15
10
5
^{3}H cts/min x 10^{-2}
^{35}S cts/min x 10^{-2}
(b)
M
C
10
5
20
40
60
80
100
FRACTION NUMBER

internal sites in the messenger RNA. No differences were observed when 49S RNA was translated in the presence of phenyl methyl sulfonyl fluoride indicating that any cleavage which occurred was catalyzed by enzymes different from trypsin or chymotrypsin. When 49S RNA was translated at 37°C, the larger polypeptides were labeled more heavily than the smaller ones (results not shown), although at that temperature, relatively less methionine label was incorporated into Sindbis-specific polypeptides.

4. Discussion

Wild-type Sindbis 26S RNA was translated *in vitro* by ribosomes of rabbit reticulocytes into two major polypeptides which comigrated in acrylamide gels with two proteins found in infected cells. These *in vivo* proteins are the nucleocapsid protein and a protein (protein B) which contains the same amino acid sequences present in the two glycoproteins of the virus (Schlesinger & Schlesinger, 1972). The polypeptide made *in vitro* which comigrated with protein B can be precipitated from the reaction mixture with antibodies (IgG fraction) directed against Sindbis virus.

Under certain conditions, 26S RNA specified, *in vitro*, a polypeptide chain with a molecular weight of 130,000 daltons. This polypeptide chain migrated with the same electrophoretic mobility as a protein (the ts2 protein) isolated from cells infected at the restrictive temperature with certain temperature sensitive mutants of Sindbis virus (ts2, ts5, ts13, and ts106) (Strauss *et al.*, 1969). The ts2 protein

contains the amino acid sequences present in all three viral structural proteins (Schlesinger & Schlesinger, 1973). The large polypeptide which appears to be the ts2 protein was the only Sindbis-specific product synthesized in vitro at the restrictive temperature in response to 26S RNA isolated from cells infected with ts2. This polypeptide was also made at the permissive temperature in response to ts2 26S RNA. A polypeptide of the same size was also synthesized in very small quantities in response to wild-type 26S RNA (results not shown). However, in the last two cases, the majority of the label in Sindbis-specific polypeptides was in the presumptive nucleocapsid and B proteins.

In light of the fact that polypeptide chain cleavage has not been previously observed in reticulocyte lysates, it is possible that the nucleocapsid and B proteins are generated in vitro as a result of internal chain termination and reinitiation signals in 26S RNA. Another possibility is that the polypeptide which comigrates with protein B is formed as a result of random termination events. The most reasonable interpretation of these results is that wild-type 26S RNA directs the synthesis of a single polypeptide chain which is cleaved at some point in time, to produce the nucleocapsid and B proteins. Such an interpretation is suggested from the approximate 1 to 1 molar ratio of the presumptive B and nucleocapsid proteins made in response to 26S RNA at 37^{o}C, and from the restriction that animal virus messenger RNA probably lack internal chain termination and initiation signals for translation (Summers & Maizel, 1968; Jacobson & Baltimore, 1968). It may be dif-

ficult, however, to distinguish one mechanism whereby a large protein is first synthesized and then cleaved, from a second mechanism where cleavage occurs in nascent unfinished chains. Since the large polypeptide directed by ts2 26S RNA at the restrictive temperature was not cleaved by shifting to the permissive temperature, we can hypothesize that the chain acquired an irreversible configuration which was not recognized by a cleavage enzyme. It is also possible that the ts2 protein serves as its own protease and that cleavage does not require an external source of a specific cleavage enzyme.

A study of the initiation of Sindbis-specific polypeptide chains in reticulocyte lysates should distinguish between the internal initiation/termination and cleavage hypotheses.

In infected cells, protein B is, at some stage, cleaved to E_1 and to PE_2, the immediate precursor to one of the viral glycoproteins (E_2) (Schlesinger & Schlesinger, 1972). Proteins E_1 and E_2 are collectively referred to as M in Figs. 2 and 6. In reticulocyte lysates, this cleavage apparently did not occur. In infected hamster cells, E_1 and E_2 are completely glycosylated, PE_2 is only partially glycosylated and protein B is not at all glycosylated (Sefton & Burge, 1973; J. Bell, personal communication). A possible interpretation of these results is that protein B is first partially glycosylated and then very rapidly cleaved. At the present time, we do not know whether or not protein B is glycosylated <u>in vitro</u>.

Approximately 8 or 9 Sindbis-specific polypeptides, ranging in molecular weight from 60,000 to 180,000 daltons, were synthesized <u>in vitro</u> in response to virion 49S RNA. The structural proteins of the

virus or their precursors were not made as such in the reaction mixture. If none of the polypeptides specified by 49S RNA contained the amino acid sequences present in the structural proteins of the virus, then the portion of the 49S RNA molecule corresponding to 26S RNA was not translated in the reticulocyte lysates. If this is true, either there is an internal initiation or termination codon in the virion RNA, or translation stops prematurely along the chain. Nothing is known of the function of any of the polypeptides made in response to 49S RNA. If they are synthesized in infected cells, they exist in quantities too small to detect. Further investigations are necessary to characterize all the proteins specified by Sindbis RNA.

Cancedda and Schlesinger (1974) have recently observed the production of nucleocapsid protein in reticulocyte lysates with added Sindbis messenger RNA.

Acknowledgements

We wish to acknowledge the expert technical assistance of Edith M. Lenches and Sharman E. S. Christoph in preparing the cultures of chick embryo fibroblasts. D. T. S. thanks his wife, Susan, for staining the reticulocytes. This investigation was supported in part by the National Science Foundation Grant No. GB31763X and by the U. S. Public Health Service Grant No. GM06965. One of us (D. T. S.) was supported by the National Institutes of Health Training Grant No. 5-T1-GM-86.

References

Adamson, S. D., Herbert, E. & Godchaux, W. III (1968). Arch. Biochem. Biophys. 125, 671-683.

Borsook, H., Fisher, E. H. & Keighley, G. (1957). J. Biol. Chem. 229, 1059-1070.

Brecher, G. (1949). Am. J. Clin. Path. 19, 895-896.

Burge, B. W. & Pfefferkorn, E. R. (1966). Virology 30, 204-213.

Burge, B. W. & Pfefferkorn, E. R. (1968). J. Mol. Biol. 35, 193-205.

Cancedda, R. & Schlesinger, M. J. (1974). Proc. Nat. Acad. Sci. U.S., in press.

Crawford, L. V. & Gesteland, R. F. (1973). J. Mol. Biol. 74, 627-634.

Dobos, P. & Faulker, P. (1970). J. Virol. 6, 145-147.

Fraenkel-Conrat, H., Singer, B. & Tsugita, A. (1961). Virology 14, 54-58.

Graziadei, W. D. III & Lengyel, P. (1972). Biochem. Biophys. Res. Comm. 46, 1816-1823.

Jacobson, M. F. & Baltimore, D. (1968). Proc. Nat. Acad. Sci. U.S. 61, 77-84.

Kennedy, S. I. T. (1972). Biochem. Biophys. Res. Comm. 48, 1254-1258.

Kerr, I. M., Cohen, N. & Work, T. S. (1966). Biochem. J. 98, 826-835.

Kingsbury, D. W. (1973). J. Virol. 12, 1020-1027.

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

Lingrel, J. B. (1972). In Protein Biosynthesis in Nonbacterial Systems (Last, J. A. & Laskin, A. I., eds.), pp. 231-263, Marcel Dekker, Inc.

Lingrel, J. B. & Borsook, H. (1963). Biochemistry 2, 309-314.

Lockard, R. E. & Lingrel, J. B. (1969). Biochem. Biophys. Res. Comm. 37, 204-212.

Lodish, H. P. & Desalu, O. (1973). J. Biol. Chem. 248, 3520-3527.

Mathews, M. B. & Korner, A. (1970). Eur. J. Biochem. 17, 328-338.

McDowell, M. J., Joklik, W. K., Villa-Komaroff, L., & Lodish, H. F. (1972). Proc. Nat. Acad. Sci. U.S. 69, 2649-2653.

Morrison, T., Stampfer, M., Baltimore, D., & Lodish, H. F. (1974). J. Virol. 13, 62-72.

Mowshowitz, D. (1973). J. Virol. 11, 535-543.

Partington, G. A., Kemp, D. J. & Rogers, G. E. (1973). Nature New Biol. 246, 33-36.

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

Pierce, J. S., Strauss, E. G. & Strauss, J. H. (1974). J. Virol., in press.

Rosemond, H. & Sreevalsan, T. (1973). J. Virol. 11, 399-415.

Schapira, G., Dreyfus, J. C. & Maleknia, N. (1968). Biochem. Biophys. Res. Comm. 32, 558-561.

Scheele, C. M. & Pfefferkorn, E. R. (1970). J. Virol. 5, 329-337.

Schlesinger, S. & Schlesinger, M. (1972). J. Virol. 10, 925-932.

Schlesinger, M. & Schlesinger, S. (1973). J. Virol. 11, 1013-1016.

Sefton, B. M. & Burge, B. W. (1973). J. Virol. 12, 1366-1374.

Smith, A. E., Marcker, K. A. & Mathews, M. B. (1970). Nature 225, 184-187.

Simmons, D. T. & Strauss, J. H. (1972a). J. Mol. Biol. 71, 599-613.

