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INTERCONVERSION OF REPLICATIVE FORMS OF SINDBIS VIRUS RNA AND THE ELECTROPHORESIS OF VIRAL RNA

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Jonathan D. Martin

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DEDICATION

This manuscript is dedicated respectfully and sincerely to Patty P. Martin, for her love, understanding, and perseverence, and to Dennis R. Roop, Steven D. Brown, and Mark L. E. Camblin, who have shared most acutely the author's graduate school experience.

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ABSTRACT

Three species of double-stranded (ds) RNA, designated RF I, RF II, and RF III in order of decreasing size, were produced by RNase treatment of extracts of chicken embryo cells infected for 6 h with Sindbis virus. These species were identifiable with those reported by Simmons and Strauss (28). At a low level of enzyme (0.001 µg/ml) the major species obtained was RF I. When the enzyme concentration was increased 10-, 100-, and 1000-fold, there was a progressive increase in the proportions of RF's II and III and a concurrent decrease in the proportion of RF I. Only RF I and replicative intermediate RNA (RI) were present in preparations of isolated ds RNA. Mild RNase treatment of these preparations converted the RI primarily to RF I. Higher enzyme levels generated greater proportions of RF II and RF III, but RF I-like molecules were the major source for these increased proportions. The generation of RF's II and III by RNase resulted in the ratio expected for these two species if they are produced by cleavage of RF I-like molecules. These results suggest a RNase-dependent conversion of RF I-like molecules to molecules of RF's II and III. The data support the model for Sindbis virus RNA replication proposed by Simmons and Strauss (28).

RF I was apparently the only naturally occurring ds form of Sindbis virus RNA. However, in the pool of partially purified RF I were cores which could be cleaved by RNase to generate RF's II and III. This finding is consistent with a replication model in which all transcription occurs on a negative strand of the size of the genome of Sindbis virus (28).

A study of the electrophoresis of Sindbis virus single-stranded RNA in polyacrylamide gels suggested an explanation for the disparity in molecular weights (MW's) previously reported for alphavirus RNA's. With HeLa cell 45S, 23S, 28S, and 18S RNA's as reference markers, the MW's of Sindbis RNA varied with the percentage of acrylamide in the gels, particularly for the virion RNA. This variation (3.2×10^6 to 4.5×10^6) may be explained in part by a deviation from linearity in the high MW-region of the calibration curves [log (MW) vs. mobility]. The break occurred in gels of 2.0 and 2.2% acrylamide but not in gels of 2.4 and 2.6% acrylamide. The most accurate determinations of MW were obtained on gels which provided strictly linear calibration curves. It is therefore important to use reference markers which calibrate the gel throughout the required range of MW. The deviation from linearity of the calibration curves for certain gels is discussed.

The rate of change in mobility of Sindbis virion RNA with gel concentration was considerably different from the changes in rates of Sindbis messenger RNA and HeLa cell RNA's used for reference. Certain theoretical considerations suggested the possibility that the virion RNA may have a secondary structure very different from those of the other RNA's.

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PART I

REPLICATIVE FORMS OF SINDBIS VIRUS RNA: INTERCONVERSION BY LIMITED RIBONUCLEASE TREATMENT

CHAPTER I

INTRODUCTION

Simmons and Strauss (28) proposed that in cells infected with Sindbis virus there are two types of replicative intermediates (RI) of the viral RNA. From these, three replicative forms (RF's) are generated by RNase treatment. RF I, which is the double-stranded (ds) form of the single-stranded (ss) genome (42S RNA), may be the enzymatic product of one population of RI (RIa). RF II, of unknown significance, and RF III, the ds form of Sindbis 26S messenger RNA, are proposed to be cleavage products of the other population of RI (RIb). This latter class of RI may be derived by discontinuous transcription of a minus-strand into 26S mRNA, which is one-third the size of the genome (27), and an as yet undetected ss RNA species that is equivalent to the other two-thirds of the genome.

To date this model has remained untested. Indeed, convincing corroborative data for the existence of three RF's have not been published. Levin and Friedman (16) obtained electrophoretic profiles of RNase-treated extracts of cells infected with Semliki Forest virus (SFV) which were suggestive of three RF's. Later, three ds forms of SFV RNA were identified in RNase-treated preparations of isolated RF and RI (19). However, the data suggested a model for SFV RNA replication that is somewhat different from that for Sindbis virus RNA replication. Bruton and Kennedy (3) found three naturally occurring SFV RF's which were of the sizes of those reported for Sindbis virus (28). Stollar et al. (32) reported that sucrose gradients of labeled extracts of Sindbis virus-infected cells contained

three RNase-resistant peaks of radioactivity. Their sizes were smaller than those reported by Simmons and Strauss (28). Finally, Segal and Sreevalsan (26) treated isolated Sindbis virus RI with RNase and obtained an electrophoretic profile of the products which suggested three ds species may have been produced.

Preliminary experiments in this laboratory suggested a means of testing the Simmons-Strauss model by verifying a prediction consequential to the model. At very low concentrations of RNase, there should be generated, from any or all RI's, ds molecules the majority of which should migrate during electrophoresis as RF I, since few minus-strand backbones would be attacked. That is, the enzyme-sensitive transcriptional gap of RIb-type molecules would be left intact, with only plus-strand tails being digested. At somewhat higher concentrations of RNase there may be nicking of the minus strand at this gap in some molecules. Therefore, some of the ds RNA, which in the former case would have migrated as RF I, now migrates as RF II and RF III.

The results obtained in this study follow the above prediction and support the model for replication of Sindbis virus RNA. There were found three ds RNA species identifiable with those obtained by Simmons and Strauss (28). Moreover, there was a RNase-dependent conversion of RF I-like molecules to molecules of RF II and RF III. These were generated in the expected ratio. RF I is probably the only naturally occurring replicative form. However, RF's II and III can be obtained by RNase treatment of either a pool of RF I and RI or of partially purified RF I.

CHAPTER II

MATERIALS AND METHODS

Media. Chick embryo cells were grown in Eagle's minimal essential medium containing Earle's salts (EMEM) and supplemented with 5% calf serum (medium and serum from GIBCO). Penicillin and streptomycin (Eli Lilly) and fungizone (GIBCO) were added to give concentrations of 100 units, 100 µg, and 2.5 µg per ml, respectively. Infection medium was EMEM with 2% heat-inactivated, dialyzed calf serum (GIBCO) and 1 µg/ml of actinomycin D (CALBIOCHEM).

Cells and virus. Cells from 10- or 11-day chicken embryos were obtained by methods used by Schluter et al. (25). Primary cultures of these cells (CE cells) were grown to confluency in 100 mm plastic tissue culture dishes (Falcon Plastics). Washed monolayers contained 1-2 × 10^7 cells/dish.

The heat-resistant strain of Sindbis virus (HR) was originally a gift of E. Pfefferkorn and has been described (4). For use in this study, HR was plaque-purified three times on CE cells.

Infection and labeling. Virus was diluted in cold infection medium so that an inoculum of 0.5 or 1.0 ml contained ∿10 plaque-forming units per cell. Twenty monolayers were washed twice with cold Earle's balanced salt solution (EBSS; GIBCO) and were inoculated. Adsorption was carried out at 4 C for 30 min. The inoculum was removed from each plate and was replaced with 10 ml of warm infection medium containing 10 µCi per ml of

[5-^{3}H]-uridine (28 Ci/mmol; New England Nuclear). Infected cultures were incubated at 37 C for 6 h.

Preparations labeled from 5.5 to 6 h post-infection (PI) were obtained by removing the medium at 5.5 h PI from plates infected as above and replacing it with 2.5 ml of fresh medium containing 40 µCi of ^{3}H-uridine per ml.

Cells also were infected and labeled essentially by the methods of Simmons and Strauss (28). Beginning at 6 h after infection, monolayers were labeled for 40 min in 2.5 ml of infection medium containing 40 µCi of ^{3}H-uridine per ml.

Preparation of RNA. RNA was extracted from cells by two methods. Method 1 involved extraction with a mixture of phenol, 8-hydroxyquinoline, and m-cresol (15). The mixture is more efficient in removing protein than is phenol alone. Method 2 was that prescribed by Simmons and Strauss (28).

Method 1. Twenty infected and labeled monolayers were washed three times with cold EBSS without phenol red. Each monolayer was lysed in situ at room temperature with 2 ml of pH 7.6 buffer (0.01 M Tris-hydrochloride, pH 7.6; 0.15 M NaCl; 0.001 M EDTA) containing 0.5% or 1.0% sodium dodecyl sulfate (SDS). Immediately after lysis the fluids were collected as two 20-ml pools. To each pool was added an equal vol of redistilled phenol saturated with buffer and containing 0.112 g of 8-hydroxyquinoline and 12.25 ml of m-cresol (Aldrich) per 100 ml of buffered phenol. The lysates were extracted by vigorous shaking at room temperature for 5 min. Phases were separated at 12,000 × g for 10 min at

0 C. The aqueous phases were removed and kept on ice while the phenol phases were reextracted with 10 ml of buffer + SDS. The aqueous phases thus obtained were pooled with the originals, and the pools were extracted 2 or 3 additional times with equal vol of fresh phenol mixture. The final aqueous phases were extracted twice with cold ether. Ether was removed by aspiration followed by perfusion with nitrogen. The aqueous phases were pooled, and 0.1 vol of 2 M sodium acetate, pH 6.0, and 2.5 vol of 95% ethanol were added. Precipitation was carried out at -20 C for at least 12 h. The precipitated RNA was collected at 12,000 x g for 30 min and was dissolved in pH 7.6 buffer to give an optical density at 260 nm of 11-13 units/ml. RNA prepared in this manner had A_{260}/A_{280} ratios of 1.9-2.1.

Method 2. Cells infected and labeled according to Simmons and Strauss (28) were extracted at 55 C by the method of Penman (22) as specified by the former authors for the preparation of Sindbis virus-specific double-stranded RNA for analysis on polyacrylamide gels. The precipitate was obtained by centrifugation as above and was dissolved in 10 ml (for 20 monolayers) of TNE buffer (0.01 M Tris-hydrochloride, pH 7.2; 0.1 M NaCl; 0.001 M EDTA) and was reprecipitated with sodium acetate and ethanol to remove SDS. The final precipitate was collected and dissolved in 10 ml of TNE buffer containing 0.01 M $MgCl_2$. One ml of a 500 µg/ml solution of DNase I (RNase-free; Worthington Biochemical) in the same buffer was added, and the preparation was incubated at 37 C for 15 min. The RNA was extracted with phenol-SDS and was precipitated with ethanol (28). The final precipitate was dissolved in TNE buffer to give 12 OD units of RNA per ml.

Chromatography on CF-11 cellulose. The double-stranded forms of Sindbis RNA were isolated by chromatography on Whatman CF-11 cellulose (10). A slurry was prepared according to the method of Bishop and Koch (2). Columns of 10- or 20-ml packed vol were washed at room temperature with 3 bed vol of 15% (v/v) ethanol in pH 7.6 buffer. RNA preparations were adjusted to 15% ethanol and were loaded. Single-stranded RNA was eluted with 3 bed vol of 15% ethanol and the remaining RNA with buffer alone. This latter fraction, containing all of the double-stranded RNA, was rechromatographed immediately after restoring the column to 15% ethanol. After this second passage, the 0% ethanol fraction contained very little single-stranded Sindbis RNA. The first 15% ethanol pool and the second 0% ethanol pool were precipitated with ethanol (without carrier) as above. The precipitates were collected at 27,000 RPM (SW27 rotor) for 2 h at 0 C. The pellets were dissolved in pH 7.6 buffer.

Treatment with RNase. Pancreatic ribonuclease A (Worthington Biochemical) was dissolved and diluted in pH 5.0 buffer (0.2 M sodium acetate buffer, pH 5.0) or in pH 7.2 buffer (0.1 M Tris-hydrochloride, pH 7.2; 0.15 M NaCl; 0.001 M EDTA). Portions of diluted RNase were added to equal vol of Sindbis RNA preparations to give final concentrations of 0.001 µg to 10.0 µg RNase/ml in either 0.1 M sodium acetate buffer, pH 5.0, or 0.05 M Tris buffer, pH 7.2. The ionic strength of either system was approximately 0.2. The mixtures were placed in a water bath at 37 C for 20 min. The reactions were terminated by the addition of 0.1 vol of 5% or 10% SDS in pH 7.6 buffer. After removing samples for TCA precipitation, most preparations were stored at -70 C. Some reaction

mixtures were precipitated with ethanol directly or were first extracted one time with chloroform-isoamyl alcohol (99:1) at room temperature.

Electrophoresis on composite gels. Polyacrylamide gels of 2.0 or 2.2% acrylamide (w/v) and modified to contain 0.5% agarose (w/v) were prepared by the method of Loening (17), which employs a Tris-phosphate buffer system ("E buffer" of Ref. 18). All reagents (Bio-Rad Laboratories) except ammonium persulfate and N,N,N',N'-tetramethylethylenediamine were mixed at 46 C. The latter two reagents (at room temperature) were added rapidly to the acrylamide-agarose mixture upon its removal from the water bath. Plexiglas tubes (9 cm × 6 mm bore) sealed at one end with parafilm were filled to near capacity. After the agar solidified, the exposed ends were covered with distilled deionized water. After standing for 30 min, the tubes were stored in a brown-glass jar at room temperature for one day and then at 4 C for up to two weeks.

Before use the gels were cut to 8 cm and sealed at the cut end with perforated dialysis tubing. The gels were subjected to electrophoresis without samples for 30 min at the same current used for electrophoresis with samples. All samples were adjusted to 25% (v/v) glycerol with an equal vol of 50% glycerol in pH 7.6 buffer. Electrophoresis was carried out at the currents and for the times given in the figure legends. For runs longer than 4 h, tap water was circulated through the jacketed, lower (anode) reservoir.

Gels were sliced on a Mickle gel slicer (Brinkman Instruments). Slices of 1 mm were digested in 0.2 ml of 30% hydrogen peroxide at 55-60 C for 8-14 h. The digested samples were counted in 4 ml of ScintiVerse

(Fisher Scientific) on a model 3375 Tri-Carb liquid scintillation spectrometer (Packard Instrument).

Salt fractionation. The naturally occurring RF of Sindbis RNA (see Chap. III, p. 37) was partially purified by salt fractionation of RNA prepared by Method 1. Two procedures for fractionation were followed: one employing 1 M NaCl (1, 26) and the other employing 2 M LiCl (26).

Fractionation with NaCl. A sample (2.5 ml) of RNA was mixed with an equal vol of 2 M NaCl in water. The mixture was placed at -20 C for 2 h and was then thawed at 4 C. The precipitate was collected at 40,000 RPM (SW 50.1 rotor) for 30 min at 0 C. The pellet was very gently rinsed with 1 ml of cold 1 M NaCl, and the wash was pooled with the supernate. The pellet was dissolved in 3 ml of pH 7.6 buffer. The salt-soluble and salt-insoluble fractions were precipitated overnight with ethanol at -20 C. The ethanol precipitates were recovered by centrifugation at 27,000 RPM (SW 27.1 rotor) for 2 h at 0 C. The salt-insoluble RNA was dissolved in 2 ml of pH 7.6 buffer, and the salt-soluble RNA, in 0.8 ml.

Fractionation with LiCl. One vol (2.5 ml) of 4 M LiCl was added to an equal vol of RNA. The preparation was placed at -20 C overnight and then was thawed at 4 C. The precipitate and supernate were obtained as above and were precipitated with ethanol. The final precipitates were collected at 40,000 RPM (SW 41 rotor) for 1 h at 0 C. Each pellet was dissolved in 0.4 ml of buffer.

Preparations obtained by either procedure were refractionated in 2 M LiCl.

CHAPTER III

RESULTS

Sindbis virus RNA. The study of double-stranded (ds) RNA produced during Sindbis virus infection of CE cells required that one be able to identify such molecules in cell extracts. The RNA extracted with phenol-hydroxyquinoline-cresol (Method 1, p. 5) from cells infected and labeled in the presence of actinomycin D for the first 6 h of infection contained the species shown in Fig. 1a. The pattern is similar to that observed by Levin and Friedman (16) and Guild and Stollar (13). The two major species, designated 42S and 26S, are single-stranded (ss) and correspond respectively to the genome of purified virions (13, 16, and Martin, data not shown) and to the messenger RNA encoding virion structural proteins (5, 6, 29). [Determination of the molecular weights of these ss RNA species is the subject of the second part of this dissertation (p. 37) and will not be discussed here.] Minor species of size 38S and 33S (13, 24) were minimally detectable in this preparation but were more prominent in others. The 16-18S species reported by Rosemond and Sreevalsan (24) for Sindbis virus and by Levin and Friedman (16) for Semliki Forest virus was not present in any of the extracts prepared in this study. The 42S and 26S molecules behaved as ss RNA during chromatography on CF-11 cellulose (2, 10; Fig. 1b). The two peaks designated DS in Fig. 1a were composed of molecules which behaved as ds RNA during chromatography (Fig. 1c). The faster-migrating species is presumably a replicative form, and the slower species is thought to be the replicative intermediates of the

Figure 1. Single- and double-stranded RNA's of Sindbis virus.

CE monolayers were infected and labeled for 6 h as described in Materials and Methods. The RNA was extracted with phenol-cresol-hydroxyquinoline at room temperature (Method 1, p. 5) and was ethanol-precipitated. The precipitate was dissolved in 8 ml of pH 7.6 buffer. (A) Ten µl of an equal-vol mixture of the RNA solution with 50% glycerol in the same buffer were applied to a 2.2% acrylamide/0.5% agarose gel, and electrophoresis was carried out at 5 mA/gel for 4 h. (B) A portion of the RNA (0.2 ml) was fractionated on a 5-ml column of CF-11 cellulose as shown in Fig. 2. The 15% ethanol eluate from the first passage (POOL A/15) was precipitated, and the product was dissolved in 0.5 ml of buffer. Ten µl were mixed with glycerol and electrophoresis was done as in (A). (C) The 0% ethanol pool from the second passage on CF-11 cellulose (POOL B/O) was precipitated and dissolved in 0.1 ml of buffer. Thirty µl were mixed with glycerol, and electrophoresis was performed as above. (D) Fifty µl of POOL B/O RNA were treated with RNase (final concentration of 0.1 µg/ml) as described in Materials and Methods (p. 7). After addition of SDS, half of the mixture was mixed with glycerol, and the final sample (100 µl) was subjected to electrophoresis.