Simmons, D. T. & Strauss, J. H. (1972b). J. Mol. Biol. 71, 615-631.

Stavnezer, J. & Huang, R. C. C. (1971). Nature New Biol. 230, 172-176.

Strauss, J. H., Burge, B. W. & Darnell, J. E. (1969). Virology 37, 367-376.

Summers, D. F. & Maizel, J. V. (1968). Proc. Nat. Acad. Sci. U.S. 59, 966-971.

Waite, M. R. F. (1973). J. Virol. 11, 198-206.

CHAPTER 4

Replication of Sindbis Virus: Multiple Forms of Double-Stranded RNA Isolated from Infected Cells

by

Daniel T. Simmons and James H. Strauss

This chapter is from the
Journal of Molecular Biology
and is reproduced with the
permission of the publisher.

J. Mol. Biol. (1972) **71**, 615–631

Replication of Sindbis Virus

II. Multiple Forms of Double-stranded RNA Isolated from Infected Cells

DANIEL T. SIMMONS AND JAMES H. STRAUSS

Division of Biology, California Institute of Technology
Pasadena, Calif. 91109, U.S.A.

(*Received 14 February 1972, and in revised form 10 July 1972*)

Three species of double-stranded RNA (replicative forms) have been isolated from cells infected with Sindbis virus after treating the extracts with pancreatic ribonuclease. Their molecular weights have been estimated to be $8{\cdot}8 \times 10^6$, $5{\cdot}6 \times 10^6$ and $2{\cdot}9 \times 10^6$ daltons (RFI, RFII and RFIII, respectively). RFII and RFIII were found to exist in a one-to-one molar ratio throughout the infection cycle, although the total amounts of these replicative forms varied. The molar ratio of RFI to the other two replicative forms was different at various times after infection. However, in the middle of the virus life-cycle, from two to six hours post-infection, this ratio was constant at 0·4 to 1.

Sedimentation analysis of pulse-labeled RNA from Sindbis-infected cells indicated that all replicative intermediates had molecular weights greater than or equal to $8{\cdot}8 \times 10^6$ daltons. Since all three replicative forms were liberated from the peak of replicative intermediates by treatment with ribonuclease, this suggested that RFII and RFIII were joined together before digestion. The constant one-to-one molar ratio of RFII and RFIII supported this hypothesis. Kinetic experiments demonstrated that while RFI and RFIII were labeled equally rapidly, RFII was labeled some 20 to 50 times more slowly.

Hybridization competition experiments showed that the minus strand of RFIII is complementary to 26 s RNA, a species of single-stranded RNA found in infected cells, and that the minus strand of RFII is complementary to the remainder of the 49 s viral genome. We postulate, therefore, that a precursor–product relation exists between RFI and 49 s RNA, between RFIII and 26 s RNA and between RFII and a third species of Sindbis-specific RNA, which we have not yet detected. Our results are consistent with the hypothesis that two classes of replicative intermediates exist in Sindbis-infected cells. One serves as the template for the synthesis of 49 s RNA, and the second manufactures two smaller segments of viral-specific RNA.

1. Introduction

The replication of viral RNA is known to involve the synthesis of and subsequent use of the complementary strand (minus strand) as a template. This complementary strand is associated with one or more plus strands of varying lengths but is largely present in a single-stranded form inside the cell (Weissman, Feix & Slor, 1968). After the removal of protein, complementary regions of the plus and minus strands join to form a partially double-stranded structure (Montagnier & Sanders, 1963; Erikson, Fenwick & Franklin, 1964) which is called a replicative intermediate, or RI†, if single-

† Abbreviations used: RI, replicative intermediate; RF replicative form.

stranded tails are present. The single-stranded tails can be removed by controlled degradation with pancreatic RNase. This produces fully double-stranded molecules (cores, duplexes, or RF's for simplicity) with single-strand breaks, since double-stranded RNA is only partially resistant to the action of RNase at moderate ionic strength (Geiduschek, Moohr & Weiss, 1962).

The replicative intermediates of Sindbis virus have been found to sediment between 20 S and 28 S (Pfefferkorn, Burge & Coady, 1967; Yin & Lockart, 1968), while those from Semliki Forest virus were reported to sediment between 16 S and 23 S (Friedman, 1968). Little has been reported on the characterization of arbovirus-specific double-stranded RNA cores. Only recently, Levin & Friedman (1971) have observed, by sucrose gradient–velocity sedimentation and by polyacrylamide gel electrophoresis, two or three species of RNase-resistant material in extracts of cells infected with Semliki Forest virus.

In the preceding paper (Simmons & Strauss, 1972), we showed that 26 S RNA, a species of single-stranded RNA made in Sindbis-infected cells, is a segment of viral RNA equal to one-third the size and containing one-third of the sequence information of the entire viral genome (49 S RNA). We report here on the existence of three species of double-stranded RNA (RF's) in RNase-treated extracts of Sindbis-infected cells. We give estimates of their molecular weights and of the molecular weights of the replicative intermediates. The relative amounts of the three RF's and their kinetics of labeling are described. Based upon our results, we present a model for the replication of Sindbis RNA, which is further supported by hybridization data. This model describes the origin of the RF's and their relation to the replicative intermediates and to the species of single-stranded RNA.

2. Materials and Methods

Most of the materials and methods used have been previously described (Simmons & Strauss, 1972). These include cell culture conditions, cell lysis, polyacrylamide gel electrophoresis, procedures for the preparation of single-stranded RNA and conditions for hybridization. Modification of these methods specifically for partially or fully double-stranded RNA are detailed below.

(a) *Lysis and extraction of the RNA from Sindbis-infected cells*

Cells infected with Sindbis virus were washed with a cold solution of 0·01 M-KCl, 0·0015 M-$MgCl_2$, 0·01 M-Tris (pH 7·4) and were lysed with Tris/NaCl/EDTA buffer (0·01 M-Tris, 0·06 M-NaCl, 0·001 M-EDTA (pH 7·2)) containing 1% sodium dodecyl sulfate. The lysate was extracted 3 consecutive times with cold phenol equilibrated with 0·1 M-Tris, pH 7·2. In turn, the phenol layers were extracted with the same vol. of cold 0·01 M-Tris, 0·06 M-NaCl (pH 7·2). The aqueous phases were combined and extracted once with fresh ether. Alternatively, the phenol/chloroform extraction technique of Penman (1966) was used at room temperature. For analysis of the RNA by polyacrylamide gel electrophoresis, it was necessary to carry out the phenol/chloroform extraction at 55°C.

(b) *Preparation and analysis of the replicative forms*

For analysis of the RF's by sucrose gradient–velocity sedimentation, the RNA was treated with 10 μg pancreatic RNase/ml. in 0·01 M-Tris, 0·1 M-NaCl, 0·01 M-$MgCl_2$, 0·001 M-EDTA (pH 7·2) and incubated for 15 min at 37°C. It was made 0·5% in sodium dodecyl sulfate and immediately subjected to sedimentation.

For the separation of the RF's by polyacrylamide gel electrophoresis, the RNA solution was incubated in the presence of 50 μg DNase/ml. as well. Sodium dodecyl sulfate was added to a final concentration of 1% and the RNA was extracted twice with phenol and once with ether at room temperature. The RNA was precipitated with ethanol, collected by

centrifugation and resuspended in a small vol. of sample buffer for electrophoresis (0·01 M-Tris, 0·005 M-sodium acetate, 0·0005 M-EDTA (pH 7·8)).

(c) *Sucrose gradient–velocity sedimentation*

A sample of the radioactively labeled RNA in Tris/NaCl/EDTA buffer containing 0·2% sodium dodecyl sulfate was layered over a 15 to 30% linear sucrose gradient in the same buffer and sedimented at 40,000 rev./min in a Spinco SW41 rotor at 23°C for 8 or 10 hr, depending upon the sample. The bottom of the tube was pierced with a size "0" insect pin, and 60 to 80 fractions (4 to 5 drops each) were collected directly in scintillation vials. One-half ml. of water and 10 ml. of Bray's scintillation fluid (Bray, 1960) were added to each vial for counting radioactivity.

(d) *Polyacrylamide–agarose gel electrophoresis*

The method used for preparing the gels has been described (Simmons & Strauss, 1972) except that the gels consisted of 1·60% acrylamide, 0·08% bis-acrylamide and 0·5% agarose, and sodium dodecyl sulfate was omitted from all buffers. These gels were sliced with a gel slicer, the slices dissolved in 1 ml. of dimethylsulfoxide and counted in 10 ml. of Bray's scintillation fluid.

For hybridization studies, double-stranded RNA was prepared from infected cell extracts as previously described (Simmons & Strauss, 1972), and separated by polyacrylamide gel electrophoresis. Gels were sliced and the RNA eluted from the slices with 0·001 M-EDTA, pH 7·0, for at least 12 hr at room temperature. Alternatively, RNA was collected from the bottom of a gel using a preparative gel apparatus. The latter method prevented the contamination of the RNA preparation with acrylamide and agarose debris and allowed the RNA to be used at a higher concentration.