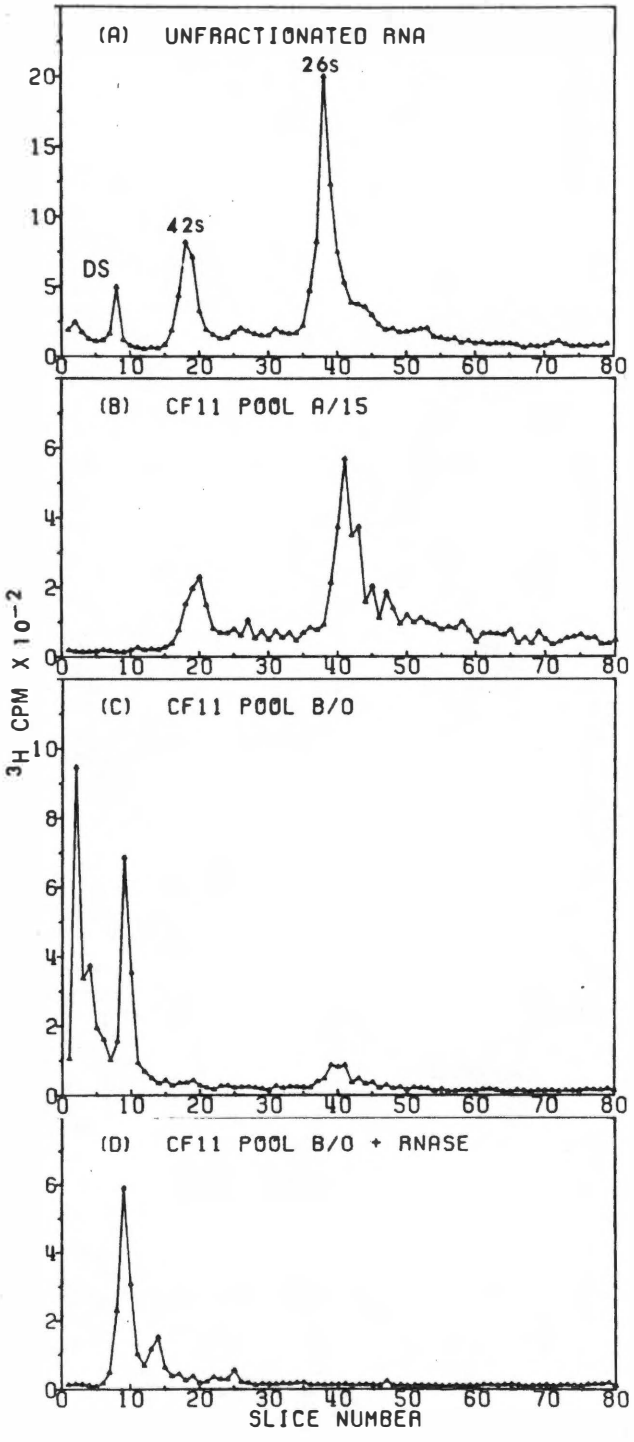

(A) UNFRACTIONATED RNA
26s
42s
DS
(B) CF11 POOL A/15
(C) CF11 POOL B/O
(D) CF11 POOL B/O + RNASE
^{3}H CPM X 10^{-2}
SLICE NUMBER

Figure 1

ss RNA's. The course of fractionation of ss RNA from ds RNA by this method is shown in Fig. 2. Single-stranded RNA was eluted from CF-11 cellulose by 15% ethanol in buffer, while ds RNA was eluted in buffer alone (0% ethanol). The ds RNA pool (POOL B/O) from two passages on CF-11 cellulose was only slightly contaminated with ss RNA (Fig. 1c). This pool contained molecules which migrated as two species after mild RNase treatment and limited electrophoresis (Fig. 1d). One of these species, presumably a replicative form, migrated identically to one of the forms in untreated ds RNA (Fig. 1a, c, and d). The products of RNase treatment of the ds RNA described here were the subject of this study.

RNase treatment at pH 5. Initial experiments involved treatment of total RNA extracts from infected cells with bovine pancreatic ribonuclease at pH 5.0. This pH was chosen because it is optimum for RNase activity at ionic strengths near 0.2 (14), that which is provided by most buffers used in extraction and analysis of alphavirus RNA. When samples of the total RNA described in the preceding section were treated with enzyme at pH 5.0, prolonged electrophoresis of the products revealed three species which survived at the lower concentrations of enzyme (Fig. 3a, b). The three peaks correspond to the three species described by Simmons and Strauss (28 and data below). The observation of interest was that of the change in proportions of the species caused by changing the concentration of RNase. In this experiment, the slowest-migrating species, designated replicative form I (RF I; Ref. 28), comprised the greatest proportion (48.7%) of the radioactivity following treatment with 0.01 µg RNase/ml (Fig. 3a). At a ten-fold higher concentration of enzyme (Fig. 3b), the

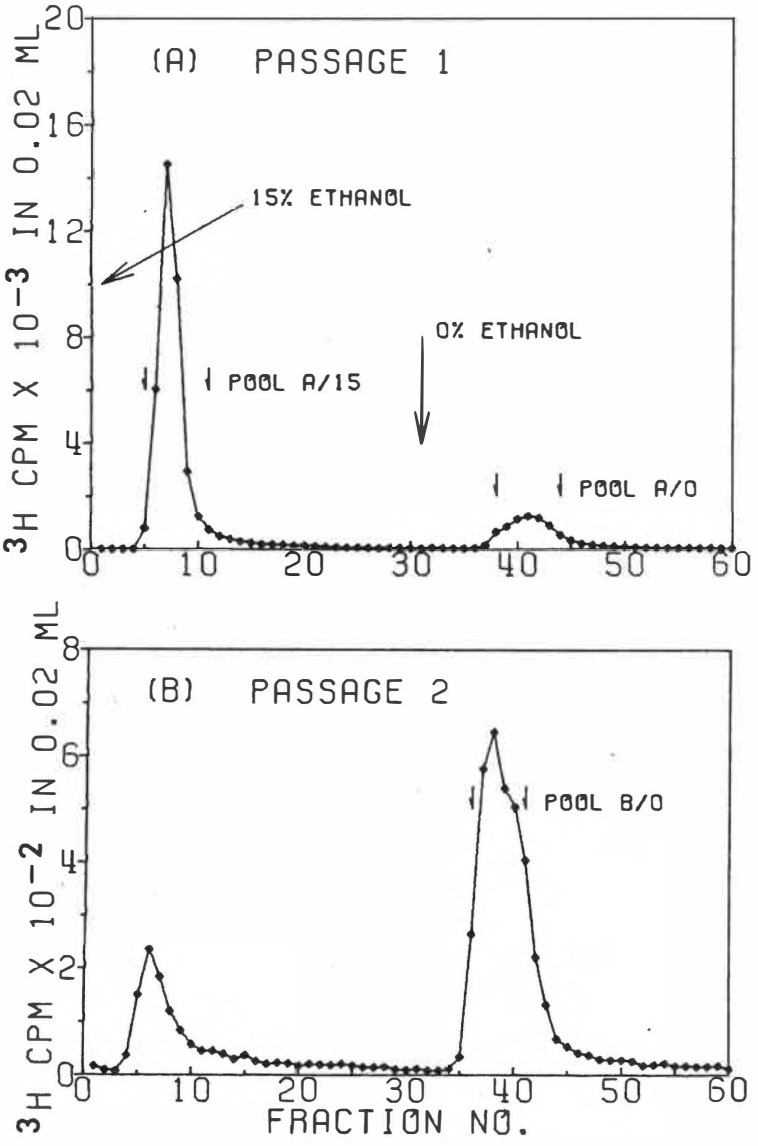


Figure 2. Chromatography of Sindbis RNA on CF-11 cellulose.

RNA was prepared and fractionated as described in the legend to Fig. 1. Fractions of 1 ml were collected at a flow rate of 1 ml/min. Samples of 20 µl were taken for assay of radioactivity. (A) POOL A/15 was the ss RNA eluted with 15% ethanol during the first passage. POOL A/O was a mixture of ss and ds RNA (data not shown) and was the starting RNA for the second passage. (B) POOL B/O was purified ds RNA eluted with buffer alone during the second passage.

Figure 3. Products of RNase treatment at pH 5.0 of Sindbis RNA.

Samples (0.5 ml) of the RNA prepared for the analyses shown in Fig. 1 (p. 11) were treated with RNase at pH 5.0 as described in Chapter II (p. 7). The final concentrations of enzyme are indicated. Treatment was stopped by the addition of SDS, and the products were precipitated with ethanol. The precipitates were collected and dissolved in 0.2 ml of 0.5% SDS in pH 7.6 buffer. After adjustment to 25% glycerol, samples were analyzed by electrophoresis on 2.2% gels at 5 mA/gel for 6 h.

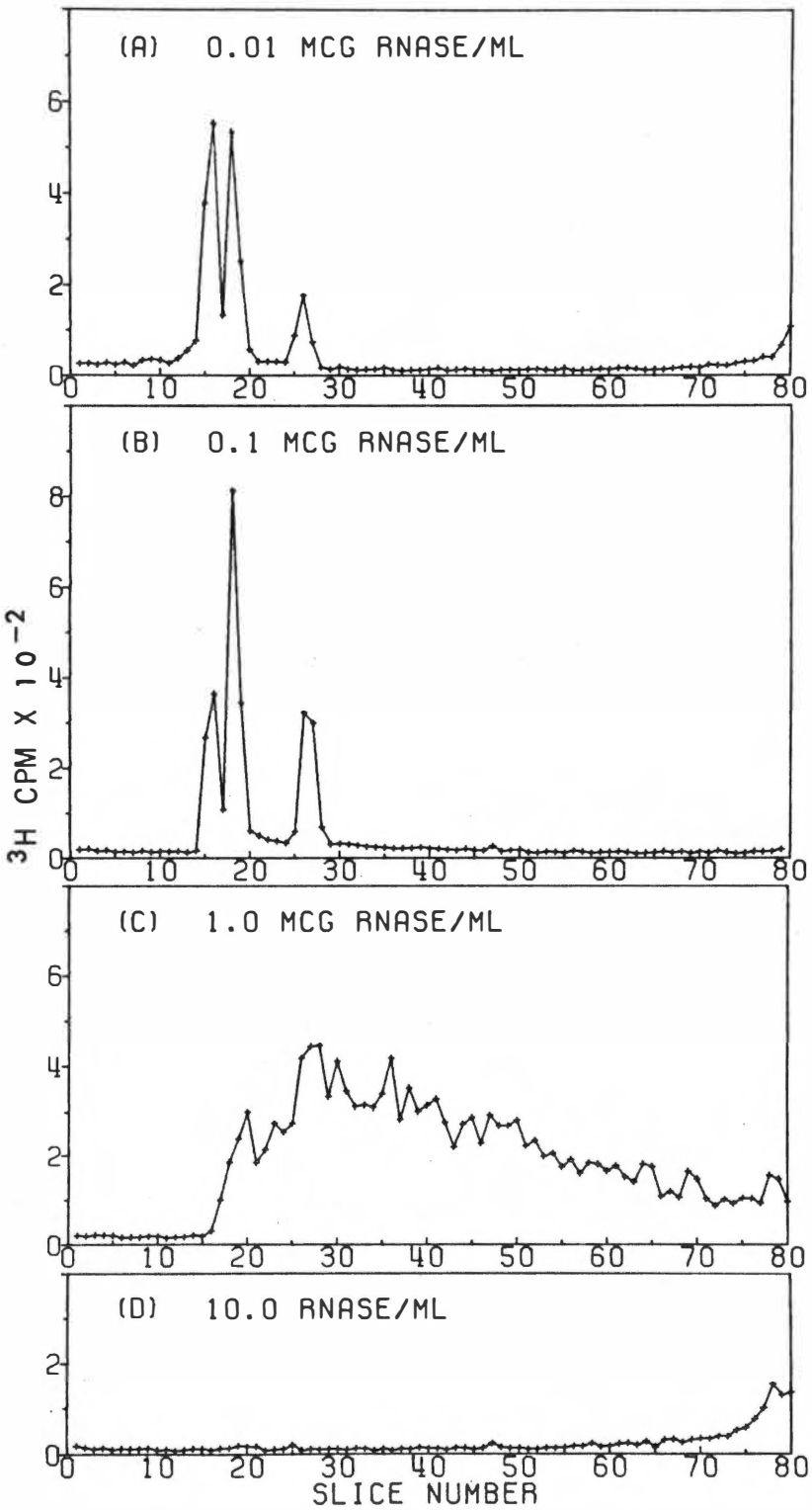

(A) 0.01 MCG RNASE/ML
(B) 0.1 MCG RNASE/ML
(C) 1.0 MCG RNASE/ML
(D) 10.0 RNASE/ML
^{3}H CPM X 10^{-2}
SLICE NUMBER

Figure 3

intermediately migrating species (RF II) was predominant (48.6% of the radioactivity), and there was an increase in the proportion of radioactivity (from 12.6% to 26.2%) contributed by the fastest-migrating species (RF III). The total CPM in the three peaks was approximately the same at both enzyme concentrations (2144 and 2054 CPM, Fig. 3a and b, respectively). It therefore seemed that RF I-like molecules were converted to molecules of RF II and RF III at the higher RNase concentration. This result suggested a means of testing the model proposed by Simmons and Strauss (28) for the synthesis of Sindbis virus RNA's. The rationale is stated in the Introduction above (p. 2).

In this experiment and others, RNase concentrations of 1.0 µg/ml or greater degraded the ds products surviving lower enzyme concentrations (Fig. 3c, d). It is known that this ribonuclease attacks double-stranded molecules (8, 9, 21).

Several experiments employing several RNA preparations were performed with various low concentrations of RNase at both pH 5 and pH 7.2 in order to examine this phenomenon in detail. Treatment at neutral pH is more common, and Simmons and Strauss (28) used a neutral pH. Because results from experiments carried out at pH 5 showed the same pattern as that seen at pH 7.2, only the latter experiments are described below. However, exemplary results obtained by enzyme treatment at pH 5 are given in the Appendix (p. 63) in a manner which parallels that used for the presentation below of the data from experiments carried out at pH 7.2.

It should be mentioned here that the presence of RNase (and SDS) during electrophoresis of the products did not affect their rates of

migration or their proportions during electrophoresis. One RNA preparation was treated with RNase (at pH 5) and the products were analyzed by brief electrophoresis without prior removal of the enzyme (Fig. 4a). A second preparation was treated similarly but was extracted with chloroform-isoamyl alcohol to remove the enzyme before electrophoresis (Fig. 4b). The two sets of products had identical rates of migration. Extraction of the reaction mixtures was therefore not necessary.

RNase treatment at pH 7.2. RNA was prepared from infected CE cells by two methods. In the first method, cells were continuously labeled for the first 6 h of infection, and the RNA was extracted at room temperature with phenol-cresol-hydroxyquinoline. The second method was essentially that of Simmons and Strauss (28). Infected cells were labeled for a 40-min period beginning at 6 h after infection. The RNA was extracted with phenol-chloroform at 55 C. Portions of these preparations, designated RNA 1 and RNA 2, respectively, were treated with various low concentrations of RNase at pH 7.2.

The course of digestion of virus-specific RNA in total RNA extracts is shown in Fig. 5. The progress of digestion by increasing concentrations of RNase was very similar for the two types of RNA preparations. At the lowest enzyme level used (0.001 μg/ml), the RNA's appeared to be 90-95% resistant, as measured by cold TCA precipitation. However, all of the viral ss RNA was degraded to heterogeneous fragments of the size range 20S to 4S (data not shown). Replicative intermediate molecules were degraded to replicative forms (see below). Thus, RNase-resistance must be assessed by examination of the product molecules. As will be shown,

Figure 4. Effect of the presence or absence of RNase on migration of ds RNA during SDS-polyacrylamide gel electrophoresis.

RNA was extracted (Method 1, p. 5) from CE cells infected and labeled for 6 h. Samples were treated with RNase (final concentration of 0.01 µg/ml) as described in Chapter II (p. 7). One preparation was extracted 1× with an equal volume of chloroform-isoamyl alcohol (99:1, v/v). Both preparations were precipitated with ethanol and then were dissolved in pH 7.6 buffer. The products were analyzed as described in the legend to Fig. 1 (p. 11).

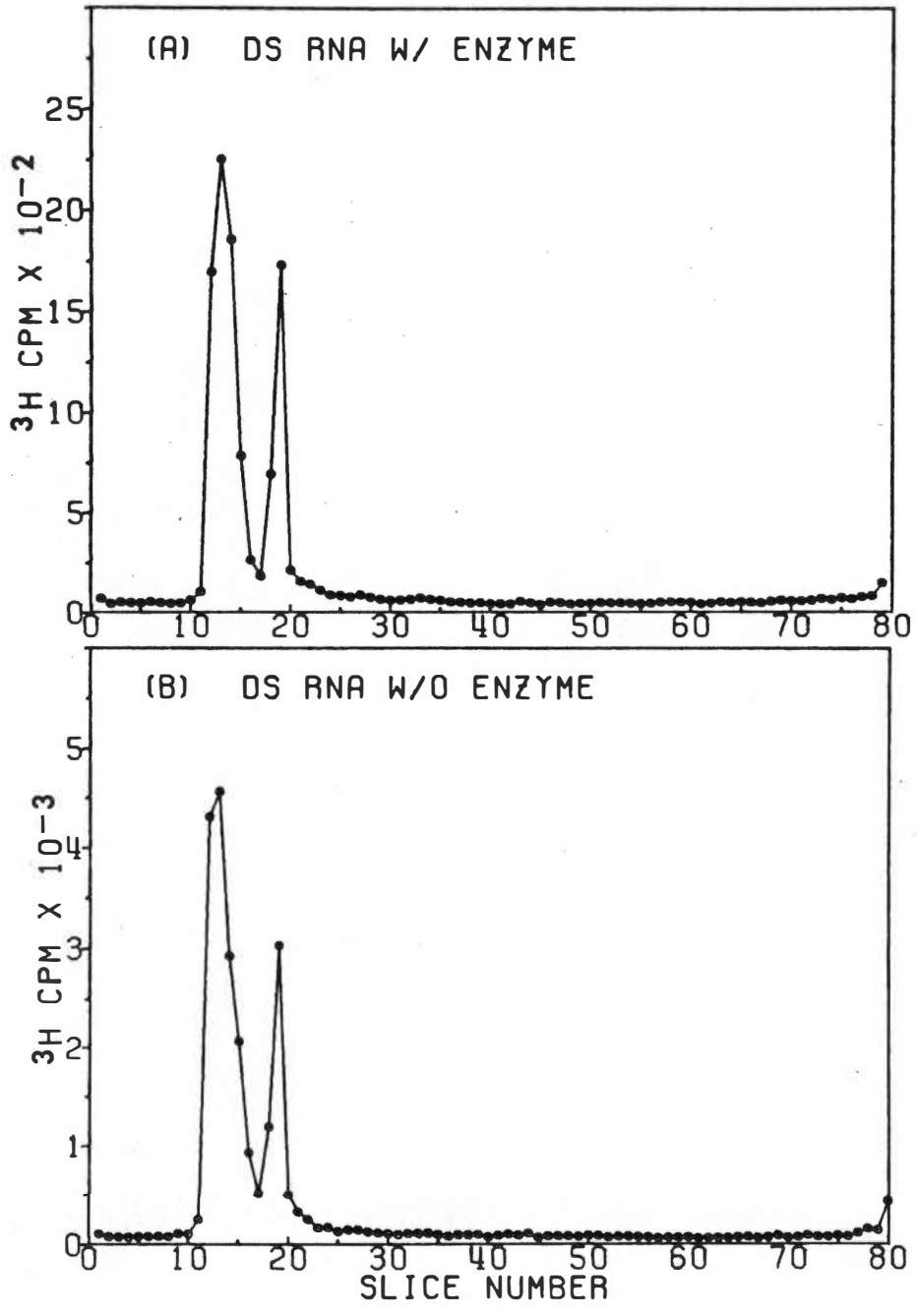

(A) DS RNA W/ ENZYME
^{3}H CPM X 10^{-2}
0
5
10
15
20
25
0
10
20
30
40
50
60
70
80
(B) DS RNA W/O ENZYME
^{3}H CPM X 10^{-3}
0
1
2
3
4
5
0
10
20
30
40
50
60
70
80
SLICE NUMBER

Figure 4

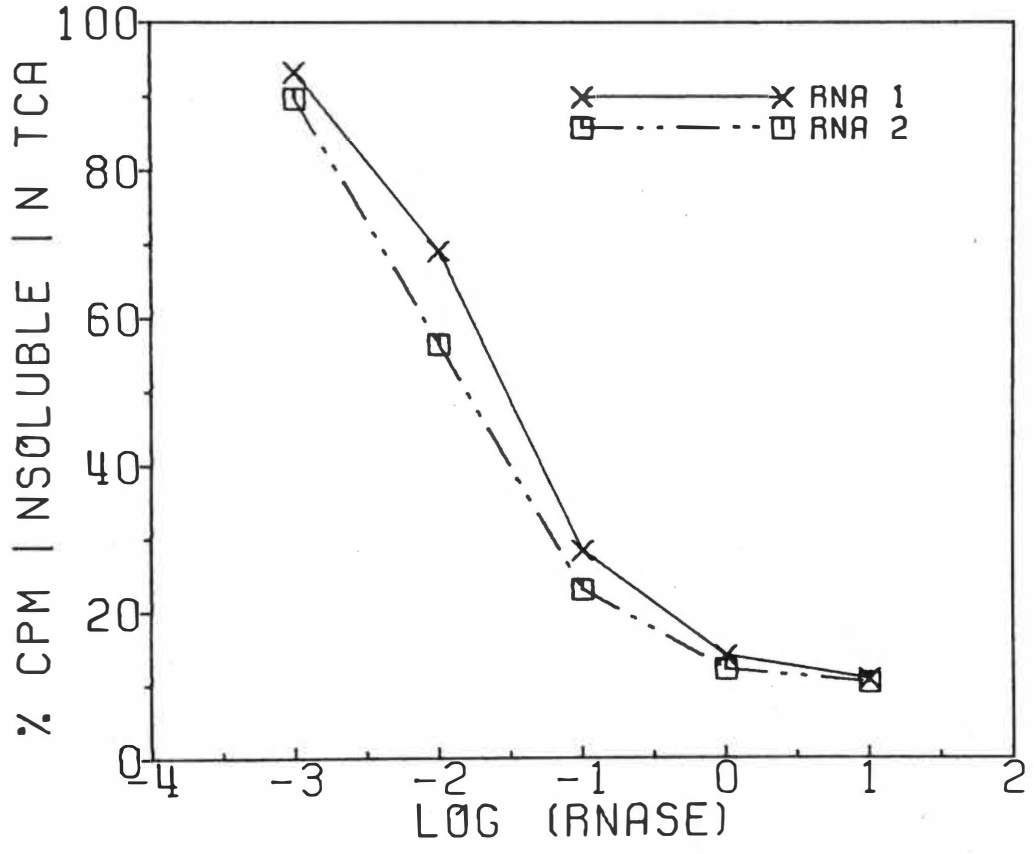


Figure 5. Digestion of Sindbis virus RNA at low concentrations of RNase at pH 7.2.

RNA was prepared by the two methods described in the text. Portions (50 µl) were treated with the indicated concentrations of enzyme as described in Chapter II (p. 7). The reactions were stopped with SDS, and 20-µl samples were taken for precipitation with cold TCA. RNA 1 was prepared by the first method as stated in the text. Each point represents the average for three experiments. RNA 2 was prepared by the second method, and each point represents two experiments.

RNase concentrations up to 1.0 µg/ml caused changes in the proportions of the three replicative forms. Single-stranded RNA was degraded to molecules of sizes 4S and smaller (data not shown). Although the RNA's appeared to be 10% resistant to the highest level of RNase used (10 µg/ml; Fig. 5), even the ds RNA's were partially degraded (Fig. 6e).

These results differ somewhat from those obtained by treatment at pH 5 in that at pH 7.2, but not at pH 5, ds molecules were intact after treatment with 1 µg RNase/ml (cf. Fig. 3c, p. 16, and Fig. 6d). It should be pointed out also that the course of digestion (Fig. 5) did not differ between RNA labeled for 6 h (RNA 1) or for 40 min (RNA 2). Therefore the observed pattern of digestion is a consequence of the properties of the RNA molecules and not of the kinetics of labeling.

Analysis on composite gels of the ds products from RNase treatment of RNA 1 gave the profiles shown in Fig. 6. After treatment with 0.001 µg RNase/ml, RF I was clearly the major product, comprising 60.4% of the total CPM in the peaks shown (Fig. 6a). This proportion decreased as higher levels of enzyme were used (Fig. 6b-d). At 1.0 µg/ml RF I contributed only 25.5% of the radioactivity. The proportions of RF II and RF III increased from 23.1% and 16.4%, respectively (Fig. 6a), to 48.0% and 26.5% (Fig. 6d). As mentioned above, 10 µg of RNase per ml degraded the ds RNA (Fig. 6e).

No replicative intermediate molecules remained after treatment at the lowest enzyme level (Fig. 6a); they were converted to replicative forms (see below). Therefore, they were not a source for the increases of RF II and RF III. It appears that the increased proportions of these

Figure 6. Products of RNase treatment at pH 7.2.

Treated samples of RNA 1 were adjusted to 25% glycerol, and samples (50 µl) were subjected to electrophoresis on 2.0% gels at 8 mA/gel for 6 h.

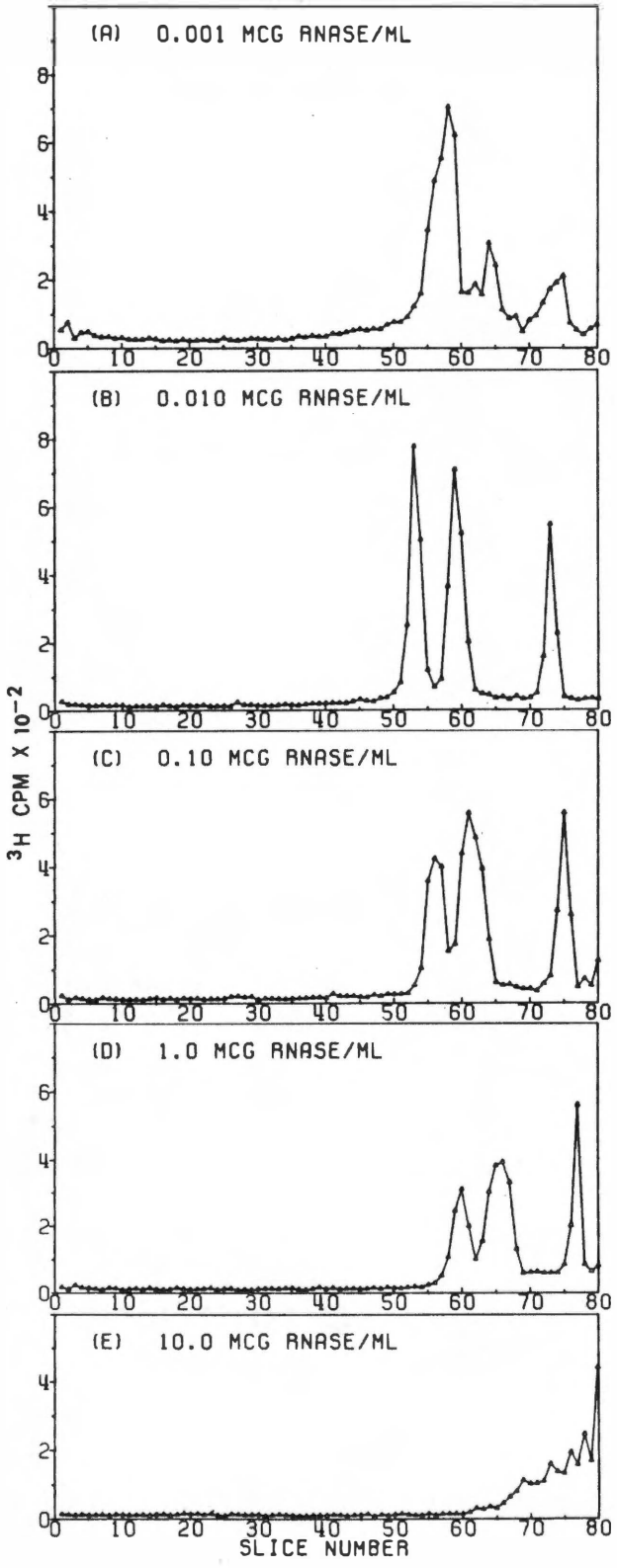

(A) 0.001 MCG RNASE/ML
(B) 0.010 MCG RNASE/ML
(C) 0.10 MCG RNASE/ML
(D) 1.0 MCG RNASE/ML
(E) 10.0 MCG RNASE/ML
^{3}H CPM X 10^{-2}
SLICE NUMBER

Figure 6

molecules may be explained by a conversion of RF I-like molecules to the other two forms, rather than simply by a specific loss of CPM from the RF I peak.

In some experiments in which an RNA preparation was digested at different RNase concentrations and analyzed on gels, the total recovery of counts was significantly different for the samples treated at different enzyme concentrations. This situation presents a potential problem in interpretation in that some of the change in proportion of the various peaks could hypothetically be attributed to a selective degradation of one peak (i.e., RF I), rather than to the conversion which is postulated above. That this is not the case is illustrated in Table 1. Two sets of gel profiles were obtained for one set of RNase-treated preparations. Similar results were obtained with other preparations. The total radioactivity in the ds RNA varied considerably (17%) over the range of enzyme concentration in one set of gels but varied minimally (6%) in the other. Moreover, the data shown in Table 1, gel set A, appear to reflect a progressive loss in total counts, as might be expected to occur if selective breakdown were taking place. However, this set of gels showed essentially the same pattern of change in the relative proportions of counts in the three peaks as did the other set (Table 1, gel set B), in which the total radioactivity was essentially constant. One can conclude therefore that the change is caused by a conversion of material migrating as RF I to molecules of RF II and RF III, rather than by a selective breakdown of RF I. This interpretation is confirmed further by additional experiments described below.

TABLE 1. Distribution of radioactivity in ds RNA after RNase treatment.

GEL SET	RNASE CONC. (µg/ml)	% OF TOTAL CPM IN RF			TOTAL CPM	MEAN + CV[a]
		I	II	III		
A	0.001	60.4	23.1	16.4	5054	
	0.01	37.8	41.6	20.6	4442	4364
	0.1	27.8	47.6	24.6	4628	±16.8%
	1.0	25.5	48.0	26.5	3334	
B	0.001	65.8	25.7	8.5	3082	
	0.01	37.4	40.2	22.5	3530	3318
	0.1	28.7	44.1	27.1	3243	± 5.9%
	1.0	27.2	50.9	21.9	3419	

[a]Coefficient of variability: the standard deviation expressed as a percentage of the mean.

Figure 7 shows graphically these changes in the proportions of the three species. In every experiment the proportion of RF I decreased and the proportions of RF II and RF III increased with increases in the concentrations of RNase. The greatest change in proportions occurred with the two lower enzyme levels. This suggests that most of the RNase-sensitive regions in RF I-like molecules are cleaved at enzyme concentrations of 0.01 μg/ml or less.

Some of the electrophoretic profiles obtained in these experiments revealed a fourth, minor peak of ds RNA which migrated between the peaks of RF II and RF III. This peak was seen consistently at all RNase concentrations which did not degrade the ds RNA. A representative profile is shown in Figure 8. The presence or absence of this peak may have contributed to some of the variability in the total CPM for the three major peaks in different experiments. The radioactivity in this peak was not included in any of the data considered here.

The results presented thus far indicate that in total RNA preparations there is a set of replicative molecules the majority of which migrate as RF I after mild RNase treatment. At higher enzyme concentrations these molecules are cleaved to form molecules which migrate as RF II and RF III. These results are predictable from the replication model of Simmons and Strauss (28). However, these RNA's were prepared by a method somewhat different from that used by Simmons and Strauss. It was therefore important to attempt to reproduce these results with RNA prepared by their method (RNA 2).

The RNA's present in such an extract (RNA 2) were analyzed by polyacrylamide gel electrophoresis before treatment with RNase (Fig. 9b).

Figure 7. Changes in proportions of RF's after treatment with different concentrations of RNase.

The CPM in each peak were obtained from replicate gels at each RNase concentration. A representative profile is shown in Fig. 6 (p. 23). The average CPM in each peak is plotted against the logs of the RNase concentrations used. The RF represented by each curve is indicated.

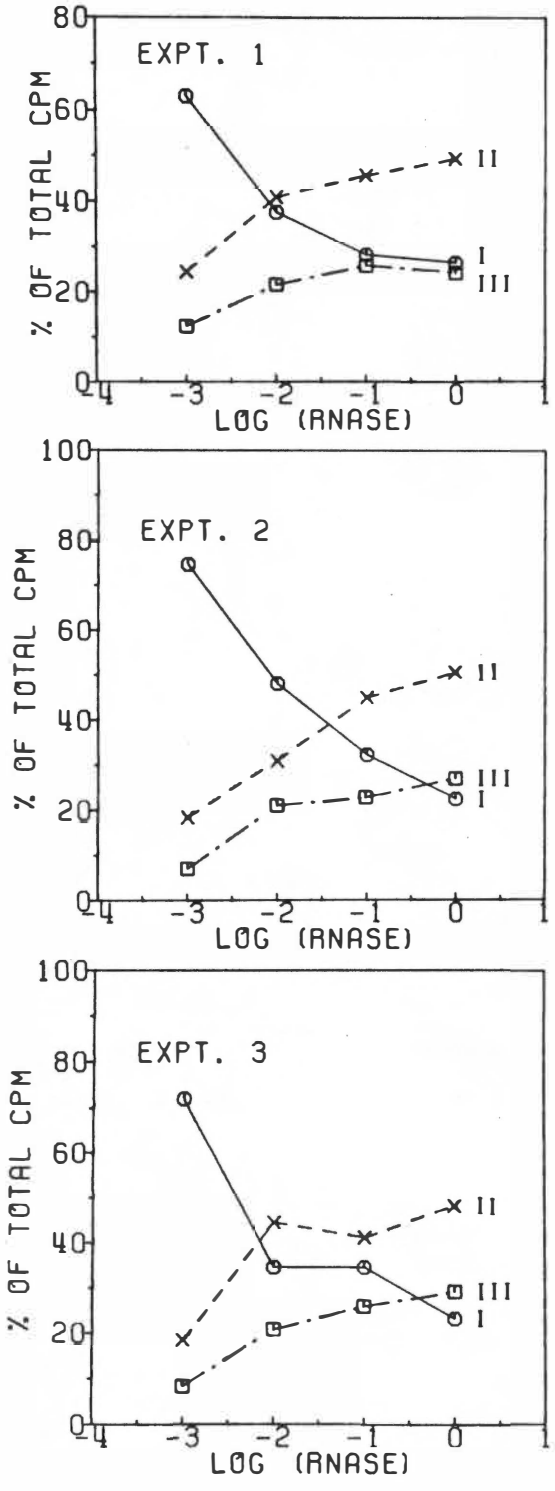

EXPT. 1
% OF TOTAL CPM
LOG (RNASE)
II
I
III
EXPT. 2
% OF TOTAL CPM
LOG (RNASE)
II
III
I
EXPT. 3
% OF TOTAL CPM
LOG (RNASE)
II
III
I

Figure 7

Figure 8. A fourth peak of ds RNA.

Treated samples of RNA 1 were obtained exactly as were those in the experiment represented in Figure 6 (p. 23). They were analyzed on 2.0% gels at 8 mA/gel for 5.5 h.

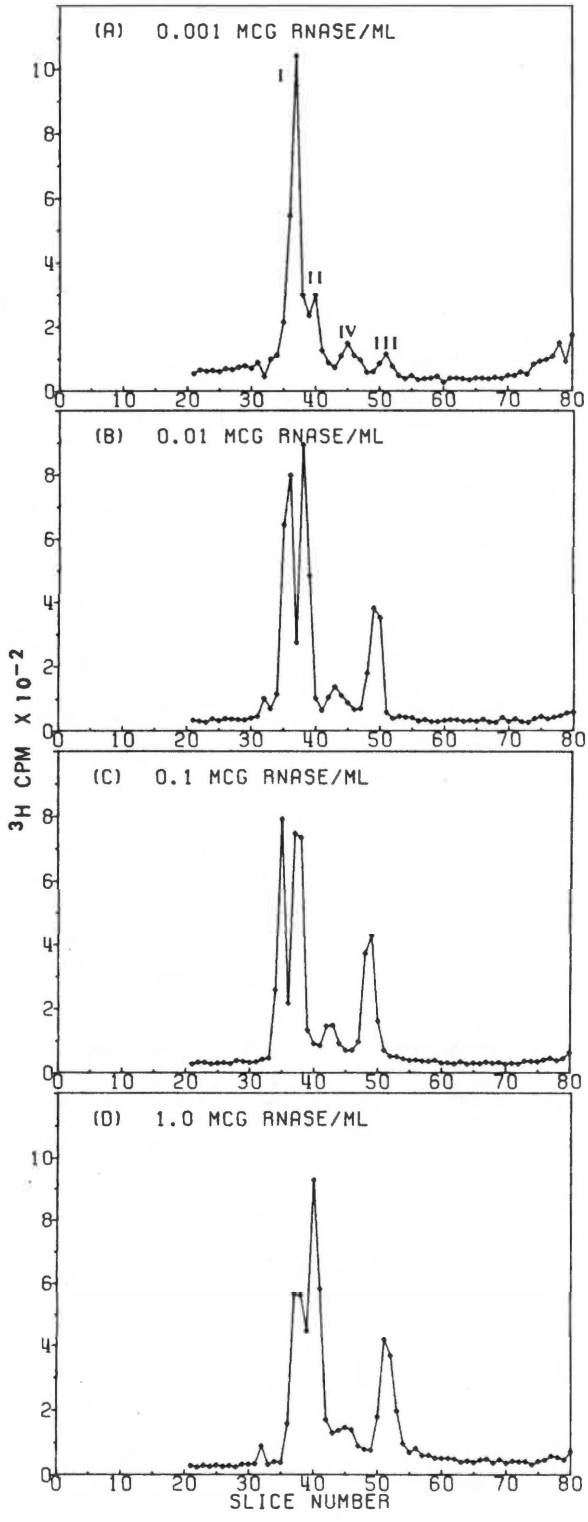

(A) 0.001 MCG RNASE/ML
I
II
IV
III
(B) 0.01 MCG RNASE/ML
(C) 0.1 MCG RNASE/ML
(D) 1.0 MCG RNASE/ML
^{3}H CPM X 10^{-2}
SLICE NUMBER