3. Results

(a) *Identification of the three replicative forms*

In RNA virus systems studied so far, the RF's (the double-stranded RNA cores remaining after treatment of RI's with RNase) have been found to have twice the molecular weight of the single-stranded RNA (or RNA's) found in the virion (Bishop & Koch, 1967; Franklin, 1967; Pons & Hirst, 1968). Since Sindbis RNA has a molecular weight of about $4{\cdot}3 \times 10^6$ daltons (Simmons & Strauss, 1972), we expected to find a single species of RF with a molecular weight of $8{\cdot}6 \times 10^6$ daltons. To our surprise, however, we found not one but three species of Sindbis-specific RF's (Fig. 1). This Figure shows the pattern obtained when RNA is isolated from hamster cells infected with Sindbis, treated with RNase and then sedimented in a sucrose gradient. The RF isolated from *Escherichia coli* infected with bacteriophage MS2 (MS2 RF, a generous gift from Dr Jane Cramer) was added as a marker. The three Sindbis RF's have sedimentation coefficients of 23·5 S (RFI), 20·1 S (RFII) and 16·0 S (RFIII); MS2 RF has a sedimentation coefficient of 14·5 S. These values were determined from other gradients with *E. coli* rRNA as markers.

Studier (1965) found that the sedimentation coefficient of double-stranded DNA varies as the 0·346 power of the molecular weight. Assuming that this relation holds for double-stranded RNA, and using the sedimentation coefficient of MS2 RF and its known molecular weight of $2{\cdot}2 \times 10^6$ daltons (Strauss & Sinsheimer, 1963; Gesteland & Boedtker, 1964) for calibration, we have determined the molecular weights of the Sindbis RF's. RFI has a molecular weight of $8{\cdot}8 \times 10^6$ daltons, RFII of $5{\cdot}6 \times 10^6$ daltons and RFIII of $2{\cdot}9 \times 10^6$ daltons. RFI has twice the molecular weight of Sindbis RNA and must, therefore, be the "classical" RF.

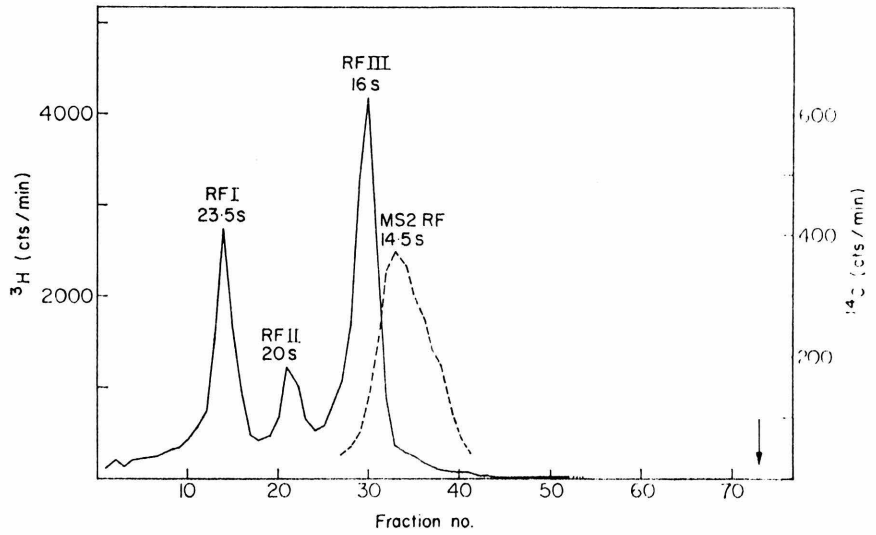


FIG. 1. Identification of the three RF's. Hamster cells (BHK21) were infected with Sindbis virus at a multiplicity of 10 plaque-forming units/cell and labeled with 100 μCi/ml. of [5-^{3}H]uridine for 8 min starting at 6 hr after infection. The RNA was purified by the sodium dodecyl sulfate/phenol method at 0°C and then treated with RNase (10 μg/ml. for 15 min at 37°C). It was sedimented in a linear sucrose gradient (15 to 30% sucrose in Tris/NaCl/EDTA buffer and 0·2% sodium dodecyl sulfate) for 10 hr at 40,000 rev./min in the Spinco SW41 rotor at 23°C (——). ^{14}C-labeled MS2 RF was added as a molecular weight marker (– – –). The arrow indicates the top of the gradient in this and all subsequent sucrose gradient patterns.

We have been able to obtain excellent separation of the three Sindbis RF's using polyacrylamide–agarose gel electrophoresis (Fig. 2). RFIII migrates most rapidly and RFI most slowly. The single-stranded viral RNA (49 s RNA) migrates approximately twice as rapidly as RFIII in a gel of this concentration. Variations in the relative sizes of the three peaks are due to different labeling conditions (see below).

(b) *Relative amounts of the replicative forms at various times after infection*

Since the infecting viral genome is 49 s RNA, RFI should be the first RF made in the infection cycle. To investigate this possibility and to determine the relative amounts of each RF at different times after infection, we examined RNA prepared from chick cells infected for varying lengths of time. This RNA had been labeled continuously starting at 1 hour after infection. Each preparation was assayed for its total and its RNase-resistant radioactivity (Fig. 3(a) and (b), respectively). Uptake of uridine was linear from 1·5 until 5 to 6 hours after infection, whereas incorporation into RNase-resistant material was linear from 1·5 to 4 hours. Notice that the net amount of RNase-resistant RNA remained constant from 4 to 10 hours. After 10 hours, the amount of label in both RNA and in RNase-resistant RNA declined because of loss of cells from the monolayers.

To determine the amounts of each RF in the preparation, the RNase-resistant RNA was analyzed by sucrose gradient–velocity sedimentation. The amounts of label in RFI and in RFII are plotted in Figure 3(b) for each time point. For clarity, the points

corresponding to RFIII (the smallest RF) are not included, but, from the following discussion, it will become clear that they fall below the respective points for RFII. In the middle of the infection cycle, from 4 to 6 hours, the amount of each of the RF's did not vary. After 6 hours, the amounts of RFII and RFIII decreased while the amount of RFI increased, although the total label in RF remained constant.

The molar ratios of RFI to RFII and of RFII to RFIII were also calculated and are plotted in Figure 3(c). Throughout the infection cycle, the molar ratio of RFII to

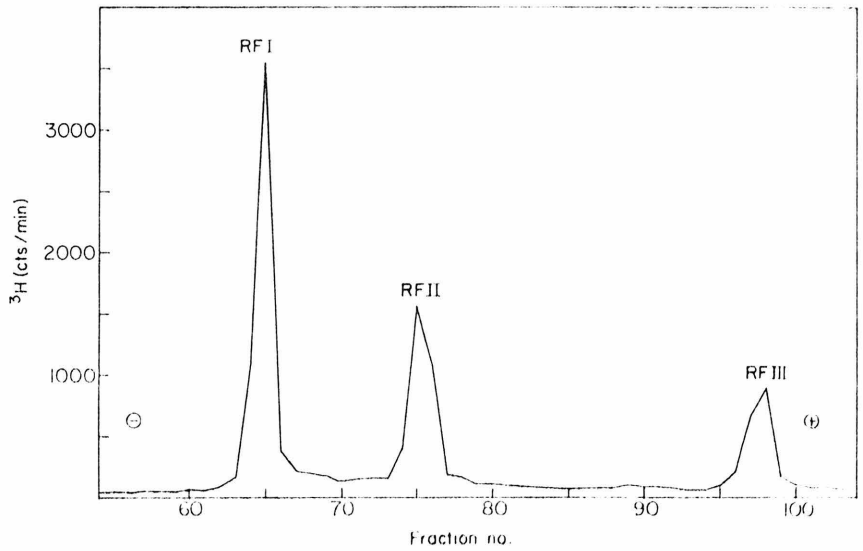


FIG. 2. Separation of the three RF's by polyacrylamide-agarose gel electrophoresis. Hamster cells were labeled with 100 μCi/ml. of [5-^{3}H]uridine for 38 min, starting at 6 hr after infection. The sodium dodecyl sulfate-lysate was extracted with phenol/chloroform at 55°C. The RNA was treated with RNase (10 μg/ml.) and DNase (50 μg/ml.) and re-extracted with phenol/ether. Electrophoresis was from left (cathode) to right (anode) for 13 hr at 10 mA in a 3/8 in. diameter gel containing 1·6% acrylamide, 0·08% bis-acrylamide and 0·5% agarose. The top of the gel is at fraction one; only the lower half of the gel is shown.

RFIII was a constant one-to-one. The significance of this result will be discussed below. It is also evident that RFI was the predominant RF early in infection, as we predicted, but interestingly enough, it was also predominant late in infection.

As a check on the relative amounts of RF present early in the infection cycle, another experiment was carried out with the label added at the beginning of the infection cycle (rather than at 60 min after infection). The same results as those given in Figure 3 were obtained.

(c) *Relative rates of RNA synthesis on the replicative form templates*

Nascent viral RNA from cells infected with single-stranded RNA viruses is located in replicative intermediates (Fenwick, Erikson & Franklin, 1964; Girard, Baltimore & Darnell, 1967; Stampfer, Baltimore & Huang, 1969). After RNase digestion, much of this newly synthesized material remains associated with the double-stranded cores or

RF's. The rate at which label is incorporated into an RF should thus be a direct measure of the rate of single-strand synthesis from this template. We therefore examined the kinetics of labeling of the three Sindbis RF's at 6 hours after infection. For the purpose of comparison the mass distribution of the RF's isolated from hamster cells at this time is shown in Figure 4. The molar ratio of RFI: RFII: RFIII was found

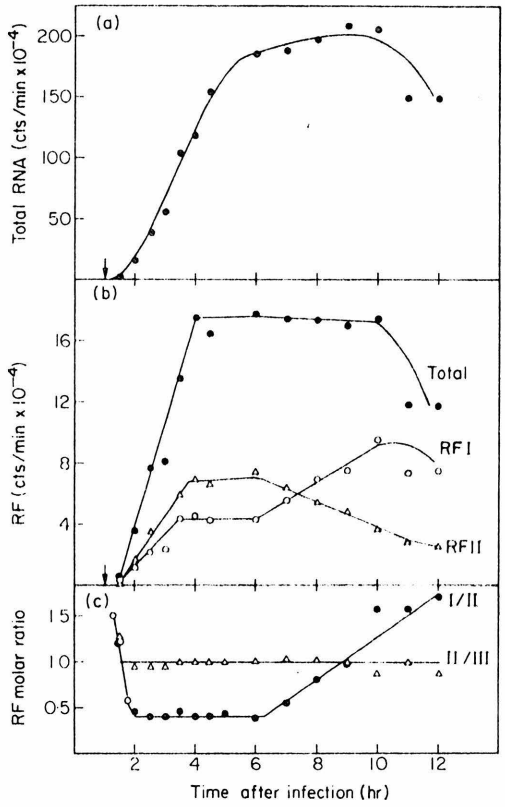


FIG. 3. Relative amounts of the RF's at various times after infection. Chick embryo cells were infected with Sindbis virus at a multiplicity of 10. At 1 hr after infection (arrows), [5-^{3}H]uridine was added to a final concentration of 20 μCi/ml., and at various times thereafter, cell monolayers were dissolved with Tris/NaCl/EDTA buffer containing 1% sodium dodecyl sulfate. The RNA was purified by the phenol/chloroform method at room temperature. Acid-insoluble radioactivity in total RNA (a) and in RNase-resistant RNA (b) was determined. In addition, a portion of each RNA sample was analyzed by sucrose gradient–velocity sedimentation following treatment with RNase to determine the relative amounts of the RF species. Total radioactivity in RFI, RFII and in RFIII (RFIII not shown) was determined (b), and the molar ratios of the various RF's were calculated (c). Explanation of the symbols is as indicated in the Figure. Values of the molar ratio of RFI to RFII are given for 2 different experiments (open and closed circles).

to be 0·4 : 1 : 1, as was the case for the RF's isolated from infected chick embryo cells (Fig. 3(c)).

When hamster cells were pulsed for 1 minute or 2 minutes at 6 hours after infection, the pattern of newly incorporated label in RF's (Fig. 5(a) and (b) and Table 1) was very different from the mass label in RF's. No RFII was detectable after a 1-minute pulse and very little was detectable after 2 minutes. In both cases, the majority of the

label was in RFIII (the smallest RF). In RF's pulsed for 8 minutes and 38 minutes (Figs 1 and 2), a rise in the relative amounts of RFII was readily observed. The kinetics of labeling of RFII are shown in Figure 6 (data for the early points are also given in Table 1). Figure 6 shows a plot of the molar ratio of radioactivity in RFII to RFIII (label in RFII to label in RFIII, normalized to their respective molecular weights) for several pulse lengths. As the pulse length increases, this ratio increases linearly and reaches an equilibrium value of one at 45 minutes. Since most of the RFIII molecules are labeled in a relatively short period of time, it takes approximately 45 minutes to completely label the RFII molecules. This represents a maximum estimate for the

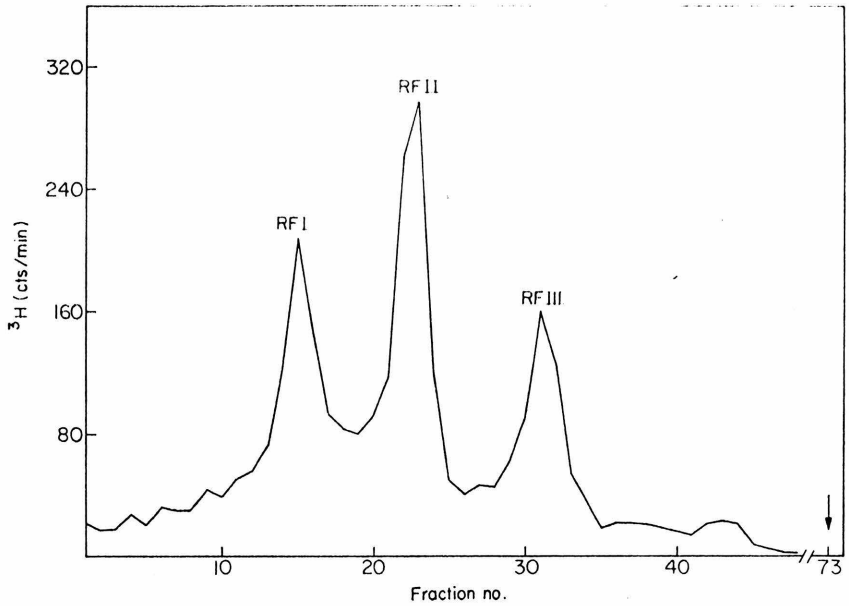


FIG. 4. RF's present at 6 hr after infection. Hamster cells were labeled continuously with 20 μCi/ml. [5-^{3}H]uridine between 1 and 6 hr after infection. The RNA was purified by the phenol/chloroform extraction method at room temperature, treated with RNase and sedimented in a sucrose gradient as described in the legend to Fig. 1.

average time required to synthesize a product molecule from an RFII template. If we assume that initiation of product chains is rate limiting and is random among the pool of RFII molecules participating in product synthesis, then the average time required for one product molecule to be synthesized from an RFII template would be approximately 30 minutes.

RFIII incorporates 2 to 3 times more label than RFI in very short pulses at this period of the infection cycle (Table 1). At this time, the mass ratio of 26 s RNA (the product molecule of RFIII, see below) to 49 s RNA (the product of RFI) is 2·5 to 1. Thus, the pulse data accurately reflect the relative rates of product synthesis. In longer pulses, the ratio of counts in RFIII to RFI changes (Table 1) in the direction of the mass ratio of these two RF's (0·83 to 1). Since the mass ratio of 26 s RNA to 49 s RNA is 2·5 to 1 and the mass ratio of RFIII to RFI is 0·83 to 1, RFIII is 3 times more active in RNA synthesis than RFI in terms of product chains per RF molecule.

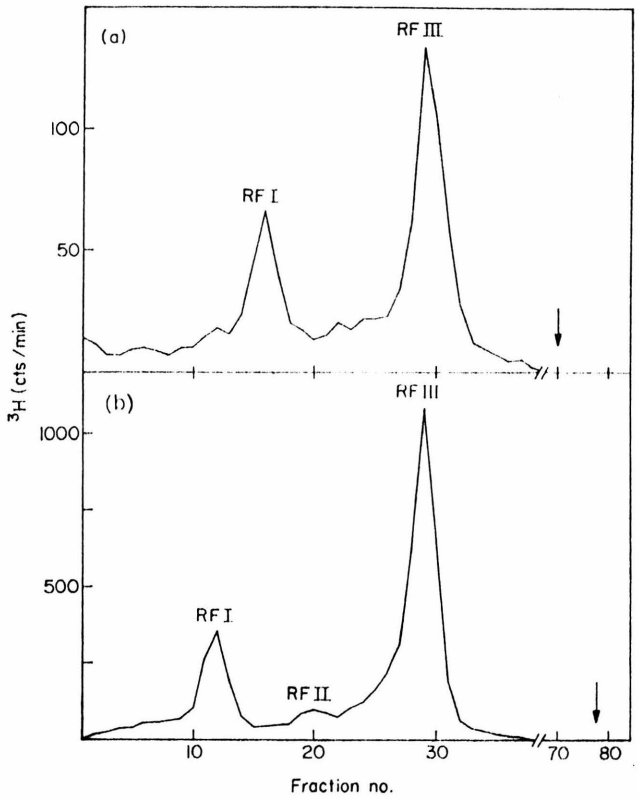


FIG. 5. One- and two-min pulse-labeled RF's. Preparation of the RNA and centrifugation is as described in the legend to Fig. 1, except that the pulse of [5-^{3}H]uridine was either 1 min (a) or 2 min (b) long.