Figure 8

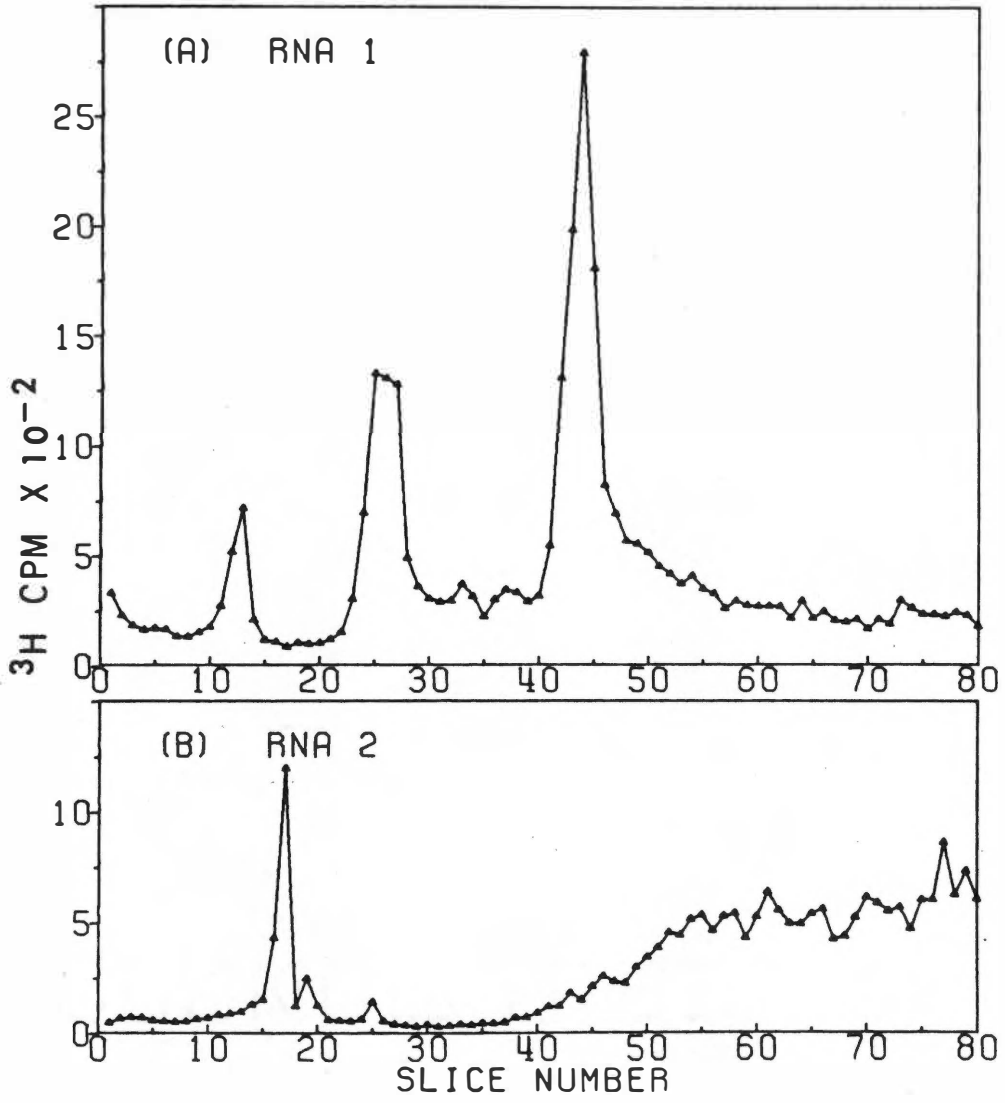


Figure 9. Comparison of RNA 1 and RNA 2.

The RNA's are identified in the text (p. 18). Equivalent samples (10 µl each) were adjusted to 25% glycerol and were analyzed on 2.2% gels at 5 mA/gel for 4 h.

Figure 9a shows a profile of RNA 1 for comparison. The ss RNA was degraded during extraction by this procedure. However, the ds RNA appeared to be intact and was sufficient for the experiments described below. [Simmons and Strauss (28) did not examine the RNA prepared by this method before it was treated with RNase.]

The electrophoretic profiles of RNA 2 following RNase treatment are shown in Figure 10. The positions of these peaks show that the species obtained from RNA 1 may be correctly identified as RF's I, II, and III. The pattern of change in proportions of the three ds RNA's from RNA 2 is similar to that obtained with RNA 1. In the profile shown, RF I decreased in the contribution of radioactivity from 67.9% at 0.001 µg RNase/ml to 31.1% at 1.0 µg RNase/ml. RF II increased in proportion from 19.5% to 43.5% and RF III, from 12.6% to 25.4%. No replicative intermediate RNA was detectable at the lowest enzyme level. The changes in proportions for each of the three ds species are plotted in Figure 11. The pattern of change is similar to that shown in Fig. 7, page 29, for RNA 1. The ds RNA in this preparation was degraded at the enzyme level of 10.0 µg/ml (data not shown), just as was seen with RNA 1.

The experiments with RNA 2, as well as those with RNA 1, show an interconversion of RF's that is consistent with the model for Sindbis RNA replication. Another prediction of the model is that the ratio of radioactivity in RF III to that in RF II should be 0.52, since their molecular weights have the same ratio. (It is assumed that they are labeled to the same specific activity, at least under the steady-state labeling conditions employed for RNA 1.) For the replicates in all

Figure 10. Products of RNase treatment of RNA 2.

This RNA is that identified in the text (p. 18). RNase treatment and electrophoresis were carried out exactly as described in the legends to Fig. 5 (p. 21) and Fig. 6 (p. 23).

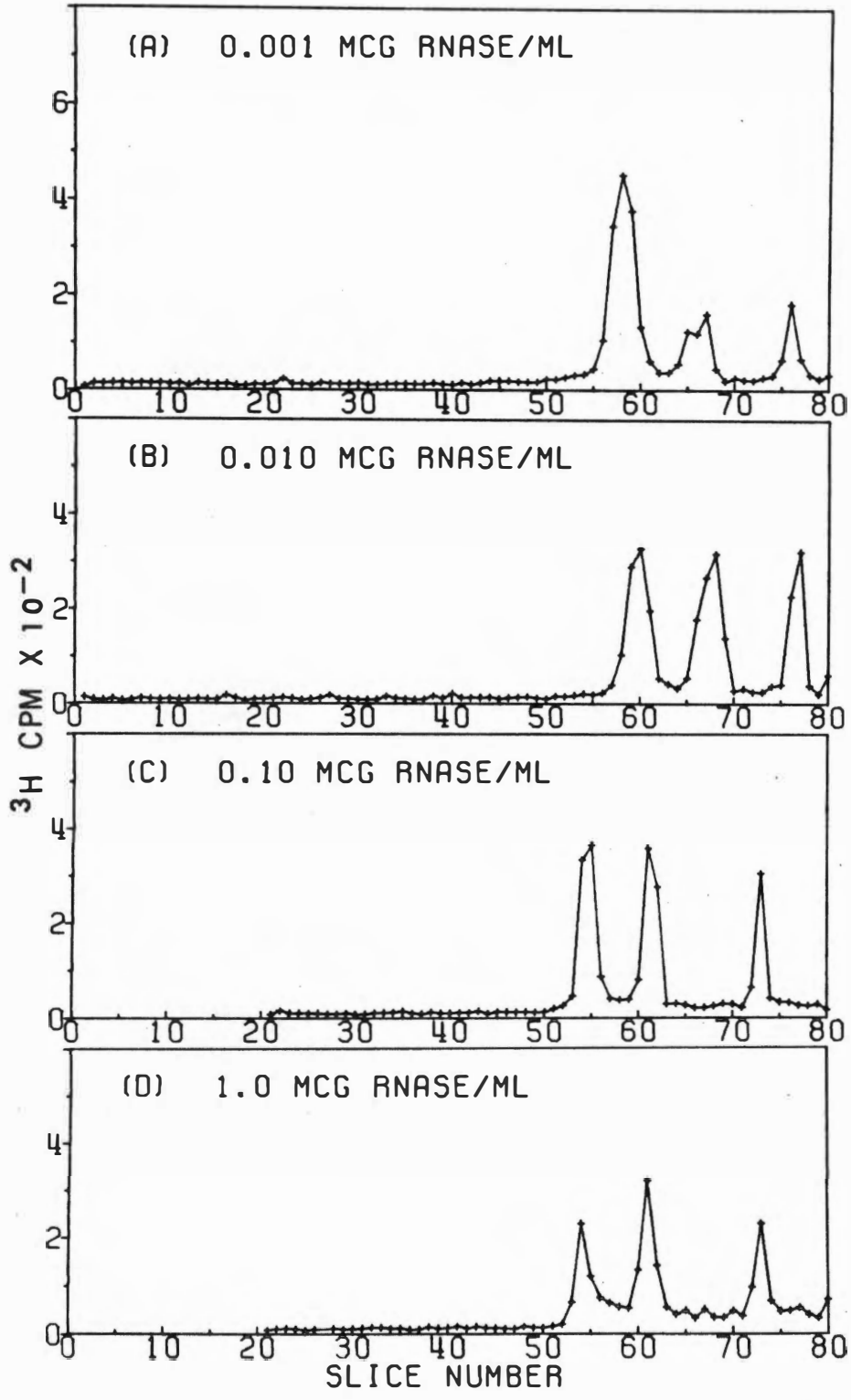

(A) 0.001 MCG RNASE/ML
(B) 0.010 MCG RNASE/ML
(C) 0.10 MCG RNASE/ML
(D) 1.0 MCG RNASE/ML
^{3}H CPM X 10^{-2}
SLICE NUMBER
0
2
4
6
10
20
30
40
50
60
70
80

Figure 10

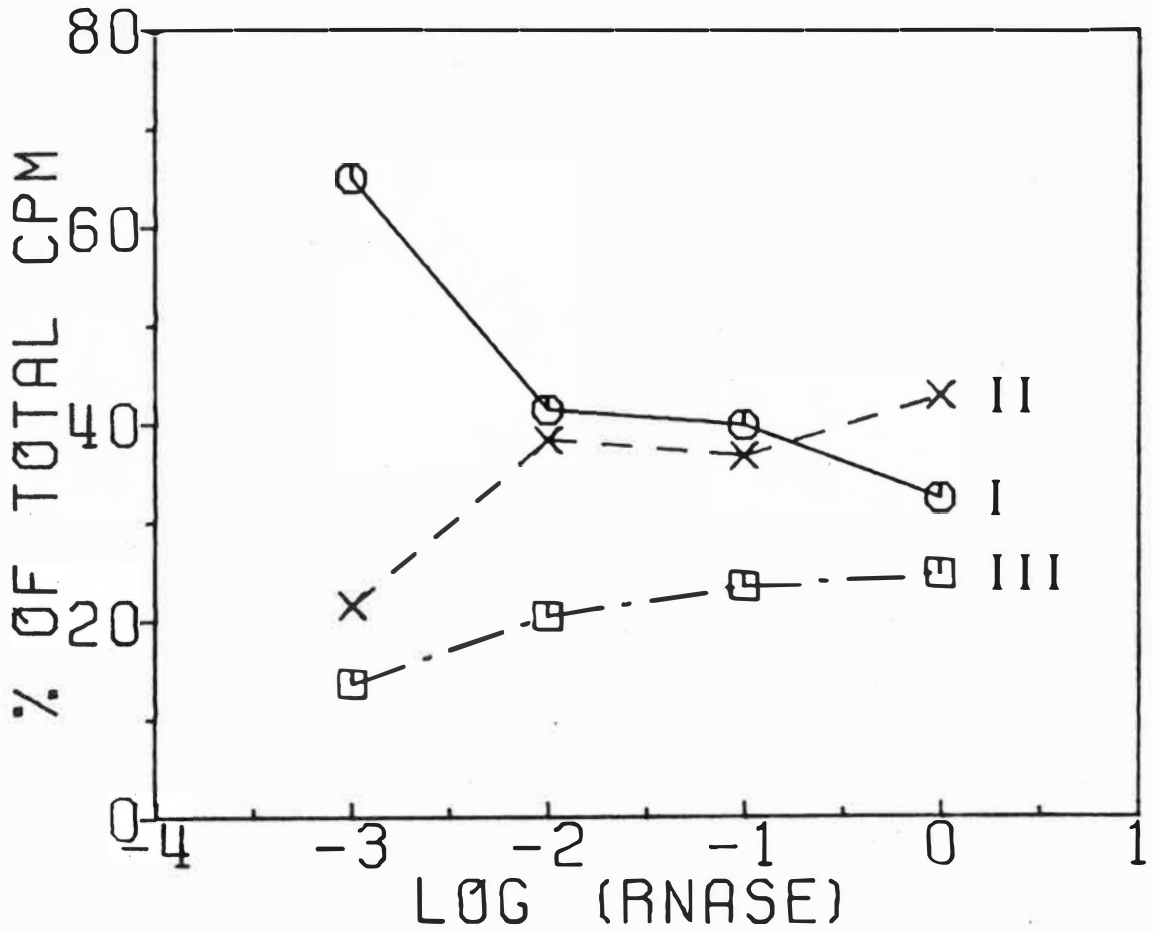


Figure 11. Changes in proportions of RF's after RNase treatment of RNA 2.

The data for RNA 2 were obtained and plotted as described for RNA 1 in the legend to Fig. 7 (p. 28). Each point represents the average obtained from replicate gels in each of two experiments.

experiments, this ratio was 0.53 ± 0.08 with RNA 1 and 0.60 ± 0.09 with RNA 2.

RNase-treatment of isolated ds RNA. It was anticipated that the interrelationships of the various forms of ds RNA in response to RNase treatment could be better examined with isolated ds RNA. Infected cells were labeled for a 30-min period ending at 6 h post-infection to obtain more highly labeled ds RNA. The RNA was extracted with phenol-cresol-hydroxyquinoline at room temperature (Method 1, p. 5). RNA containing ds regions was isolated on CF-11 cellulose and was characterized as in Fig. 1, page 12. Portions of the isolated material were subjected to electrophoresis with or without prior RNase treatment (Fig. 12). After prolonged electrophoresis, the untreated ds RNA had migrated almost totally as RF I and replicative intermediate RNA (RI; Fig. 12a); RF II was minimally detectable, and there were only a few CPM in the region of RF III. This suggests that the only naturally occurring RF is RF I. Treatment with 0.001 µg RNase/ml (at pH 7.2) converted most of the RI to RF I (Fig. 12b). The small amounts of RF II and RF III could have been generated from either the preexisting RF I or from RI molecules. It is apparent that very little radioactivity was lost due to RNase treatment (re. Table 2), probably because most of the label was in the newly synthesized ends of molecules and these were conserved in the ds RNA products of digestion. Figure 12c shows that a 10-fold higher enzyme level converted all of the RI to replicative forms and also converted many of the RF I-like molecules to RF II and RF III. The amount of RI remaining after treatment with 0.001 µg RNase/ml (Fig. 12b) was

Figure 12. RNase treatment of isolated ds RNA.

RNA was prepared as described in the text. Four ml of the total RNA was fractionated on a 20-cm column of CF-11 cellulose as described in Chapter II (p. 7). The 0% ethanol eluate from the second passage was precipitated and collected as described. The pellet was dissolved in 0.2 ml of pH 7.6 buffer. Samples (25 µl) were treated with pH 7.2 buffer alone (A) or with the indicated concentrations of RNase (C-D) as described in Chapter II (p. 7). Equal vol of 50% glycerol were added, and samples of 95 µl were analyzed by electrophoresis on 2.0% gels for 6 h at 8 mA/gel.

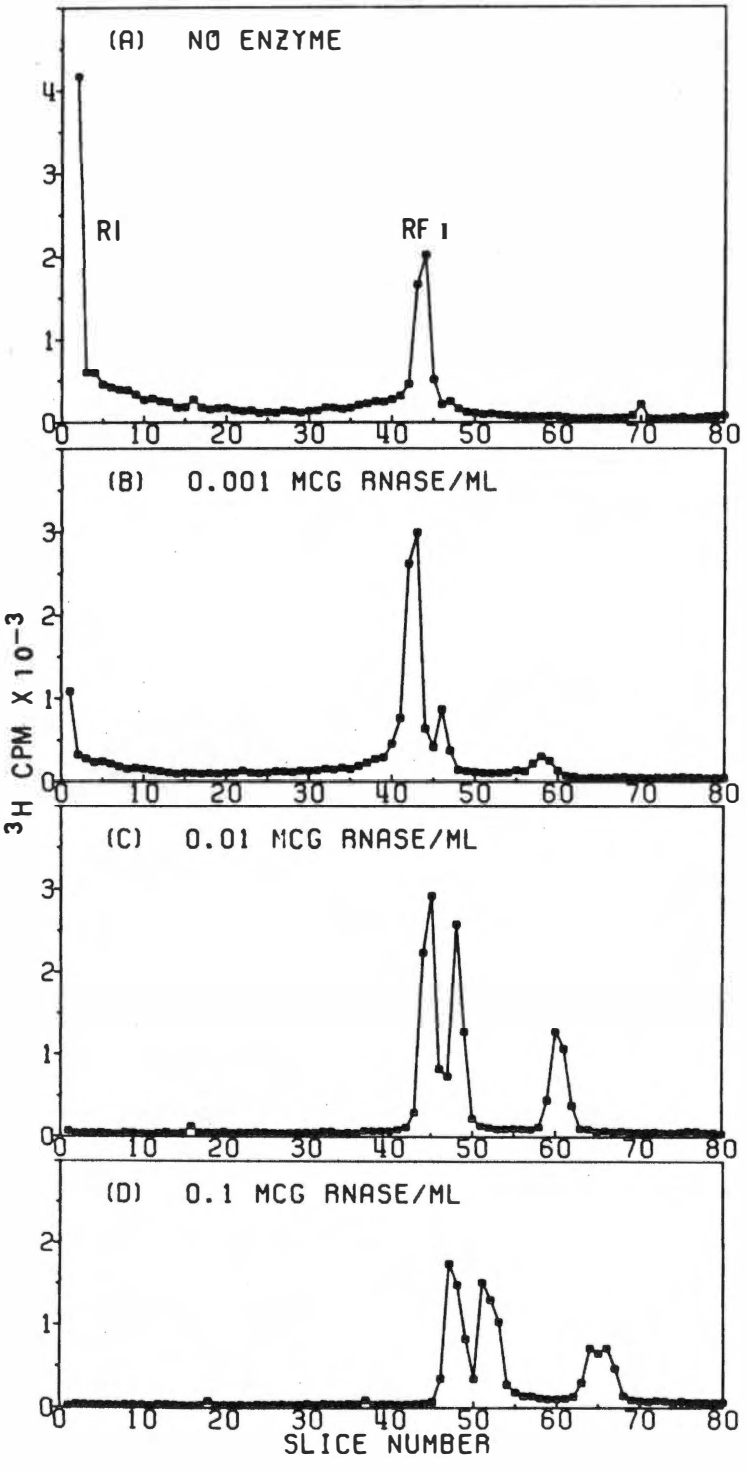

(A) NO ENZYME
RI
RF I
(B) 0.001 MCG RNASE/ML
(C) 0.01 MCG RNASE/ML
(D) 0.1 MCG RNASE/ML
^{3}H CPM X 10^{-3}
SLICE NUMBER

Figure 12

TABLE 2. Distribution of radioactivity in isolated ds RNA after RNase treatment.