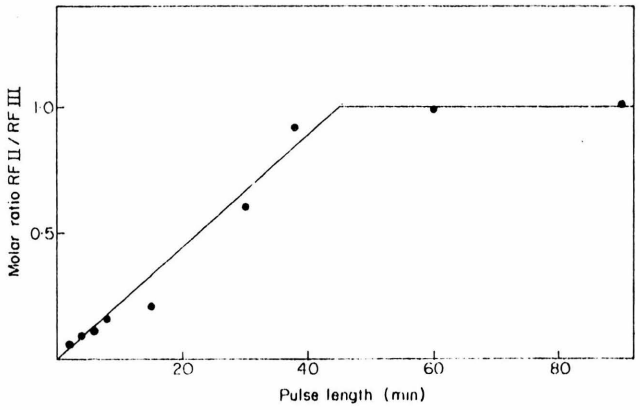


FIG. 6. Relative labeling kinetics of RFII and RFIII. Hamster cells were infected and labeled as described in the legend to Fig. 1, except that the pulse length, beginning at 6 hr after infection, was varied between 2 and 90 min. RF's were prepared and analyzed by sucrose gradient–velocity sedimentation (2-, 4-, 6-, 8-, 15-, 60- and 90-min samples) or by polyacrylamide gel electrophoresis (30- and 38-min samples). The molar ratio of labeled RFII to labeled RFIII was determined in each case and plotted *versus* the length of the pulse. This Figure includes data from 4 different experiments.

TABLE 1

Uridine incorporation into Sindbis RNA's during short pulses

Length of pulse (t) (min)	Total ^{3}H (cts/min incorporated)	% RNase resistance	RFI : RFII : RFIII (ratio of ^{3}H cts/min)	t_{III} (sec)
1	2,000	89	1 : — : 2·17	43
2	24,000	42	1 : 0·25 : 3·08	40
4	64,000	30	1 : 0·30 : 1·82	50
6	112,000	27	1 : 0·31 : 1·52	64
8	240,000	18	1 : 0·50 : 1·64	54

BHK21 cells were pulse-labeled at 6 hr after infection with [^{3}H]uridine for the length of time shown. Total cts/min in RNA, in RNase-resistant RNA and in each of the RF's were determined. The average time to synthesize one single-stranded RNA molecule from RFIII (t_{III}) was calculated by using the following relations:

$$\frac{\%\,\text{RNase resistance}}{100} = \frac{\text{RNase-resistant cts/min}}{\text{RNase-resistant cts/min} + \text{RNase-sensitive cts/min}},$$

$$= \frac{\text{RFI}+\text{RFII}+\text{RFIII}}{\text{RFI}+\text{RFII}+\text{RFIII}+\text{RFI}(X-1)+\text{RFII}(Y-1)+\text{RFIII}(Z-1)},$$

$$= \frac{\text{RFI}+\text{RFII}+\text{RFIII}}{X\,\text{RFI}+Y\,\text{RFII}+Z\,\text{RFIII}},$$

$$\text{and } t_{III} = t/Z$$

where X, Y, Z are, respectively, the number of product molecules made in time t from each labeled RFI, II and III, and where RFI, RFII, RFIII are the cts/min in these RF's. Y is negligible compared to X or Z, and X is approximated by $Z/3$.

RFIII is labeled much more rapidly than RFII, indicating that proportionately more product is synthesized from RFIII templates than from RFII templates. In the shortest pulse in which RFII was detectable (2 min), there was 12 times as much radioactivity in RFIII as in RFII (Table 1).

The rate of synthesis of RNA on RFIII templates was determined in two ways. In one experiment, cells were labeled with uridine for one hour starting at 5 hours after infection. The amount of label incorporated into 26 s RNA was 43 times greater than the radioactivity incorporated into RFIII. If only the plus strand of the RFIII template was labeled in this experiment, the average time required to synthesize a 26 s RNA molecule from an RFIII template was 85 seconds (60 min divided by 43). This is an upper limit on the time required. If 26 s RNA is unstable and turns over or if the minus strand in RFIII is labeled in whole or in part under these conditions, the actual time to synthesize a 26 s RNA molecule will be less.

We can also estimate the time for synthesis of a 26 s RNA molecule from the data in Table 1. Total incorporated radioactivity, the percentage RNase resistance, and the distribution of radioactivity among the 3 RF's is shown for RNA labeled during 1-, 2-, 4-, 6- and 8-minute pulses. For this calculation we assume, as a first approximation, that the pool size of radioactive uridine was constant during the pulse and that each growing chain releases the previously synthesized plus strand upon termination (or, to state it differently, that the first strand equivalent of label incorporated into the RF is RNase-resistant, as indicated by the 89% RNase resistance of the label in the one minute pulse: further incorporation of label in longer pulses leads to the appearance of RNase-sensitive RNA). The equations in Table 1 were derived using these assumptions and the fact that the radioactivity incorporated per molecule of RF is the same for RFI and for RFIII and is significantly greater than for RFII. From these equations,

the time required to synthesize a product molecule on RFIII is approximately 50 seconds. If the pool size of radioactive uridine increased during the pulse, this estimate will be too high, and the actual time required will be less than 50 seconds.

It is difficult to tell whether the pool size of labeled uridine is constant during a pulse under our conditions. In the experiment in Table 1 and in other experiments (not shown), radioactivity is incorporated linearly between 1 minute and 6 minutes after the start of the pulse, suggesting a constant pool size during this time. After 6 minutes, however, the incorporation is no longer linear.

We conclude that it takes on the order of 1 minute to synthesize a product molecule on an RFIII template. Since RFII and RFIII are found in equimolar proportions and it takes on the order of 30 minutes to synthesize the product of RFII template, we would expect on the order of 20 to 50 times more product molecules from RFIII as from RFII.

(d) *Replicative intermediates*

In all previously described RNA virus systems, each RF is derived from RI molecules possessing the same double-stranded backbone as the RF. These RI's vary from those

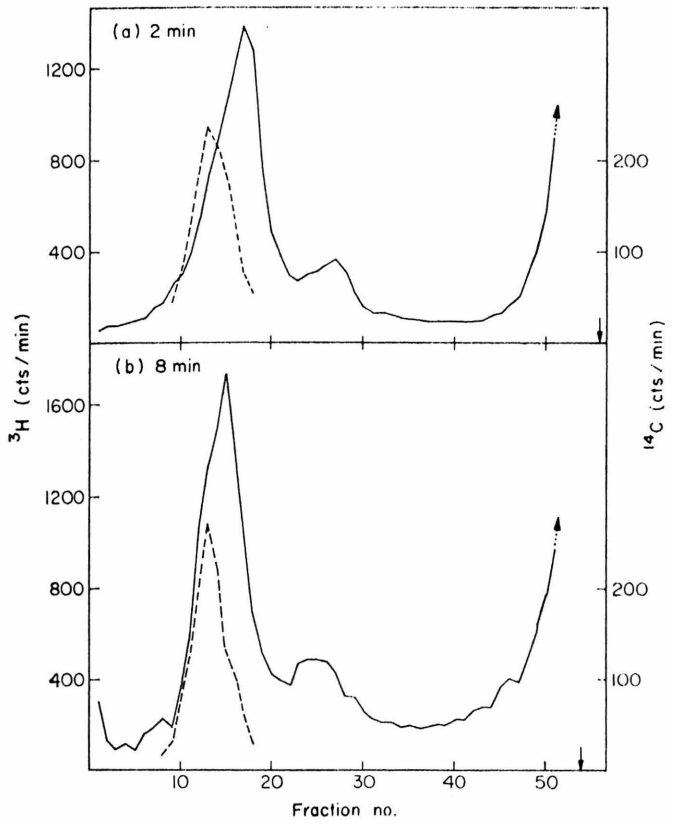


FIG. 7. Analysis of 2- and 8-min pulse-labeled RNA. Hamster cells were infected and labeled as in Fig. 1, except that the pulse length was 2 or 8 min beginning 6 hr after infection. The RNA was prepared by the cold sodium dodecyl sulfate/phenol method and sedimented in a 15% to 30% sucrose gradient for 8 hr at 40,000 rev./min (——). ^{14}C-labeled 26 s RNA, a Sindbis-specific species of single-stranded RNA, was added as a marker (– – – –). The size of the sample used was varied so that approximately equal amounts of radioactivity were layered on each gradient.

having very short single-stranded RNA "tails" and a sedimentation coefficient virtually indistinguishable from the RF, to molecules with long tails which sediment appreciably faster. Since there are three species of RF's derived from Sindbis-infected cells, we might expect, by analogy, that there would be three sizes of RI's each synthesizing single-stranded RNA separately. However, when RNA was pulse-labeled for 2 minutes or for 8 minutes and sedimented in the presence of Sindbis 26 s RNA as marker, most of the label was found in only one broad peak (Fig. 7(a) and (b)); the RF patterns from these pulse experiments were shown in Fig. 5(b) and Fig. 1, respectively). The sedimentation coefficient of the maximum of this peak was 23 s for RNA labeled in a 2-minute pulse and 25 s for RNA labeled in an 8-minute pulse. The shift in sedimentation coefficient of the maximum from 23 s to 26 s is undoubtedly due to the accumulation of labeled single-stranded 26 s RNA molecules which mask the RI maximum near 23 s. The smaller, more slowly sedimenting peak was almost entirely RNase-sensitive (see Fig. 8) and most probably represents degradation products (we have some evidence that pulse-labeled RNA is more labile than pre-existing RNA).