EXPT.	RNASE CONC. (µg/ml)	CPM (AND % OF TOTAL CPM) IN				TOTAL CPM	MEAN ±CV[a]
		RI	RF I	RF II	RF III		
1	0	7608 (52.6)	6503 (45.0)	247 (1.7)	103 (0.7)	14461	
	0.001	2574 (17.3)	9376 (63.0)	1443 (9.7)	1478 (9.9)	14871	13982
	0.01		5720 (40.7)	4999 (35.6)	3330 (23.7)	14048	±7.3%
	0.1		4752 (37.9)	4749 (37.9)	3044 (24.3)	12546	
2	0	8281 (59.9)	4992 (36.1)	502 (3.6)	40 (0.3)	13816	
	0.001	2741 (20.8)	8172 (61.9)	1551 (11.7)	740 (5.6)	13206	13192
	0.01		6471 (46.5)	4368 (31.4)	3084 (22.1)	13922	±7.3%
	0.1		4463 (37.1)	4384 (37.1)	2979 (25.2)	11826	

[a]Coefficient of variability, as defined in Table 1, page 26.

insufficient to account for all of the increase in RF's II and III following treatment with 0.01 μg RNase/ml (Fig. 12c). The source for most or all of these molecules must have been molecules which migrated as RF I. The change in proportions is further evident after treatment with 0.1 μg RNase/ml (Fig. 12d).

These conversions were quantitative in each of two experiments (Table 2). The total radioactivity in the RNA forms examined (RI and RF's) varied minimally. Thus there was a quantitative conversion of most of the RI molecules to RF molecules when the RNA was treated with 0.001 μg RNase/ml. At the next higher enzyme level (0.01 μg/ml), all of the RI was converted to RF's. In addition, some of the RF I-like molecules which were generated at the lowest level of RNase were converted to RF's II and III, as argued above.

It should be noted that the migration rates of the RNA's shown in Figure 12 appear to be slower than those shown in Figure 6, page 24 and Figure 10, page 35. However, this is an effect produced by the low-percentage acrylamide gels used (data not shown) and is dependent on the age of the gels. The gels represented in the latter two figures were used within 2 d after having been made, whereas those represented in Figure 12 were used 4 d after they were made. This problem was not experienced with gels of higher percentages of acrylamide.

RNase treatment of salt-fractionated RNA. The experiment described in the preceding section revealed that replicative intermediate RNA could be converted by RNase to molecules migrating primarily as RF I (Fig. 12a and b). However, during the process of this conversion, RF II and RF III

were generated, also. Segal and Sreevalsan (26) have reported that isolated RI (free of any RF's) is digested by RNase to three RF's. It was therefore of interest to determine if any of the naturally occurring molecules migrating as RF I are the uncleaved cores of RF's II and III. That is, will RNase treatment of RF I generate any molecules of RF II and RF III, or is the only source of the latter molecules the pool of RI?

The common method for separation of RI from RF's is that of salt frationation (1, 3, 19, 21, 26). Two variations of the procedure were attempted: one in which fractionation was done in 1 M NaCl (1) and one in which it was done in 2 M LiCl (26). It was expected that completely ds RNA would remain soluble in the salt solutions, whereas ss RNA and RI would precipitate.

When a total infected cell extract (Fig. 13a) was fractionated in 1 M NaCl, most of the ss RNA precipitated (Fig. 13b). However, almost all of the RI remained in the supernatant fraction, as did RF RNA (Fig. 13c). A small amount of Sindbis 26S RNA also remained soluble. Therefore, this preparation was refractionated in 2 M LiCl (Fig. 13d). It then appeared to contain primarily RF material with some contamination with RI. Prolonged electrophoresis revealed that 73% of the radioactivity was in RF I, with the remainder being almost evenly distributed between RF II and RI (re. Table 3, expt. 1, 0 µg RNase/ml, p. 45).

The above results suggested that fractionation in 2 M LiCl might achieve better separation of RF RNA from RI. Figure 14b is the electrophoretic profile of the RNA insolbule in 2 M LiCl: This fraction contained all of the ss RNA and most of the RI. However, the soluble

Figure 13. Fractionation of Sindbis RNA in 1 M NaCl.

A sample of the RNA designated RNA 1 (p. 18) was fractionated in 1 M NaCl as described in Chapter II (p. 9). (A) A portion of RNA 1 (20 µl) in 25% glycerol was subjected to electrophoresis on 2.2% acrylamide/0.5% agarose gels at 5 mA/gel for 4 h. (B) Electrophoresis of 10 µl of the salt-insoluble RNA. (C) Electrophoresis of 10 µl of the salt-soluble RNA. (D) The salt-soluble RNA was fractionated in 2 M LiCl. A portion (10 µl) of this salt-soluble fraction was analyzed as above.

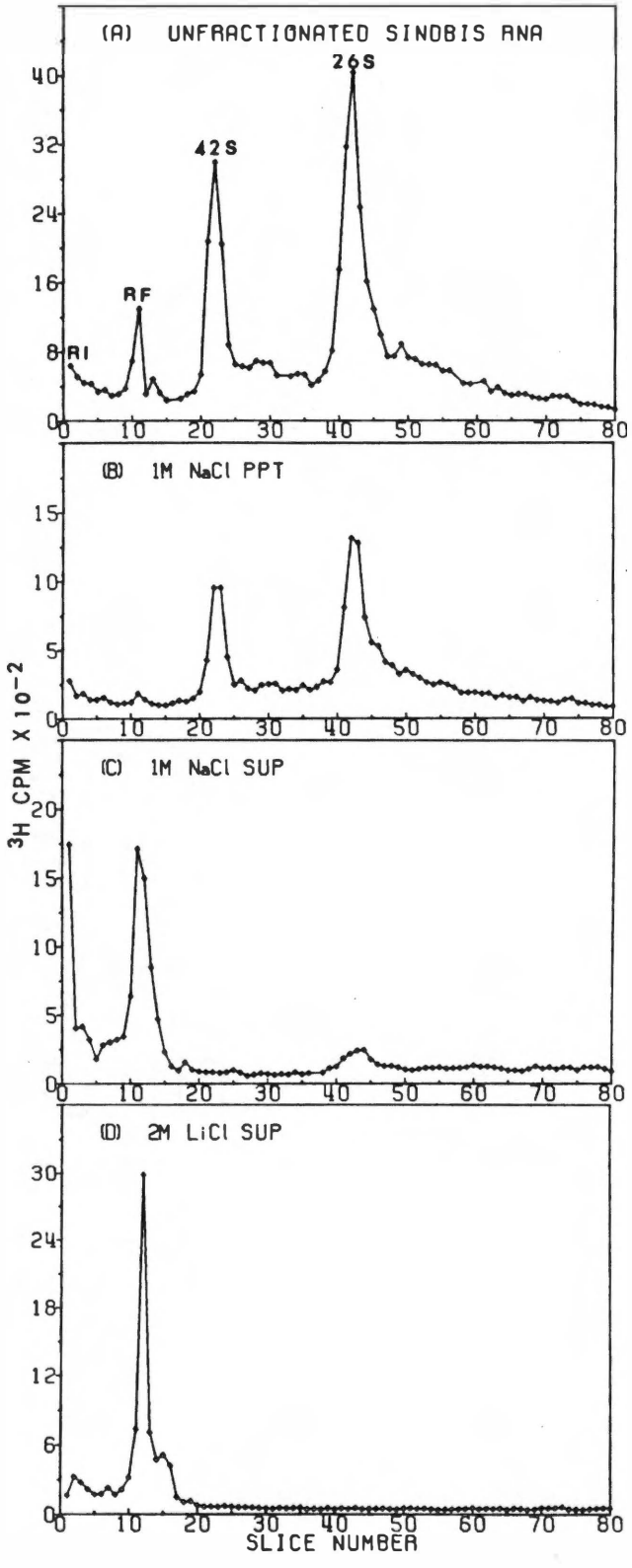

(A) UNFRACTIONATED SINOBIS RNA
26S
42S
RF
RI
(B) 1M NaCl PPT
(C) 1M NaCl SUP
(D) 2M LiCl SUP
^{3}H CPM X 10^{-2}
SLICE NUMBER

Figure 13

Table 3. Distribution of radioactivity after RNase treatment of partially purified RF I.

EXPT.	RNase conc. (μg/ml)	CPM (AND % OF TOTAL CPM) IN				TOTAL CPM
		RI	RF I	RF II	RF III	
1	0	1593 (11.3)	10283 (73.2)	2167 (15.4)		14043
	0.001	1003 (7.2)	10048 (72.4)	2130 (15.4)	693 (5.0)	13874
	0.01		4741 (33.1)	7192 (50.2)	2391 (16.7)	14324
2	0	2054 (16.4)	8453 (67.6)	2003 (16.0)		12510
	0.001	897 (7.2)	9436 (75.4)	1666 (13.3)	523 (4.2)	12522
	0.01		5416 (42.3)	5023 (39.3)	2350 (18.4)	12789
3	0	1036 (16.9)	4361 (71.3)	718 (11.7)		6115
	0.001	177 (2.5)	5878 (83.8)	956 (13.6)		7011
	0.01		2721 (37.0)	3423 (46.6)	1208 (16.4)	7352

Figure 14. Fractionation of Sindbis RNA in 2 M LiCl.

A portion of a second RNA extract prepared by Method 1 (Chapter II, p. 5) was fractionated in 2 M LiCl (Chapter II, p. 9). (A) Unfractionated RNA (2.5 µl) was analyzed as described in the legend to Figure 13. (B) Electrophoresis of salt-insoluble RNA (2.5 µl). (C) Electrophoresis of salt-soluble RNA (12.5 µl). This latter RNA preparation was refractionated in 2 M LiCl (re. Fig. 15a, p. 50).

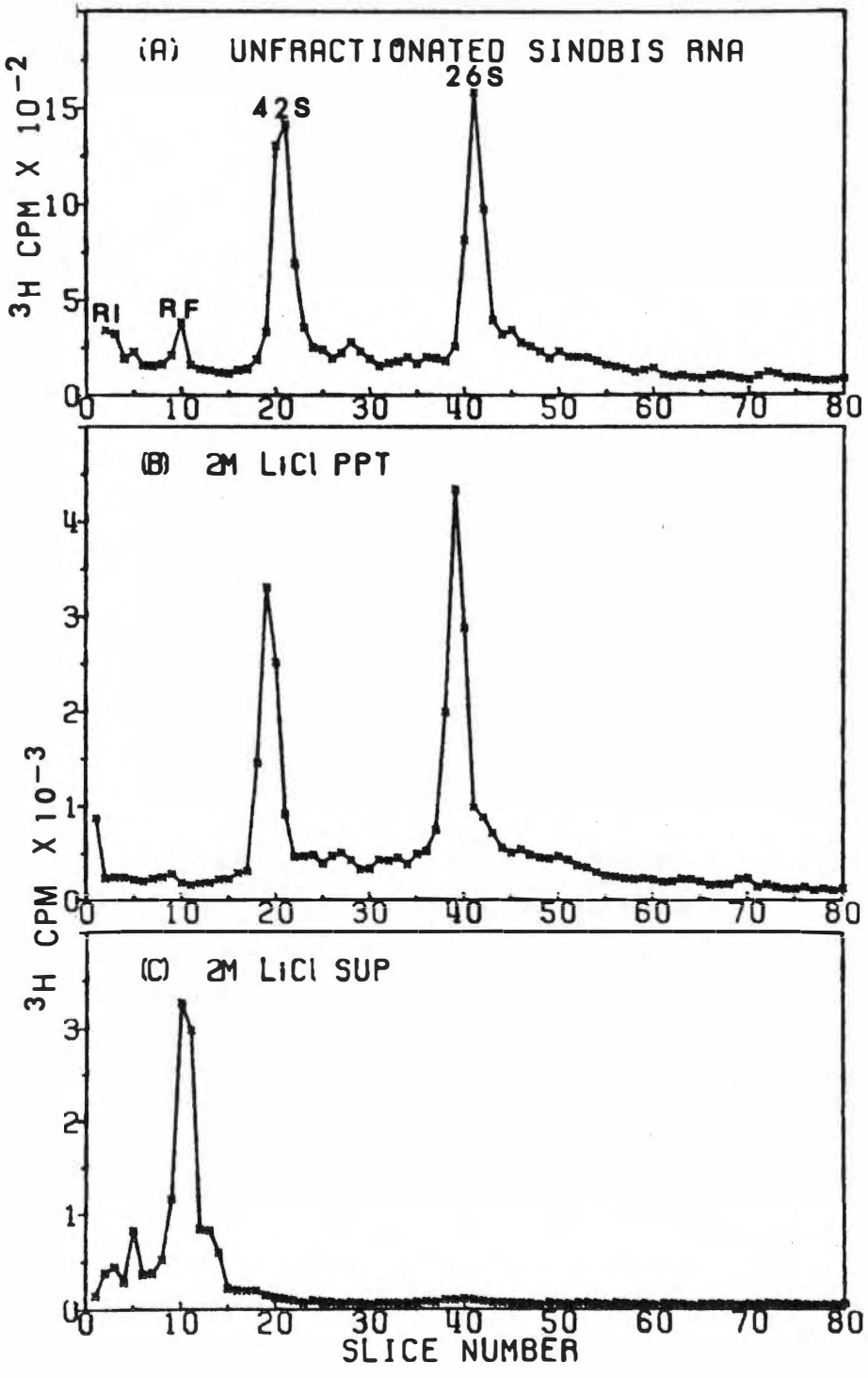

(A) UNFRACTIONATED SINDBIS RNA
42S
26S
RI
RF
^{3}H CPM X 10^{-2}
(B) 2M LiCl PPT
^{3}H CPM X 10^{-3}
(C) 2M LiCl SUP
SLICE NUMBER

Figure 14

RNA (Fig. 14c) included some RI (17.2% of the radioactivity), in addition to the RF RNA (82.8% of the radioactivity). This remained the case even after a second fractionation (Fig. 15a and Table 3, expt. 3, 0 µg RNase/ml).

RF I was purified, therefore, only partially. However, by either fractionation procedure, 68-73% of the RNA migrated as RF I. [Note that RF I is 36-45% of the total ds RNA (Table 2, p. 40)]. RNase treatment of such preparations caused cleavage of molecules migrating as RF I into molecules of RF II and RF III (Fig. 15). The critical enzyme concentration was 0.01 µg/ml (Fig. 15c). At this enzyme level almost 50% of the total radioactivity was in RF II. The distributions of the radioactivity after RNase treatment in three experiments are given in Table 3. In the first two experiments, RI was only partially sensitive to the lowest enzyme level used (0.001 µg/ml). The products were RF III (expt. 1) and RF I + RF III (expt. 2). In light of previous results (Table 2), these distributions of products were unexpected. However, in the third experiment, almost all of the RI were converted primarily to RF I, as expected, by 0.001 µg RNase/ml. More importantly, all three experiments clearly demonstrated that with 0.01 µg RNase/ml RF I-like molecules were cleaved to molecules of RF II and RF III. Thus these latter two species can be generated from both the pool of RI and the pool of naturally occurring RF I.

It should be noted that RF RNA isolated by salt fractionation includes some 12-16% of the material as RF II (Table 3), whereas the ds RNA prepared by chromatography on CF-11 cellulose contains only 2-4% RF II

Figure 15. RNase treatment of salt-soluble RNA (RF I).

RNA was prepared by Method 1 (Chapter II, p. 5) and was processed through two fractionations in 2 M LiCl (Chapter II, p. 9). Samples (25 µl) were treated with pH 7.2 buffer alone (A) or with the indicated concentrations of RNase (B-C) as described in Chapter II (p. 7). The products were analyzed as described in the legend to Fig. 12 (p. 38).

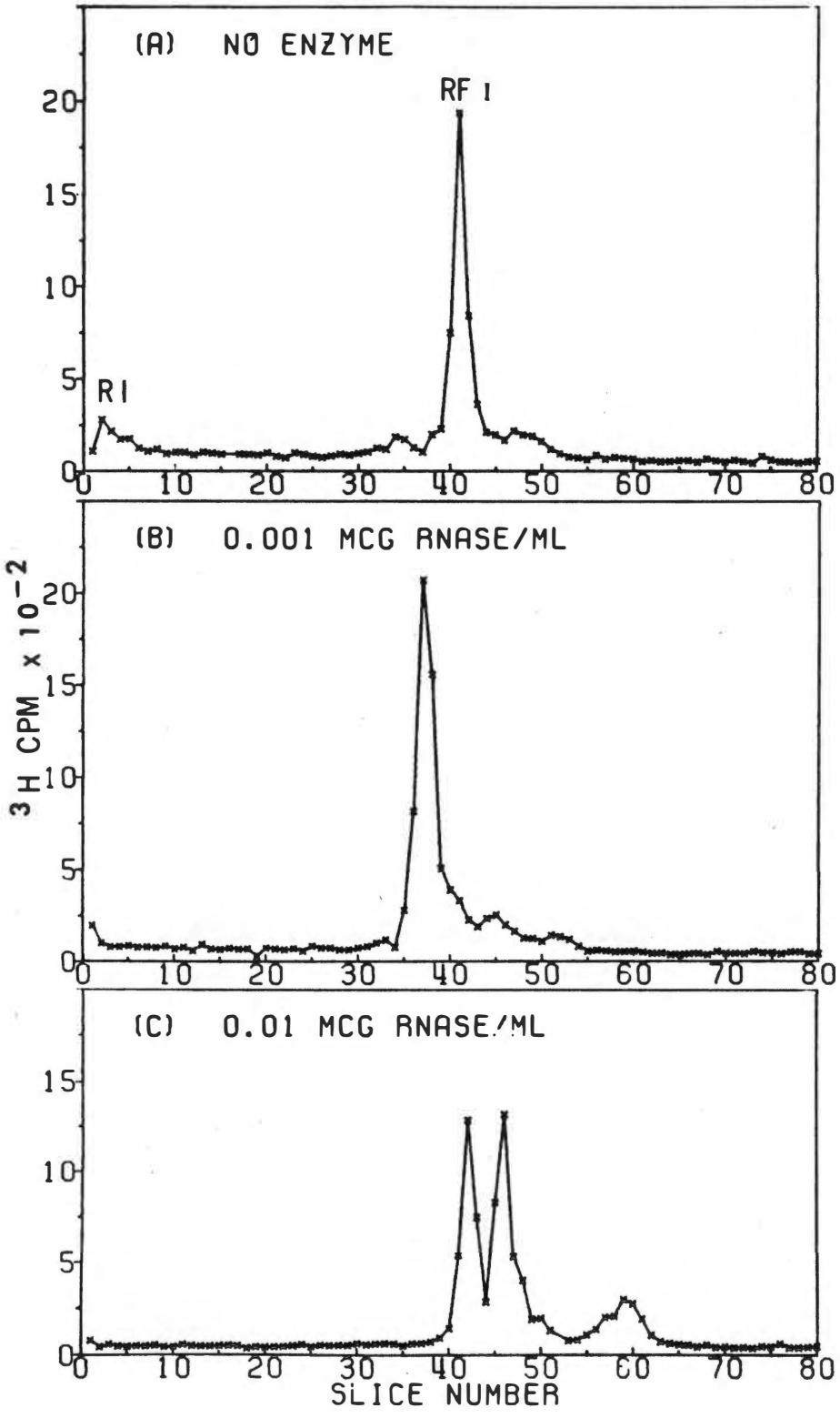

(A) NO ENZYME
RF I
RI
(B) 0.001 MCG RNASE/ML
(C) 0.01 MCG RNASE/ML
^{3}H CPM x 10^{-2}
SLICE NUMBER

Figure 15

(Table 2, p. 40). It seems reasonable that RF II obtained by either procedure is a product of degradation. The freeze-thaw cycles employed in salt fractionation evidently caused more breakage of the RNA.

CHAPTER IV

DISCUSSION

The results of this study support the model for Sindbis virus RNA synthesis proposed by Simmons and Strauss (28). They reported that RNase-treated extracts of infected cells contain three replicative forms of viral RNA. The authors proposed that these are generated from two populations of replicative intermediates. One population (RIa) is comprised of molecules from which copies of the viral genome are synthesized. RNase-treatment of this population yields the replicative form of the genome (RF I). The other population (RIb) contains molecules having minus-strand templates of the size of the genome but which are transcribed discontinuously. The transcriptional products are proposed to be the 26S viral messenger RNA, which is a unique fraction (one third) of the genome (27), and an as yet undetected ss RNA which represents the other two thirds of the genome. When RIb molecules are treated with RNase, two RF's are generated, presumably by cleavage at the transcriptional gap. One (RF III) is the ds form of the messenger RNA, and one (RF II) is that of the undetected ss RNA. It was observed that RF II has a molecular weight twice that of RF III and that the two species occur in equal-molar concentrations throughout infection.

Preliminary results during this research confirmed the finding of three RF's following RNase treatment of infected-cell extracts. However, it was observed that their proportions were RNase-dependent (Fig. 3a and b, p. 16). This observation suggested a means of examining the Simmons-

Strauss model by testing a prediction of the model. Very mild RNase-treatment should degrade only the free ss tails of all RI molecules, generating species which migrate during polyacrylamide gel-electrophoresis as RF I. Treatment at higher enzyme concentrations should not only digest the tails but should cleave the transcriptional gap on the minus strands of RIb. Hence, more of the total ds RNA should then migrate as RF II and RF III. In effect, some molecules which would migrate as RF I after treatment at the lower enzyme levels are converted to molecules of RF II and RF III by treatment at the higher enzyme levels. According to the model, the ratio of the radioactivity in RF III to that in RF II should be near 0.5 at all enzyme concentrations which yield these two species.

The results presented above are consistent with the Simmons-Strauss model and show an RNase-dependent conversion of molecules which migrate as RF I to molecules which migrate as RF's II and III. When total RNA extracts from infected cells (Fig. 6, p. 24) or isolated Sindbis ds RNA (Fig. 12, p. 39) were treated with 0.001 µg RNase/ml, most of the product molecules migrated as RF I. The proportions of RF II and RF III increased when the enzyme concentration was raised (Fig. 7, p. 29).

Very similar observations (Figs. 10 and 11, pp. 35 and 36) were made in experiments with RNA extracted as prescribed by Simmons and Strauss (28). The extraction (Method 2, p. 6) involved the method of Penman (22), which is a hot phenol-chloroform extraction. In this study the ss RNA was partially degraded by the procedure (Fig. 9b, p. 32). This may have been due to the fact that Simmons and Strauss specified lysis of the cells in a low-salt (0.06 M NaCl) buffer, whereas Penman

lysed nuclei in a high-salt (0.5 M NaCl, 0.05 M $MgCl_2$) buffer and then added EDTA to 0.1 M for extraction. Beyond several trials, no attempts were made to improve upon this method because Bishop and Koch (2) reported that poliovirus RI RNA was lost during hot phenol extraction. Despite the partial degradation observed, the preparation did contain ds RNA which migrated as three species after digestion with RNase (Fig. 10, p. 35). The untreated RNA extract did not contain RI molecules. They apparently either were lost during extraction or were degraded to RF RNA. The three ds forms obtained from this preparation migrated to the same positions as those obtained from preparations extracted with phenol-cresol-hydroxyquinoline at room temperature (Method 1, p. 5; Fig. 6, p. 24). It may be concluded, therefore, that the species obtained in experiments with either type of RNA preparation were identical and that the conversions observed were not a peculiarity of the extracts obtained by Method 1.

The suggestion that raising the level of enzyme converts RF I-like molecules to RF's II and III requires that there must be an actual increase in the amounts of RF's II and III, in addition to the decrease in amount of RF I. Several analyses indicated that this was true. While total radioactivity for the three peaks varied over the range of RNase concentration in some electrophoretic profiles (Table 1, gel set A., p. 26), there was little variation in others (Table 1, gel set B). At least in the latter cases it was clear that the only source for the increase in radioactivity of RF II and RF III was that originally found in RF I. No detectable RI material was available at the lowest enzyme

level to serve as a source for RF's at the higher enzyme levels (Figs. 6 and 10, pp. 24 and 35). Furthermore, this conversion of RF I-like molecules to RF's II and III was shown directly by RNase treatment of isolated ds RNA (Fig. 12, p. 39, and Table 2, p. 40).

As predicted by the Simmons-Strauss model, any cleavage of RF I to RF II and RF III should result in equal-molar amounts of the products. Indeed such a distribution is found in RNase-treated extracts taken throughout infection (28). Since the ratio of the molecular weight of RF III to that of RF II is 0.52 (28), the corresponding ratio of their radioactivities should be the same. It is assumed, of course, that their specific activities are the same. The experiments described above yielded the ratio 0.53 ± 0.08 for RNA extracted by our method and the ratio 0.60 ± 0.09 for RNA prepared according to Simmons and Strauss (28). These results together with those discussed above suggest that the transcriptional gap postulated by these authors may be real. The gap apparently begins at the terminus of a transcript two-thirds the size of the genome and ends at the initiation site for 26S mRNA, which is one-third the size of the genome.

Infected cell extracts were treated with RNase at pH 5 and pH 7.2, and the patterns of change in the proportions of RF's in response to changes in RNase concentration were similar. Treatment at the acid pH was employed because it is the optimum for enzyme activity at ionic strengths near 0.2 (14). Such ionic strengths are provided by the common buffers used in alphavirus RNA extraction and analysis. However, RNase treatment at neutral pH is more common; Simmons and Strauss (28) employed

a buffer at pH 7.2. Only one significant difference was observed between the acid and neutral treatments. At pH 5, 1.0 µg of RNase per ml was sufficient to partially degrade the ds RNA (Fig. 3, p. 16), whereas at pH 7.2 a concentration greater than this (in the order of 10 µg/ml) was required for degradation (Fig. 6, p. 24). This difference probably was an expression of the higher enzyme activity at the lower pH.

Most of the conversion observed appears to occur at RNase concentrations lower than 0.1 µg/ml (Figs. 7 and 11, pp. 29 and 36). The majority of RFI-like molecules which can be cleaved are done so at low enzyme levels. At somewhat higher levels all molecules may be equally resistant to degradation by the enzyme, thus producing a plateau of the changes in proportions. Such a plateau is suggested by the data in Figs. 7 and 11. At still higher concentrations (near 10 µg/ml) the three forms are apparently equally susceptible to enzyme attack (Fig. 6e). There must be a critical enzyme level which only partially degrades all RF molecules. This effect may have been seen with 1.0 µg RNase/ml at pH 5 (Fig. 3c).

Degradation of alphavirus ds RNA by bovine pancreatic ribonuclease has not been studied in detail. In the past, the presence of viral ds RNA in the infected cell was determined by acid-precipitation of RNase-treated aliquots of extracts (7, 23). A 20S "RNase-resistant" species was identified by enzyme treatment of fractions taken from sucrose gradients of infected cell extracts (30, 31, 33). The integrity of the products of RNase treatment was examined in only one of these studies (7). The 20S component has also been identified by centrifugation of extracts

treated with 1-2 µg RNase/ml at various temperatures for various lengths of time but at neutral pH (11, 12, 30). Analysis on polyacrylamide gels of the products of mild RNase treatment (0.02 or 1.0 µg/ml) presented profiles suggestive of three ds RNA species (16, 26). These reports are consistent with the observations from this research in terms of RNase-resistance of Sindbis ds RNA.

Martin and Burke (19) obtained three ds forms of Semliki Forest virus RNA by treatment of isolated RF and RI with 10 µg RNase/ml in high-salt buffers (0.3-0.45 M NaCl). It is probably that the high salt concentration rendered the ds RNA more refractory to degradation by the high enzyme concentration (1). Simmons and Strauss (28) treated their extracts with 10 µg RNase/ml in the presence of 0.01 M $MgCl_2$ (and 0.001 M EDTA). Since Mg^{++} is a potent inhibitor of RNase (20) it may be surmised that sufficient Mg^{++} ions were free to inhibit the enzyme, which therefore did not cause degradation of the three RF's. Edy et al. (8) have reported the random breakdown of the ds RNA of Penicillium chrysogenum virus into large fragments by treatment with RNase under conditions which approximate those used in this study. Erikson and Franklin (9) found that the ds RNA produced by phage R17 was nicked by even mild RNase treatment. Furthermore, Semliki Forest virus RF RNA synthesized in vitro is fragmented by 3 µg of RNase per ml but not by 0.1 µg/ml (21). Therefore, the observation of degradation by 10 µg RNase/ml in this study appears to be consistent with results reported in the literature.

Bruton and Kennedy (3) identified three ds forms of Semliki Forest virus RNA in extracts which were not treated with RNase. It is

interesting to note that the only naturally occurring replicative form of Sindbis virus RNA seems to be that of the genome, RF I (Fig. 8a, p. 31). This result was expected. As is evident from the studies by Simmons and Strauss (27, 28), all transcription must occur on minus strands of the size of the genome; there are no minus strands of size 26S (27) and therefore no naturally occurring RF III. It is probable that the RF II seen in the salt-fractionated RNA of this study (Table 3, p. 45) and the RF II and RF III reported by Bruton and Kennedy (3) were artifacts produced during salt fractionation.

One might conclude that RF's II and III are generated solely from the pool of RI. Segal and Sreevalsan (26) obtained evidence for the generation of three RF's from isolated RI, but they presented no evidence that the two smaller RF's could be obtained by enzyme treatment of RF I-like material. However, Martin and Burke (19) produced three RF's by RNase treatment of both RI and naturally occurring RF RNA of Semliki Forest virus. This study has shown similar results with Sindbis virus replicative intermediates (Fig. 12 and Table 2, pp. 39 and 40). and replicative forms (Fig. 15, page 50, and Table 3). The naturally occurring ds RNA migrating as RF I contains the core molecules that can be cleaved, presumably at the transcriptional gap, to RF II and RF III.

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LITERATURE CITED

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APPENDIX

Figure A-1. Digestion of Sindbis virus RNA at low concentrations of RNase at pH 5.

Two preparations of RNA were obtained by Method 1 (Chapter II, p. 5) from CE cells infected and labeled for 6 h. Samples (0.1-0.5 ml) were treated at pH 5 with the indicated concentrations of enzyme as described (Chapter II, p. 7). The reactions were stopped with SDS, and portions (20 µl) were taken for precipitation with cold TCA. All points represent averages of two experiments. Preparation B is designated RNA 1 in the text (Chapter III, p. 18) and in Fig. 5 (p. 21).

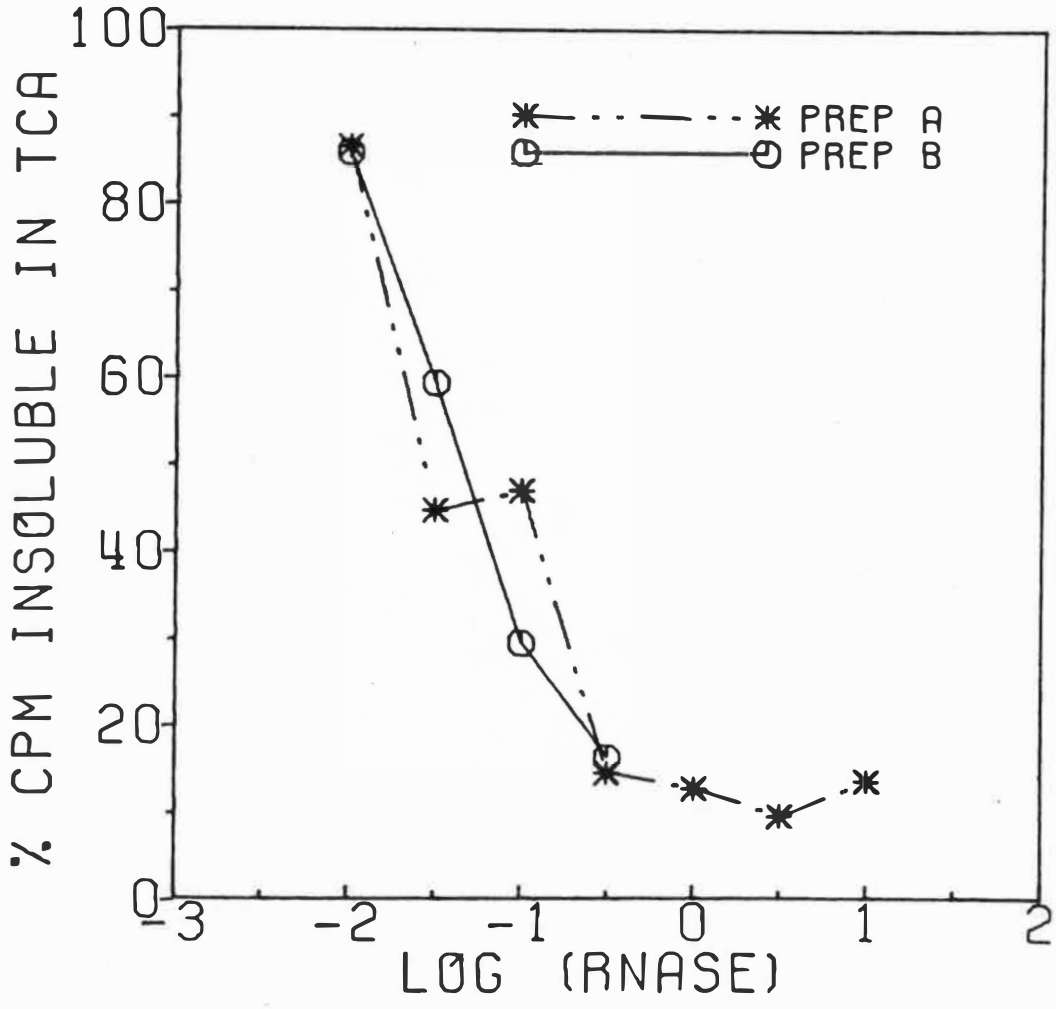

% CPM INSOLUBLE IN TCA
100
80
60
40
20
0
-3
-2
-1
0
1
2
LOG (RNASE)
PREP A
PREP B

Figure A-1

Figure A-2. Products of RNase treatment at pH 5.

Samples (0.5 ml) of RNA (preparation B of Fig. A-1) were treated with RNase at pH 5, and the enzyme was removed by extraction with chloroform-isoamyl alcohol. The products were precipitated with ethanol and were dissolved in 0.15 ml of 0.5% SDS in pH 7.6 buffer. An equal vol of 50% glycerol in the same buffer was added, and portions (20 µl) were analyzed by electrophoresis on 2.2% acrylamide/0.5% agarose gels for 5 h at 6 mA/gel and then for 3 h at 8 mA/gel.

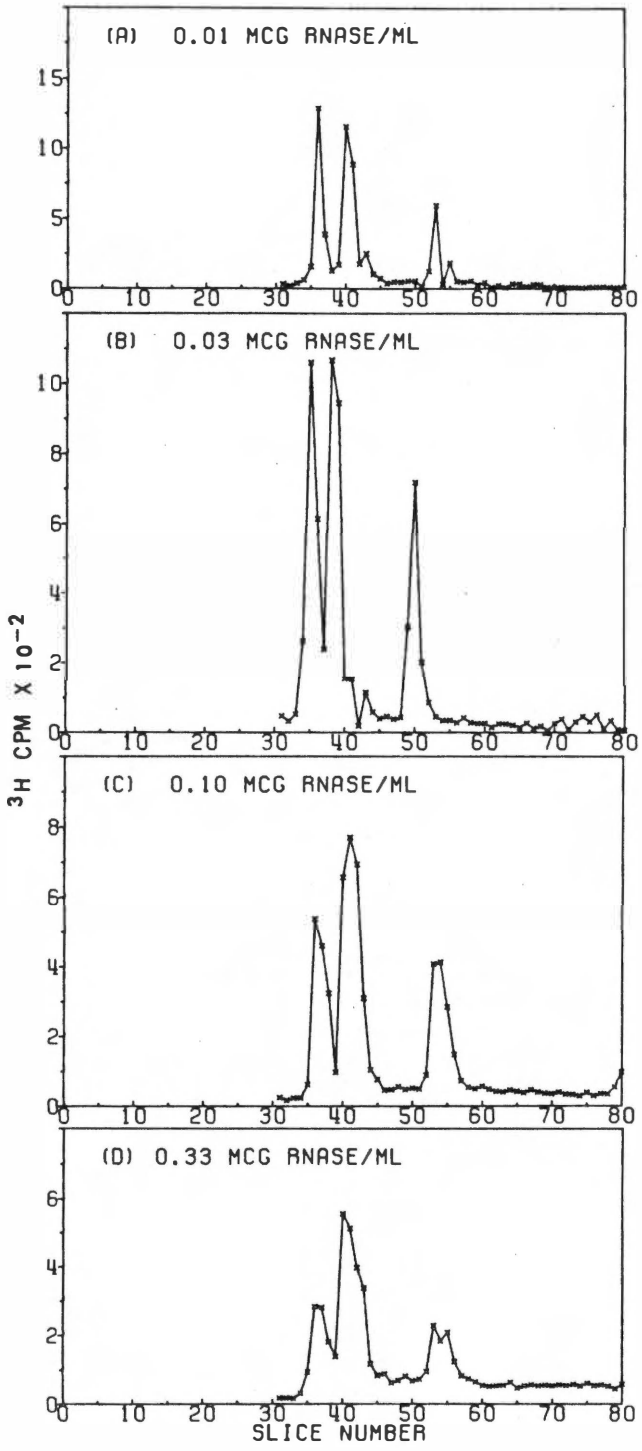

(A) 0.01 MCG RNASE/ML
(B) 0.03 MCG RNASE/ML
(C) 0.10 MCG RNASE/ML
(D) 0.33 MCG RNASE/ML
^{3}H CPM X 10^{-2}
SLICE NUMBER

Figure A-2

Figure A-3. Changes in proportions of RF's after treatment at pH 5 with different concentrations of RNase.