It is possible to define the position of RI's on these gradients by assaying for RNase resistance. For this purpose, RNA was pulse-labeled for 6 minutes and sedimented in a sucrose gradient. A sample of each fraction was counted for total radioactivity and selected fractions assayed for RNase-resistant label (Fig. 8). The RI's were found as one asymmetrical peak between 23 and 30 s. Since RFI has a sedimentation coefficient of 23 s, the double-stranded backbones of all RI's must be at least as large as RFI. The two smaller RF's (II and III) probably result from specific cleavage of RI molecules sedimenting in the 23 to 30 s region since no classes of RI's smaller than

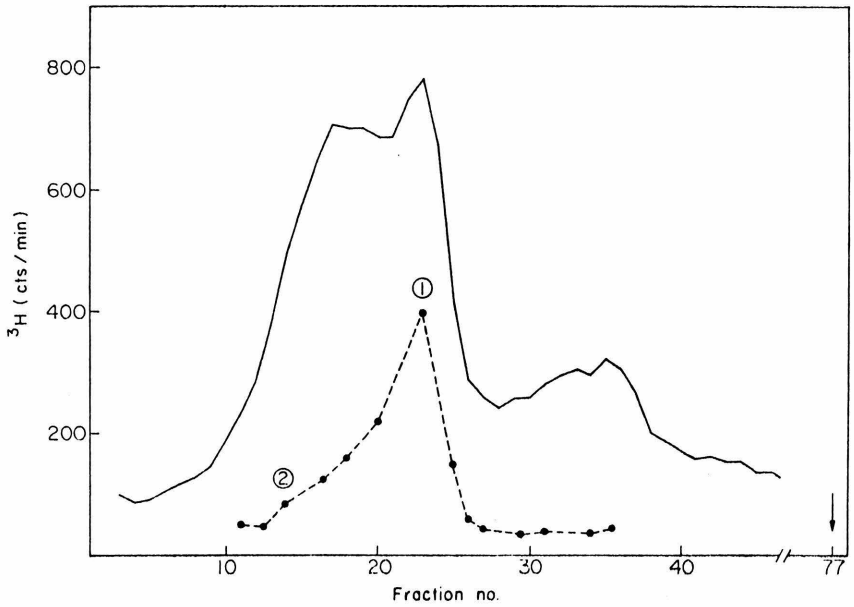


FIG. 8. Identification of RI's in RNA pulse-labeled for 6 min. RNA was prepared and sedimented as in Fig. 7, except that the pulse was 6 min long. The total radioactivity present in each fraction was determined (——). In addition, selected fractions were assayed for RNase-resistant radioactivity (– – – –).

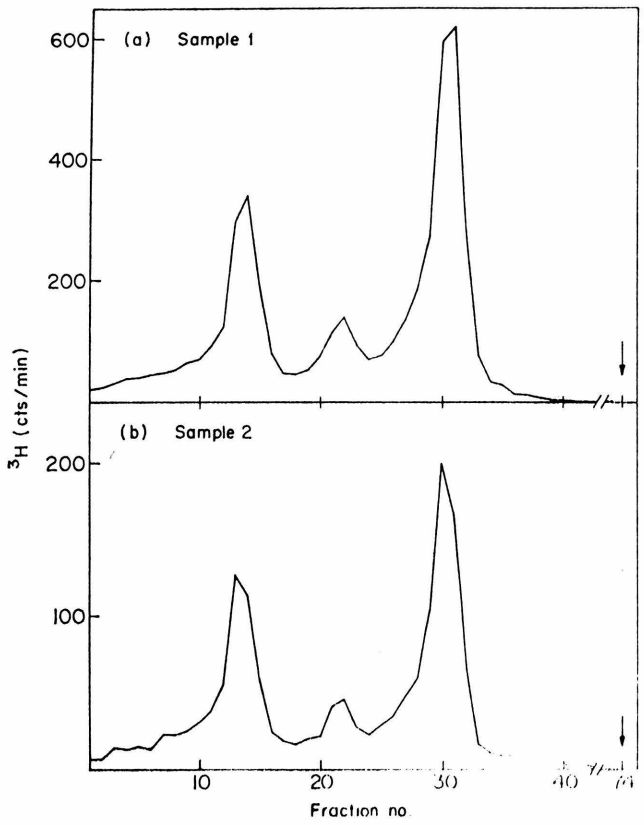


FIG. 9. Analysis of the RF's derived from different regions of the RI peak. The RNA in samples (1) and (2) of Fig. 8 was concentrated by ethanol precipitation, then treated with RNase and analyzed as in Fig. 1.

RFI exist. To test this hypothesis, the RNA from the 23 s region (sample 1 of Fig. 8) and the 30 s region (sample 2) were treated with RNase and resedimented (Fig. 9(a) and (b)). The distribution of the three RF's from these two points was nearly identical. This shows that all RF's can be derived from RI's in the same peak, and are found in the same proportions throughout the peak. Therefore, before RNase digestion, RF's II and III are joined together in an RI of the same size as the RI which yields RFI. This conclusion is strongly supported by the invariant one to one molar ratio of RFII to RFIII throughout the infection cycle (Fig. 3).

(e) *Hybridization results*

The molecular weights of Sindbis 49 s and 26 s single-stranded RNA (Simmons & Strauss, 1972), and of the three RF's, as well as the kinetics of labeling of the RF's strongly suggests: (a) that RFI is a double-stranded form of 49 s RNA, and its minus strand is a template for the synthesis of 49 s RNA, (b) that RFIII is a double-stranded form of 26 s RNA, and the minus strand of this RF is the template for the synthesis of 26 s RNA, and (c) that the minus strand of RFII is the template for the synthesis of a minor species of single-stranded RNA whose molecular weight is about $2{\cdot}8\times10^6$ daltons. Hybridization competition experiments with RFII and RFIII and with 26 s

and 49 s RNA were carried out to test this hypothesis. RF's were separated by preparative gel electrophoresis. Preparations of the single-stranded RNA species were obtained as previously described (Simmons & Strauss, 1972). Each annealing tube contained 15 ng of RFII or of RFIII, 1·5 ng of ^{32}P-labeled 26 s or 49 s RNA and various amounts (0 to 300 ng) of competing 26 s RNA. When RFIII (the smallest RF) was used as the source of minus strands (Fig. 10(b)), added 26 s RNA competed effectively with nearly all of the hybridized ^{32}P label, whether it was in 26 s or in 49 s

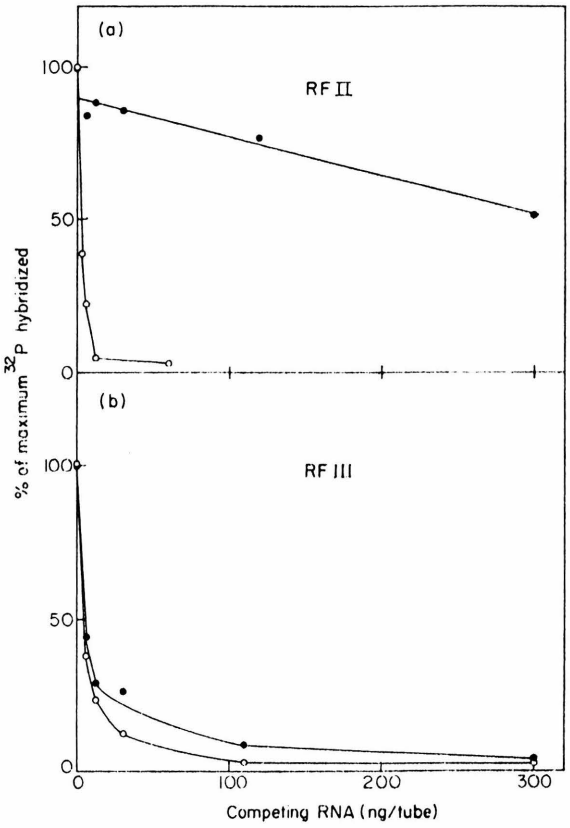


FIG. 10. Hybridization competition results. RF's were prepared from hamster cells infected with Sindbis and separated by preparative gel electrophoresis (see Materials and Methods). Single-stranded species of Sindbis-specific RNA (26 s and 49 s), labeled with either ^{32}P or ^{3}H were prepared from infected chick embryo fibroblasts as previously described (Simmons & Strauss, 1972). Conditions for hybridization are also described in the same reference. Each annealing mixture contained 15 ng of RF, 1·5 ng of ^{32}P-labeled RNA (either 49 s or 26 s) and varying amounts (0 to 300 ng) of competing 26 s RNA (^{3}H-labeled). In (a), the source of the minus strand was RFII and in (b), it was RFIII (from a different RF preparation). In both (a) and (b), the solid circles represent annealing mixtures containing ^{32}P-labeled 49 s RNA, and the open circles represent those containing ^{32}P-labeled 26 s RNA.

RNA. This result can be compared to the one obtained previously (Simmons & Strauss, 1972), with mixtures of RF's (i.e. RFI), where 26 s RNA was able to compete efficiently with only one-third of the hybridized 49 s RNA. Therefore, 26 s RNA contains all the information in the plus strand of RFIII. The inverse is probably also true since RFIII has twice the molecular weight of 26 s RNA.

Figure 10(a) shows that 26 s RNA was not able to compete effectively with the ^{32}P-labeled 49 s RNA for the complementary strand in RFII. The slight competition which did occur at very small concentrations of 26 s RNA could be explained by a 2% contamination (by weight) of the RFII preparation with RFIII. The remainder of the hybridized 49 s RNA was competed only with large amounts of 26 s RNA, probably because of contaminating fragments of 49 s RNA in the 26 s RNA preparation as previously described (Simmons & Strauss, 1972). The control curve in Figure 10(a) shows that 26 s RNA competes effectively with itself for the contaminating RFIII molecules. Thus, RFII does not contain any of the information in 26 s RNA and represents, therefore, a molecule genetically distinct from RFIII.

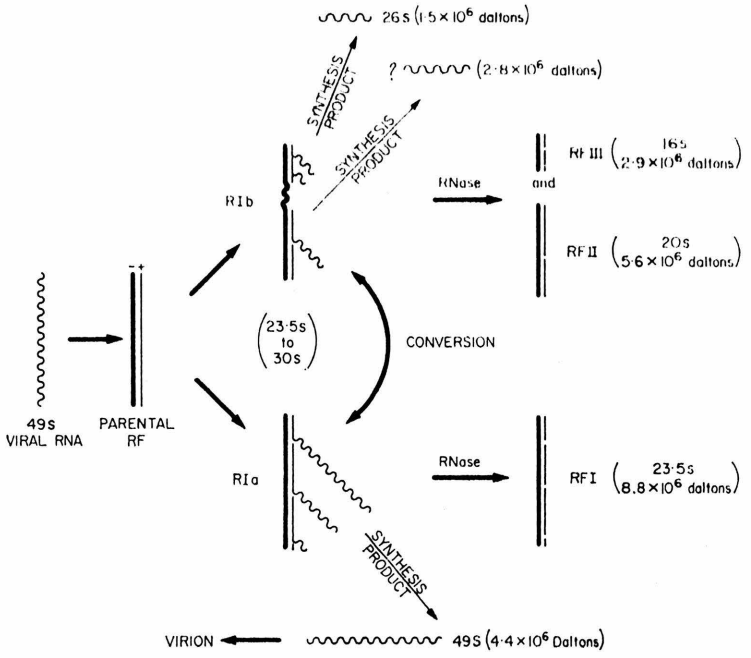


FIG. 11. Model for the replication of Sindbis RNA. Data from both the preceding paper (Simmons & Strauss, 1972) and this article have been used in the construction of this model. Wavy lines represent single-stranded molecules and single-stranded regions of the RI's. Double-stranded molecules are shown as straight lines. The minus strand is shown as a heavy line, the plus strand as a thin line.

4. Discussion

Our results on the replication of Sindbis-specific RNA are summarized in the model shown in Figure 11. We postulate the existence of two different types of replicative intermediates extracted from Sindbis-infected cells; these are called RIa and RIb. Both types have a nearly identical distribution of sedimentation coefficients (23 to 30 s). The complementary strand (minus strand) in RIa is the template for the synthesis of 49 s RNA (49 s RNA is then packaged into virions). When RIa is treated with RNase, only the single-stranded tails are degraded, and we are left with RFI. RIb differs from RIa in that it has a gap in a specific region of the plus strand, and the

single-stranded region in the minus strand across from this gap is susceptible to RNase. Thus the enzyme removes the single-stranded tails and also cleaves the minus strand to produce two pieces of double-stranded RNA: RFII and RFIII. RIb synthesizes at least two species of single-stranded RNA. One of these is 26 s RNA, which is made from that part of RIb which corresponds to RFIII. The other RNA is synthesized from the part of RIb which corresponds to RFII. Its identity is unknown, but its expected molecular weight is $2{\cdot}8 \times 10^6$ daltons. Recently, Levin & Friedman (1971) have detected a minor species of single-stranded RNA from Sindbis-infected cells which has an estimated molecular weight of $2{\cdot}5 \times 10^6$ daltons, and which may well be the product of the RFII template. In any event, this RNA is made in small quantities, as little as one molecule for every 20 to 50 molecules of 26 s RNA. Therefore, there must be an active and direct control mechanism which activates the synthesis of only part of the viral genome.

Probably, both 26 s RNA and the minor species of RNA of molecular weight $2{\cdot}8 \times 10^6$ daltons serve as messengers during infection. Specifically, we predict that 26 s RNA is the messenger for the virus structural proteins (the core and membrane proteins). It is translated as one large polypeptide chain, possibly the large polypeptide chain made in large amounts by certain temperature-sensitive mutants of Sindbis virus (Strauss, Burge & Darnell, 1969), which is then cleaved in a number of steps involving other polypeptides made in large quantities in infected cells (nos 2, 4, 9, 10 and possibly others). The minor RNA species is most likely the messenger for other virus-specific proteins which are only required in small amounts. The virion RNA (49 s RNA) must also possess messenger activity, at least during the early stages of virus infection, because Sindbis virions contain no RNA polymerase (Baltimore, Huang & Stampfer, 1970). Preliminary experiments have suggested that the changes in the relative amounts of the RF's which occur during the early and late parts of the infection cycle are due to a reversible, but controlled, conversion between RIa and RIb. In terms of a successful infection, the significance of this conversion is to allow for the synthesis of large amounts of 26 s RNA during the middle of the infection cycle and 49 s RNA near the end of the cycle. The conversion is easily explained by a mechanism whereby a replicase enzyme on a growing chain reads through or is prevented from reading through the region of the gap. The presence of two replicase enzymes with different specificities towards termination sequences on the complementary strand could account for such a conversion, or, alternatively, read-through could be a probability event influenced by mass action phenomena involving one or more species of RNA or of viral-specific proteins.

This model for the replication of Sindbis-specific RNA would seem directly applicable to the replication of other group A or group B arboviruses. In the group B arbovirus systems studied so far, molecules apparently corresponding to the 26 s and 49 s RNA of group A arboviruses are made during the infection, although in different relative amounts (Stoller, Schlesinger & Stevens, 1967; Trent, Swensen & Qureshi, 1969).

This mechanism may also operate after infection by other RNA viruses such as vesicular stomatitis virus and the paramyxoviruses (Newcastle disease virus and Sendai). Infection by vesicular stomatitis virus produces, in addition to the viral genome, smaller pieces of single-stranded RNA which serve as messenger. This messenger is complementary to the infecting viral RNA (Mudd & Summers, 1970), and appears to be made from one class of replicative intermediates (Stampfer *et al.*, 1969).

The replication of Newcastle disease virus involves the synthesis of at least three species of single-stranded RNA made in huge excess over the viral RNA and complementary to at least part of the genome (Bratt & Robinson, 1967).

Animal virus messenger RNA's probably lack internal chain termination and reinitiation signals for translation (Summers & Maizel, 1968; Jacobson & Baltimore, 1968). When faced with this restriction and with the need to manufacture large amounts of structural proteins, an RNA-containing virus may utilize a control mechanism at the level of RNA synthesis in order to manufacture virus-specific proteins in unequal amounts and thus conserve the protein synthetic machinery of the cell. This control mechanism involves production of messenger RNA of a size less than the entire genome length and may occur in two ways. In one case, the viral genome consists of segments synthesized from separate templates; the messenger RNA's correspond in size to these segments. These messengers may be present in the cell in different amounts and may be translated with unequal efficiency. Reoviruses and myxoviruses (influenza) are in this category. The RNA from influenza virus is in at least seven distinct single-stranded pieces (Duesberg, 1968; Lewandowski, Content & Leppla, 1971) each of which is synthesized from its own replicative intermediate (Pons & Hirst, 1968). The genome of reovirus and similar viruses consists of segments of double-stranded RNA which act as templates for the synthesis of the corresponding species of single-stranded RNA in infected cells (Bellamy & Joklik, 1967). In the second control mechanism, the genome is synthesized as an intact molecule from one template, and additional pieces of RNA to serve as messenger are made unequally from a second template. This second category includes the arboviruses, vesicular stomatitis virus and the paramyxoviruses.

We are pleased to acknowledge the expert technical assistance of Edith M. Lenches and Mildred Quisenberry Hoover. We thank Drs Ellen G. Strauss and John S. Pierce for their helpful editorial advice for the construction of this manuscript. This investigation was supported in part by the National Science Foundation grant no. GB13508 and by the U.S. Public Health Service grant no. GM06965. One of us (D. T. S.) was supported by the National Institutes of Health training grant no. 5-T1-GM-86.

REFERENCES

Baltimore, D., Huang, A. S. & Stampfer, M. (1970). *Proc. Nat. Acad. Sci., Wash.* **66**, 572.
Bellamy, A. R. & Joklik, W. K. (1967). *J. Mol. Biol.* **29**, 19.
Bishop, J. M. & Koch, G. (1967). *J. Mol. Biol.* **242**, 1736.
Bratt, M. A. & Robinson, W. S. (1967). *J. Mol. Biol.* **23**, 1.
Bray, G. A. (1960). *Analyt. Biochem.* **1**, 279.
Duesberg, P. H. (1968). *Proc. Nat. Acad. Sci., Wash.* **59**, 930.
Erikson, R. L., Fenwick, M. L. & Franklin, R. M. (1964). *J. Mol. Biol.* **10**, 519.
Fenwick, M. L., Erikson, R. L. & Franklin, R. M. (1964). *Science*, **146**, 527.
Franklin, R. M. (1967). *J. Virol.* **1**, 64.
Friedman, R. M. (1968). *J. Virol.* **2**, 547.
Geiduschek, E. P., Moohr, J. W. & Weiss, S. B. (1962). *Proc. Nat. Acad. Sci., Wash.* **48**, 1078.
Gesteland, R. F. & Boedtker, H. (1964). *J. Mol. Biol.* **7**, 43.
Girard, M., Baltimore, D. & Darnell, J. E. (1967). *J. Mol. Biol.* **24**, 59.
Jacobson, M. F. & Baltimore, D. (1968). *Proc. Nat. Acad. Sci., Wash.* **61**, 77.
Levin, J. G. & Friedman, R. M. (1971). *J. Virol.* **7**, 504.
Lewandowski, L. J., Content, J. & Leppla, S. H. (1971). *J. Virol.* **8**, 701.
Montagnier, L. & Sanders, F. K. (1963). *Nature*, **199**, 664.