The RNA's are identified in the legend to Fig. A-1 (p. 64). The CPM in each peak were obtained from replicate gels at each RNase concentration. In the experiments with preparation A, the products of enzyme treatment were precipitated with ethanol before analysis. The ds RNA obtained in experiment 1 with preparation B was extracted as described in the legend to Figure A-2. The products in experiment 2 were analyzed without prior extraction or precipitation. The RF represented by each curve is indicated.

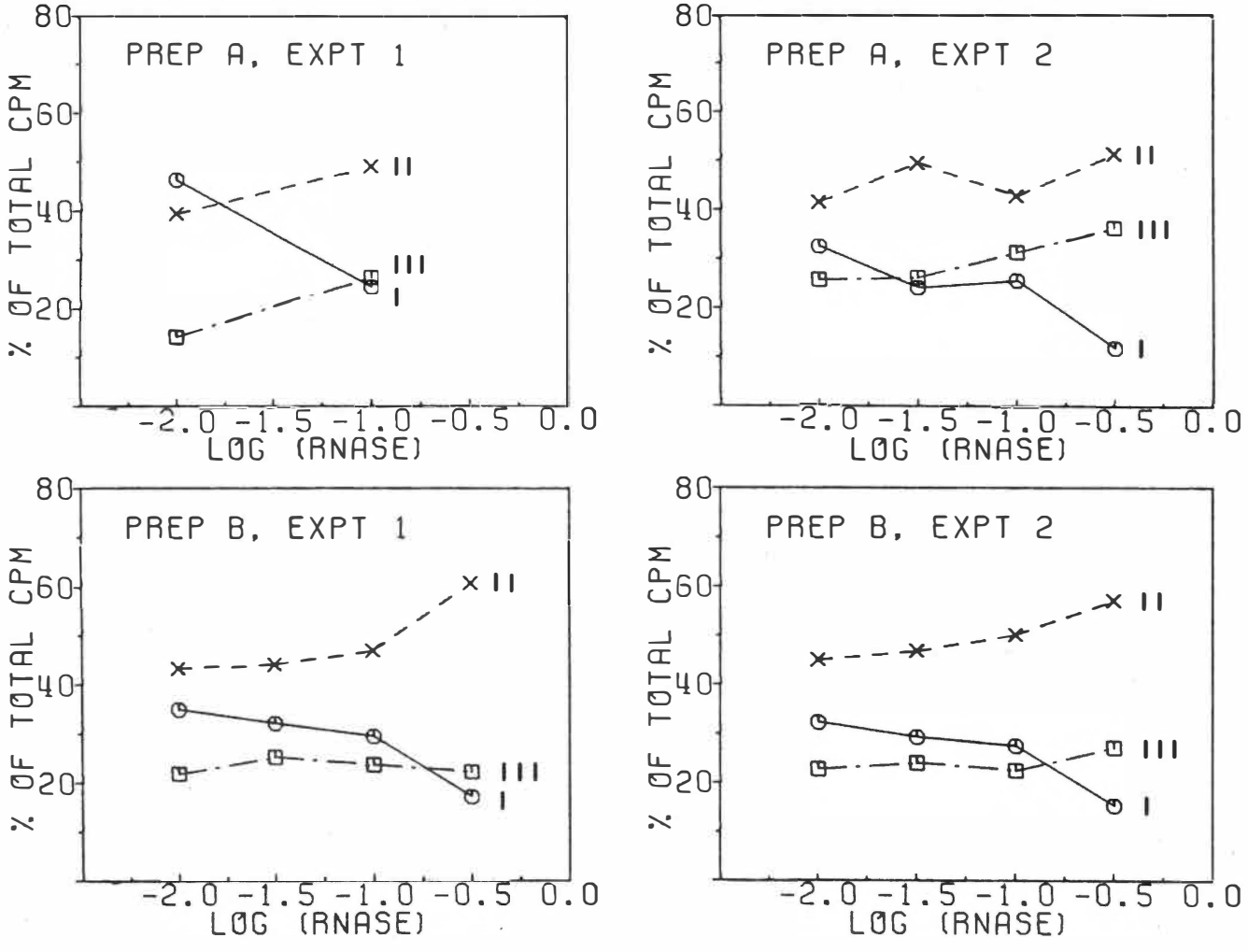

PREP A, EXPT 1
PREP A, EXPT 2
PREP B, EXPT 1
PREP B, EXPT 2
% OF TOTAL CPM
80
60
40
20
-2.0 -1.5 -1.0 -0.5 0.0
LOG (RNASE)
II
III
I

Figure A-3

PART II

ANOMALOUS BEHAVIOR OF SINDBIS VIRUS RNA DURING POLYACRYLAMIDE GEL ELECTROPHORESIS

CHAPTER I

INTRODUCTION

The RNA species produced by alphaviruses have been fractionated most clearly by polyacrylamide gel electrophoresis (PAGE). The molecular weights have been determined primarily by comparing the mobilities of the viral RNA's to those of rRNA reference markers. With respect to 18S and 28S chicken embryo (CE) rRNA, the virion RNA of Sindbis virus was assigned the molecular weight (MW) range of 3.89×10^6 to 4.45×10^6 (5). The MW of 3.4×10^6 for Semliki Forest virion RNA was derived by a similar approach (3). Cells infected with either virus contain two major species of virus-specific single stranded RNA: one is the virion RNA and the other is the messenger RNA encoding the virion structural proteins. The respective RNA's of the two viruses comigrated during PAGE, and MW's of 4×10^6 for the virion RNA and 1.8×10^6 for the messenger RNA were obtained with CE 45S precursor RNA and 28S and 18S rRNA as markers (9). Others have reported MW's of 1.7×10^6 to 1.8×10^6 for the messenger RNA (3, 5). However, Simmons and Strauss (18) used bacterial 16S and 23S r RNA as markers and obtained MW's of $4.3 \pm 0.3 \times 10^6$ for Sindbis virion RNA and 1.6×10^6 for the viral messenger RNA. Thus the reported MW's vary from 3.4×10^6 to 4.4×10^6 in the case of virion RNA but vary from only 1.6×10^6 to 1.8×10^6 for the messenger RNA.

The study summarized here suggested a possible explanation for the disparity in MW determinations by PAGE for alphavirus RNA's and further suggested that the use of rRNA's alone as reference markers does not

provide sufficient calibration for the gel system. Specifically, the MW's vary with the concentration of acrylamide monomer used in making the gels. The variation is probably a consequence of the secondary structure of the RNA's, but it is complicated by the observation that in certain gels (2.0-2.2% acrylamide[1]) the relationship of log (MW) to mobility is not strictly linear. However the relationship is linear in gels of other concentrations of acrylamide (2.4-2.6%).

The technique of fractionation of RNA and estimation of MW's of various RNA species by PAGE was established several years ago (2, 10, 11, 13, 14, 16). Indeed certain conclusions concerning secondary structure of the molecules may be inferred from their behavior during PAGE (4, 7, 11). The apparent MW as a function of mobility is linear for a variety of RNA's of MW's less than about 2×10^6 (10, 11, 14). However, RNA's of higher MW's presumably depart from this linearity during migration in gels of high acrylamide content (> 2.5%; ref. 14 as cited in ref. 7).

This anomalous behavior of high-MW RNA was examined in this study in somewhat more detail. HeLa cell 45S, 32S, 28S, and 18S RNA's were used to construct calibration curves [log (MW) vs. relative distance migrated] for gels of 2.0-2.6% acrylamide. Although there is some departure from linearity by the 45S RNA, it occurs in 2.0-2.2% gels but not in 2.4-2.6% gels. The break from linearity occurs within a narrow range of molecular weight (4.5×10^6 - 2.4×10^6).

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[1]For convenience, percent acrylamide as used in this report is the percentage (w/v) of acrylamide alone and does not include the percentage (w/v) of cross-linking agent (bis-acrylamide). The latter reagent was used at a constant percentage (w/w) of acrylamide (5%).

CHAPTER II

MATERIALS AND METHODS

Preparation of RNA from HeLa cells. Cells which were to be labeled with ^{3}H were a gift from K. R. Ambrose. HeLa cells were grown to subconfluency in 100-mm tissue culture dishes (Falcon Plastics) in 10 ml of fortified M199: M199 (Flow Laboratories) with 2X amino acids, 10% fetal calf serum, and antibiotics. At this time 10 of 20 dishes each received 0.1 ml (100 μCi) of [5-^{3}H]-uridine (28 Ci/mmole; New England Nuclear). The dishes were incubated at 37 C in a CO_2-incubator. After 17-1/2 h the medium was removed from the 10 cultures without lable and was replaced with 5 ml of Earle's balanced salt solution (EBSS; GIBCO) containing 20 μCi of ^{3}H-uridine per ml. Incubation was continued for 30 min. The 20 cultures were then extracted as one pool with phenol-cresol-hydroxyquinoline at room temperature (Method 1; Chapter II, Part I, p. 5). The RNA preparation was dissolved in pH 7.6 buffer (Chapter II, Part I, p. 5). The ^{3}H-labeled preparation contained 19.3 OD units of RNA per ml.

Preparation of Sindbis virus RNA from infected cells. Chicken embryo (CE) cell cultures were infected with plaque-purified Sindbis virus in the presence of actinomycin D. Infection, labeling with ^{3}H-uridine, and extraction of the RNA have been described previously (Chapter II, Part I). The two RNA preparations tested contained infectious RNA (data not shown).

Purification of Sindbis virus. Twenty confluent monolayers of CE cells in 100-mm tissue culture dishes were infected with plaque-purified

Sindbis virus at a multiplicity of 0.5 plaque-forming unit per cell. Each dish received 10 ml of M199 containing: 2% heat-inactivated, dialyzed calf serum (GIBCO); antibiotics, including actinomycin D (1 μg/ml; CALBIOCHEM); and 5 μCi of ^{3}H-uridine per ml. The cultures were incubated at 37 C in a CO_2-incubator for 9 h. At that time—and at 3-h intervals thereafter—the medium was removed and placed on ice. The cultures were given fresh medium (including label) and were returned to the incubator. Harvests were collected at 9, 12, 15, 18 and 21 h post-infection.

Immediately after collection, each harvest was treated as follows: The medium was clarified at 4100 × g for 20 min at 0 C. Thirty-two ml volumes of the clarified medium in polyallomer tubes were underlayed with 4 ml of 30% sucrose (w/w) in TNE buffer (0.05 M Tris, pH 7.6; 0.1 M NaCl; 0.001 M EDTA) containing 0.5% heat-inactivated fetal calf serum. The tubes were centrifuged at 26,000 RPM (SW27 rotor) for 1 h at 4 C. The pellets were obtained by decanting the supernate and were suspended in a total vol of 2 ml of TNE + serum. The suspension from each harvest was frozen at -70 C.

When harvesting was completed, 3-ml portions of the suspensions of virus were applied to 33-ml gradients of 20-60% sucrose (w/w) in TNE + serum. The virus was banded at 25,000 RPM (SW27 rotor) for 16 h at 2 C. The gradients were fractionated, and the fractions containing the peak of radioactivity (at a density of 1.18 g/cm^{3}) were pooled. The pool was stored at -70 C.

Preparation of RNA from purified Sindbis virus. The pool (24 ml) of purified virus obtained as above was adjusted to 0.5% SDS and was extracted by Method 1 (Chapter II, Part I, p. 5). The RNA was dissolved in 2 ml of pH 7.6 buffer. The preparation contained 0.6 OD units/ml, and the RNA was infectious (data not shown).

Polyacrylamide gel electrophoresis (PAGE). Details of this procedure have been described elsewhere (Chapter II, Part I). Gels of 2.4% and 2.6% acrylamide (w/v) were prepared by the method of Loening (2, 11). Composite gels of 2.0%, 2.2%, and 2.4% acrylamide (w/v) and 0.5% agarose (w/v) were prepared by a modification of the method. The concentration of bis-acrylamide was 5% of that of acrylamide throughout all experiments. All gels were 0.6 × 8 cm. Preelectrophoresis of the gels was carried out at 5 mA/gel for 30 min. Samples adjusted to 25% glycerol were applied, and electrophoresis was continued for 4 h.

Gel slices were prepared for counting of radioactivity as described (Chapter II, Part I). The samples were counted on a model 3375 Tri-Carb liquid scintillation spectrometer (Packard Instrument) or on an Isocap/300 liquid scintillation system (Searle Analytic).

CHAPTER III

RESULTS

PAGE of HeLa cell reference RNA's. In the past, the calibration of gels for PAGE of Sindbis RNA was done with only two reference RNA's, either CE rRNA (5) or bacterial rRNA (18). By this approach, the calibration of the region to which the virion RNA migrates is subject to doubt since it lies outside the region calibrated by rRNA. In the one report (9) in which CE 45S ribosomal precursor RNA was included as a reference, the MW's of the respective HeLa cell RNA's were assumed, and this assumption may not have been accurate.

In order to be more certain of calibrating gels throughout the regions to which Sindbis RNA's migrated, a mixture of HeLa cell RNA labeled for 18 h and for 30 min was used. Such a mixture contained five species of RNA (Fig. 1). Four of these served as reference markers, the MW's for which have been determined by equilibrium sedimentation (12): 45S ribosomal precursor (MW of 4.5×10^6), 32S ribosomal precursor (2.4×10^6), 28S rRNA (1.9×10^6), and 18S rRNA (0.7×10^6). The fifth species seen in the profiles is presumably the 36S ribosomal precursor observed by Weinberg et al. (20). Figure 1a-d are the electrophoretic profiles of the RNA's in gels of 2.0, 2.2, 2.4, and 2.6% acrylamide, respectively.

The important results from PAGE of these RNA's in different gels are the relationships between log (MW) and mobility (i.e., the calibration curves). Because all other factors of true mobility were held constant, mobility is expressed here in terms of relative distance migrated (RDM),

Figure 1. PAGE of HeLa cell RNA.

A mixture of HeLa cell RNA labeled for 18 h and for 30 min was prepared as described in Materials and Methods (Chapter II, p. 73). Some of the RNA was adjusted to 25% glycerol, and portions were subjected to electrophoresis as described (p. 75). Gels A and B were composite gels of 2.0 and 2.2% (w/v) acrylamide, respectively, and 0.5% agarose. Gels C and D were 2.4 and 2.6% in acrylamide, respectively. Sample volumes were 10 µl (A, C, D) or 20 µl (B). Species used as reference markers are identified as 45S, 32S, 28S, and 18S.

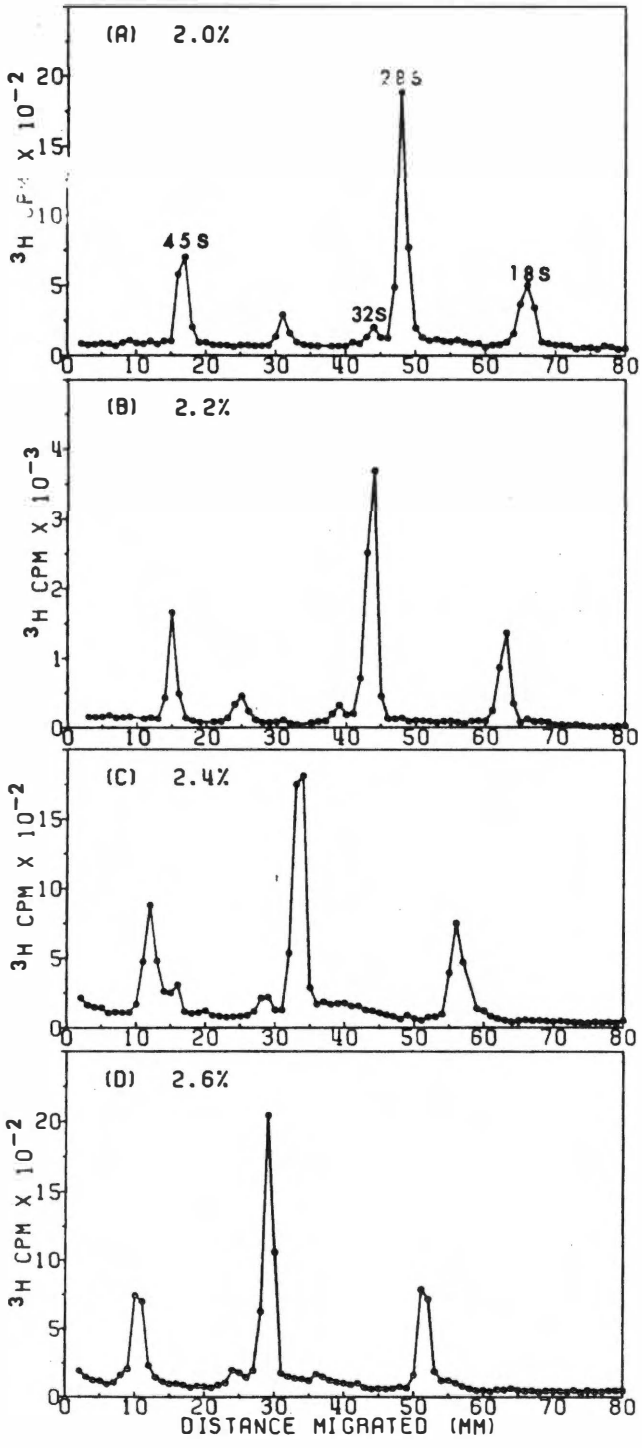

(A) 2.0%
28S
45S
32S
18S
^{3}H CPM X 10^{-2}
(B) 2.2%
^{3}H CPM X 10^{-3}
(C) 2.4%
^{3}H CPM X 10^{-2}
(D) 2.6%
^{3}H CPM X 10^{-2}
DISTANCE MIGRATED (MM)

Figure 1

a fraction of the total length of the gel. The plots of log (MW) vs. RDM for each of the gel concentrations used are given in Fig. 2. The observation of interest was that the curves deviate from linearity at the high-MW end for the gels of 2.0 and 2.2% acrylamide but not for the 2.4 and 2.6% gels. The observed deviation from linearity is also evident from the correlation coefficients for the lines computed by linear least-squares analysis (Table A-1, in the Appendix). Thus, a linear extrapolation of the line defined by 28S and 18S RNA alone can be erroneous in certain gels. As will be shown, more accurate MW's of high-MW RNA's are made only in those gels yielding linear calibration curves.

It should be noted that the 2.0 and 2.2% gels contained agarose whereas the 2.4 and 2.6% gels did not. However, the break in the calibration curve is not caused by the agarose. Composite gels of 2.4% acrylamide/0.5% agarose provided a linear calibration curve (data not shown). The linearity of the curves at the low-MW end has been observed in gels with (14) and without (11) agarose.

The data from which the curves in Fig. 2 were derived are summarized in Table A-1 of the Appendix.