Mudd, J. A. & Summers, D. F. (1970). *Virology*, **42**, 328.
Penman, S. (1966). *J. Mol. Biol.* **7**, 117.
Pfefferkorn, E. R., Burge, B. W. & Coady, H. M. (1967). *Virology*, **33**, 239.
Pons, M. W. & Hirst, G. H. (1968). *Virology*, **35**, 182.
Simmons, D. T. & Strauss, J. H. (1972). *J. Mol. Biol.* **71**, 599.
Stampfer, M., Baltimore, D. & Huang, A. S. (1969). *J. Virol.* **4**, 154.
Stollar, B., Schlesinger, R. W. & Stevens, T. M. (1967). *Virology*, **33**, 650.
Strauss, J. H., Burge, B. W. & Darnell, J. E. (1969). *Virology*, **37**, 367.
Strauss, J. H. & Sinsheimer, R. L. (1963). *J. Mol. Biol.* **7**, 43.
Studier, F. W. (1965). *J. Mol. Biol.* **11**, 373.
Summers, D. F. & Maizel, J. V. (1968). *Proc. Nat. Acad. Sci., Wash.* **59**, 966.
Trent, D. W., Swenson, C. C. & Qureshi, A. A. (1969). *J. Virol.* **3**, 385.
Weissman, C., Feix, G. & Slor, H. (1968). *Cold Spr. Harb. Symp. Quant. Biol.* **33**, 83.
Yin, F. H. & Lockart, R. Z., Jr. (1968). *J. Virol.* **2**, 728.

Note added in proof: We have no evidence as to whether 26 s RNA represents the 5′ end or the 3′ end of 49 s RNA. In Figure 11 the 3′ end was arbitrarily chosen. It has been suggested to us by several persons that the 5′ end might be a more reasonable hypothesis, since in that case 26 s and 49 s RNA could share the same initiation sequence on the minus strand. The less efficient initiation sequence for the minor species of single-stranded RNA would then be located internally.

ADDITIONAL DATA

In Fig. 7-9 in this Chapter, Sindbis replicative intermediates (RI's) were shown to consist of double-stranded RNA of a similar size as RFI and of single-stranded tails of various lengths. Replicative intermediates corresponding to RF's II and III were not detected in RNA from infected cells. The evidence indicated that RF's II and III were joined together in one RI molecule before treatment of the RNA with RNase. The following experiment is an attempt to demonstrate the same conclusion in a different way.

Total RNA from cells infected with Sindbis virus was subjected to sedimentation in a sucrose gradient and the fractions containing ribonuclease-resistant RNA (20S-30S) were pooled. These fractions contained labeled Sindbis 26S RNA as well as replicative intermediates.

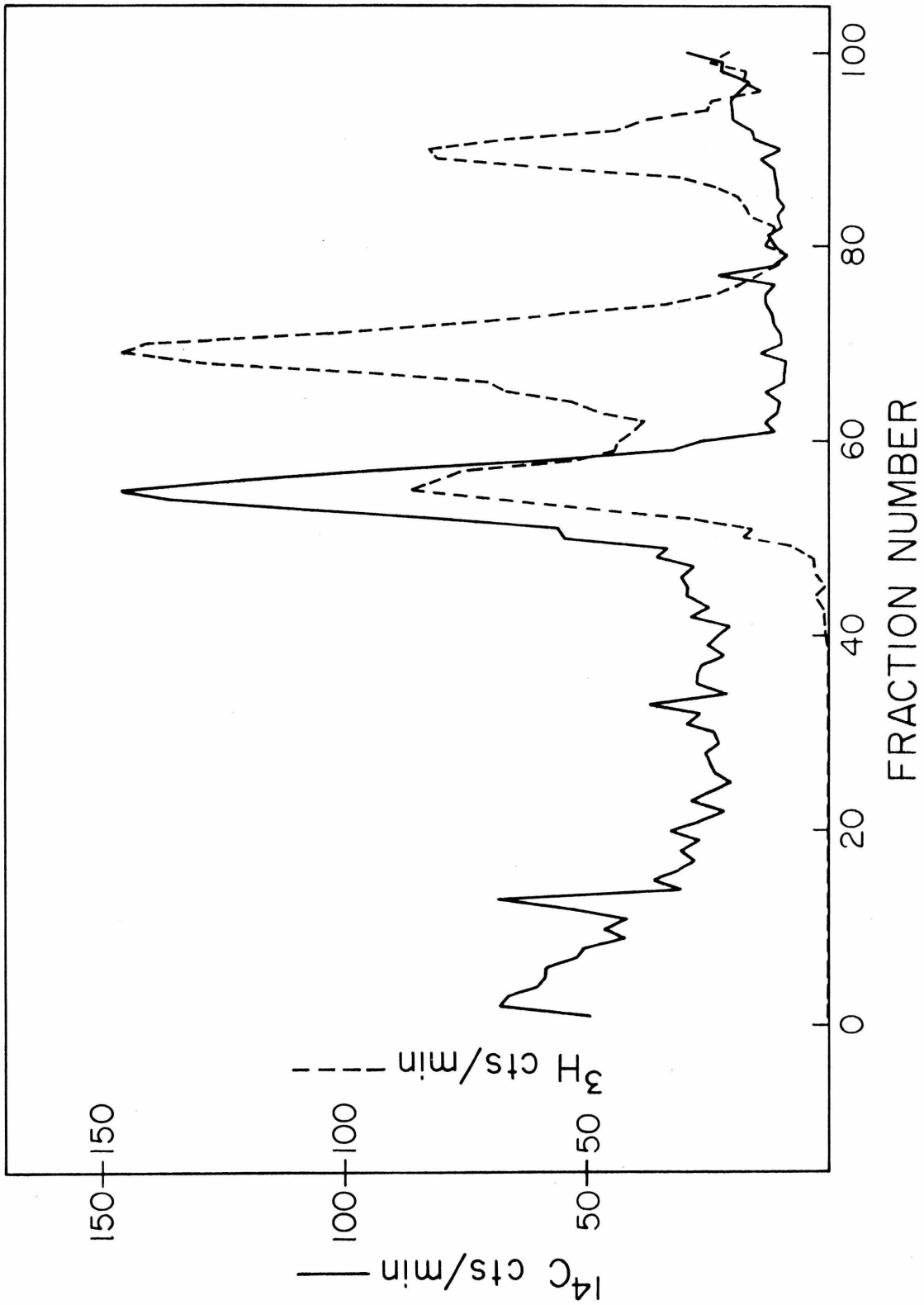
The sample of RNA was subjected to acrylamide gel electrophoresis in the presence of Sindbis RF's as markers (the RF's consisted of a mixture of RF's I, II, and III). The results are shown in Fig. 1. During electrophoresis, single-stranded 26S RNA migrated much faster through the gel than the smallest RF (RFIII) and had therefore migrated off the gel. A portion of the Sindbis-specific RI's comigrated in the gel with the RFI marker. In addition some of the label in RI's was distributed heterogeneously in the top half of the gel. Few or none of the RI's comigrated with the RFII or RFIII marker.

These observations are most easily interpreted if it is assumed that the RI's which comigrate in the gel with RFI consist of double-stranded RNA the same size as RFI but lack or have very short

Fig. 1. Infected cells were labeled with ^{14}C-uridine from 1 hr to 4 hr postinfection and the RNA extracted with phenol-chloroform at 0^{o}C. The RNA was subjected to sucrose gradient-velocity sedimentation and the fractions containing ribonuclease-resistant labeled RNA (20S-30S) were pooled and used as the source of replicative intermediates. The sample of RNA was subjected to electrophoresis in a gel consisting of 1.2% acrylamide, 0.06% bis-acrylamide, and 0.67% agarose. Sindbis RF's which had been labeled with [5-^{3}H] uridine from 1 hr to 4.5 hr after infection were added as markers. The direction of electrophoresis was from left to right for 11 hr at 3.5 mA.

(———) ^{14}C-labeled RI's

(-----) ^{3}H-labeled Sindbis RF's


150
100
50
^{14}C cts/min
^{3}H cts/min
0
20
40
60
80
100
FRACTION NUMBER

single-stranded regions. Furthermore, the RI's which migrate more slowly in a heterogeneous distribution near the top of the gel are retarded in the gel by proportionately greater total lengths of single-stranded tails. We can argue that RI's corresponding to RF's II and III do not exist as such since some of them would be expected to have very short single-stranded tails and migrate with the RFII or RFIII marker. These results indicate, therefore, that all Sindbis-specific RI's contain double-stranded regions of a size similar to RFI and that treatment of RI's with RNase converts a fraction to RFI and another fraction to RF's II and III.