MW's of Sindbis virus RNA's as determined by PAGE. Figure 3a and c are the electrophoretic profiles of the virus-specific RNA's produced in CE cells infected with Sindbis virus. The two major species, designated V and M, are single-stranded and correspond to the virion RNA and messenger RNA, respectively. (The other major peak is viral double-stranded RNA, a replicative form.) The electrophoretic profile of the

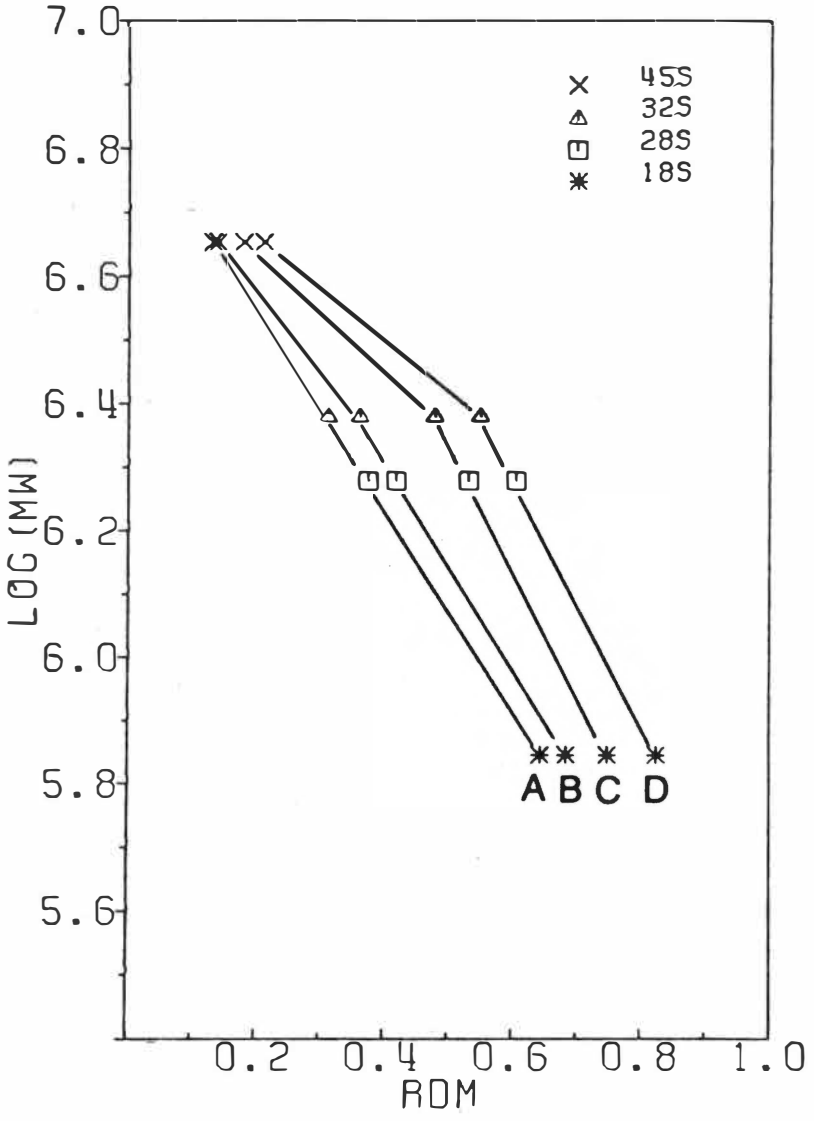


Figure 2. Relationship at different gel concentrations of log (MW) to RDM.

Mobilities of the various RNA's are expressed as relative distance migrated (RDM), the fraction of the gel length (8 cm). Each point represents several determinations (Table A-1, Appendix, p. 97). The molecular weights were taken from reference 12. Acrylamide concentrations were 2.6% (A), 2.4% (B), 2.2% (C), and 2.0% (D).

Figure 3. PAGE of Sindbis virus RNA from infected CE cells and from purified virus.

The RNA preparations were obtained as described in Materials and Methods (Chapter II, p. 73). Samples adjusted to 25% glycerol were analyzed as described (p. 75). (A) RNA (10 µl) from infected cells, 2.2% composite gel. (B) RNA (10 µl) from purified virus, 2.2% composite gel. (C) RNA (10 µl) from infected cells, 2.4% gel. (D) RNA (20 µl) from purified virus, 2.4% gel. Arrows indicate the positions (left to right) of 45S, 32S, 28S, and 18S HeLa RNA's fractionated in parallel gels. V: virion RNA; M: messenger RNA.

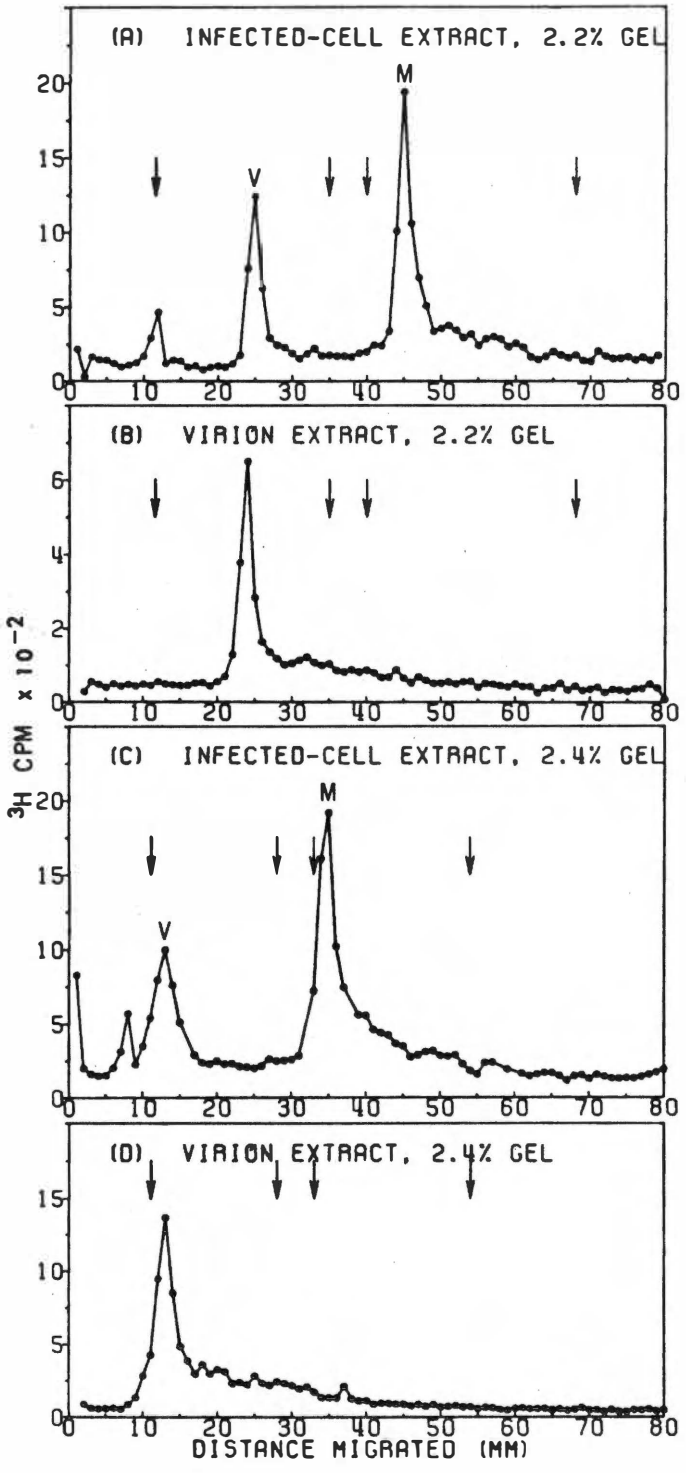

(A) INFECTED-CELL EXTRACT, 2.2% GEL
(B) VIRION EXTRACT, 2.2% GEL
(C) INFECTED-CELL EXTRACT, 2.4% GEL
(D) VIRION EXTRACT, 2.4% GEL
M
V
^{3}H CPM x 10^{-2}
DISTANCE MIGRATED (MM)

Figure 3

RNA extracted from purified virus is shown in Figure 3b and d. The profiles shown are those obtained with 2.2 and 2.4% gels, as indicated.

The MW's for virion RNA and for intracellular virion and messenger RNA's were determined from calibration curves obtained as described above. The weights derived at each gel concentration used are given in Table 1. It can be seen that the MW's vary with gel concentration. The MW of virion RNA varied considerably, from 3.2×10^6 to 4.5×10^6. The weights obtained from the 2.4 and 2.6% gels are similar to those obtained, probably fortuitously, with reference to just 18S and 28S rRNA (5) or just 16S and 23S rRNA (18). The weight to be taken as the most accurate is discussed below.

By comparison, the MW of the messenger RNA varies to a lesser extent: 1.6×10^6 to 1.9×10^6. This may be a consequence of the fact that it migrates in a region that is accurately calibrated for all gel concentrations.

Comparison of PAGE of HeLa cell RNA and PAGE of Sindbis RNA. It was of interest to compare the behavior of Sindbis RNA during PAGE to that of HeLa cell RNA since the MW's of the former seemed to vary with gel concentration (Table 1). It was also desirable to make the comparisons without consideration for apparent MW's, thereby avoiding the differences which are implicit to the variations already observed. Such a comparison can be made with plots of mobilities vs. gel concentrations. As is explained in the Discussion (Chapter IV, p. 88), the important parameter of mobility here is distance migrated (D).

TABLE 1. MW's of Sindbis virus RNA's determined by PAGE at different gel concentrations.

RNA SPECIES[a]	GEL CONC. (%)	N[b]	MEAN RDM[c]	MEAN MW
Intracellular:				
virion	2.0	5	0.406 ± 0.015	3.28 ± 0.19
	2.2	5	0.325 ± 0.018	3.22 ± 0.16
	2.4	6	0.174 ± 0.021	4.09 ± 0.22
	2.6	3	0.133 ± 0.014	4.40 ± 0.17
messenger	2.0	5	0.635 ± 0.010	1.62 ± 0.08
	2.2	5	0.557 ± 0.014	1.70 ± 0.28
	2.4	6	0.446 ± 0.026	1.65 ± 0.10
	2.6	3	0.379 ± 0.031	1.88 ± 0.23
Purified virion	2.0	2	0.418	3.20
	2.2	1	0.304	3.20
	2.4	3	0.167 ± 0.019	4.20 ± 0.26
	2.6	1	0.125	4.50

[a]RNA from infected cells (3 preparations) or from purified virus (1 preparation).

[b]Number of determinations.

[c]Relative distance migrated, as defined in the text (p.).

The plot of log D vs. total concentration (T) of acrylamide monomer (acrylamide and bis-acrylamide) is shown in Fig. 4 for each of the RNA species examined. (The significance of this manner of plotting is discussed below.) The symbols represent averages of experimental measurements, given in Table 2; the lines were computed by linear least-squares analysis. Table 2 also contains the coefficients of correlation for the experimental values. It can be seen from the latter statistics that the experimental results provide a closely fitting theoretical curve.

Two observations are readily apparent from the curves in Fig. 4. First, the change in mobility with gel concentration (i.e., the slope) is very similar for 45S, 32S, and 28S HeLa cell RNA's and for 26S Sindbis RNA; the slope of the curve for 18S rRNA is somewhat dissimilar to the others. (The slopes for the respective curves are given in Table 2.) Second, the change in mobility of Sindbis virion RNA (shown here for the intracellular RNA) is considerably different from that of the other RNA species. (It can be inferred from Table 1 that RNA extracted from purified virus behaves similarly.) An interpretation of these observations is presented in the discussion which follows.

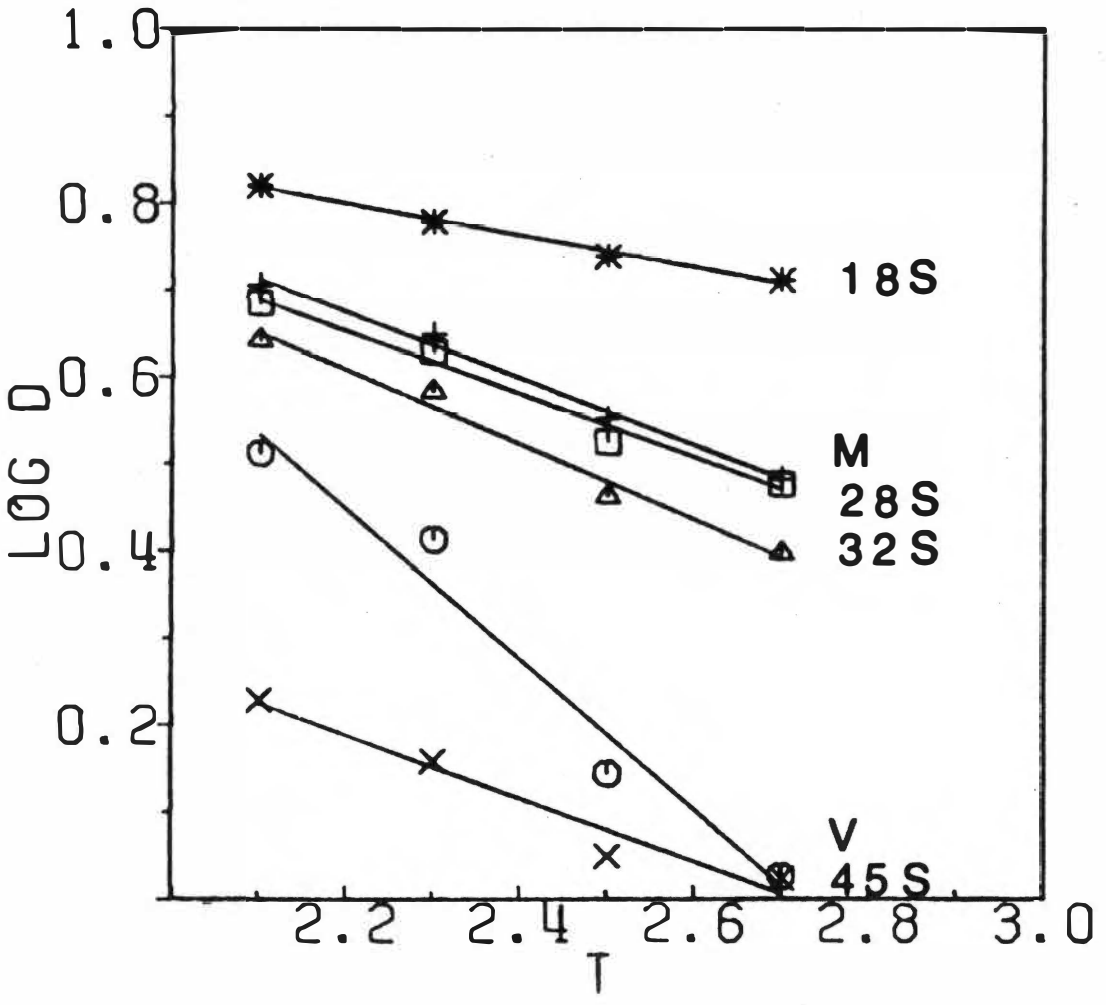


Figure 4. Relationship of log D to T.

Mobilities of the various RNA's are expressed as distance migrated (D) in cm. Gel concentration (T) is the total concentration of acrylamide monomer (acrylamide + bis-acrylamide). The points were determined experimentally and represent the means of several determinations (Table 2, p. 87). The lines are those computed by linear least-squares analysis. V: Sindbis virion RNA (intracellular); M: Sindbis messenger RNA.

TABLE 2. Mean values of D at different T's for HeLa cell and Sindbis RNA's.

RNA	T[a]	N[b]	D[c]	SLOPE[d]	CC
45S	2.1	2	17.0	-0.362	0.980
	2.3	9	14.4		
	2.5	3	11.2		
	2.7	1	10.6		
V[e]	2.1	5	32.5	-0.862	0.983
	2.3	5	26.0		
	2.5	6	13.9		
	2.7	3	10.6		
32S	2.1	2	44.0	-0.430	0.991
	2.3	9	38.3		
	2.5	3	29.0		
	2.7	1	25.0		
28S	2.1	2	48.5	-0.366	0.990
	2.3	9	42.6		
	2.5	3	33.5		
	2.7	1	30.0		
M[e]	2.1	5	50.8	-0.384	1.000
	2.3	5	44.6		
	2.5	6	35.7		
	2.7	3	30.3		
18S	2.1	2	66.0	-0.182	0.995
	2.2	9	59.9		
	2.3	3	54.8		
	2.7	1	51.5		

[a]Total acrylamide, as defined in the text (p.).

[b]Number of determinations.

[c]Mean of distance migrated.

[d]The curve fitting log D vs. T, its slope, and coefficient of correlation (CC) of the experimental values were computed by linear least-squares analysis.

[e]Sindbis virion (V) and messenger (M) RNA.

CHAPTER IV

DISCUSSION

The apparent molecular weights of the genomes of Sindbis virus and Semliki Forest virus obtained by polyacrylamide gel electrophoresis have varied over the range 3.4×10^6 to 4.4×10^6 according to different reports (3, 5, 9, 18). The reported MW's of the viral messenger RNA's range from 1.6×10^6 to 1.8×10^6. In the studies cited, electrophoresis was carried out in gels of 1.85% to 3% acrylamide, and, except for one study (9), only two rRNA reference markers were used for calibration. The results obtained in this study suggest a possible explanation for the disparities of reported MW's. [This disparity is probably not due to differences in the sizes of the respective RNA's of the two viruses since they have been shown to comigrate (9)].

The RNA's of Sindbis virus were subjected to electrophoresis in gels of 2.0-2.5% acrylamide. HeLa cell 45S, 32S, 28S, and 18S RNA's were used as reference markers since their MW's have been determined by a method independent of PAGE (equilibrium sedimentation; ref. 12). The apparent MW of Sindbis virion RNA varied from 3.2×10^6 in 2.0% gels to 4.5×10^6 in 2.6% gels; the apparent MW of the messenger RNA varied to a lesser extent, from 1.6×10^6 to 1.9×10^6 (Table 1, p. 84). Thus, variability can be observed to be a consequence of electrophoresis in gels of different acrylamide concentration. This was true in spite of the fact that the gels were calibrated over the entire range of MW being examined. The comparatively small range of MW's obtained for the messenger RNA is

probably a consequence of accurate calibration of the region to which it migrates. The low-MW end of the calibration curve is strictly linear for all of the gels described here (Fig. 2, p. 80, and ref. 11) and for a wide range of gel concentrations (14). The greater variation in the apparent MW of virion RNA may be in part a consequence of inaccurate calibration at that end of the curve (Fig. 2). For gels of 2.0 and 2.2% acrylamide, the upper end of the calibration curve deviates from linearity, and this deviation is significant (Table A-1, in the Appendix). However, the curves for 2.4 and 2.6% gels appear to be strictly linear throughout the range of MW provided by the reference RNA's (Fig. 2). It is therefore important to calibrate gels throughout the required range of MW and to use gels which provide strictly linear calibration curves. As is indicated below, such gels provide more accurate estimates of MW.

The results discussed above may present a dilemma as to which values of MW are the most accurate estimate for Sindbis virus RNA's. Intuitively they should be the ones derived from strictly linear calibration curves. Electron microscopy of the virion RNA indicated an MW of $4.65 \pm 0.4 \times 10^6$ (8). Electrophoresis in agarose gels under denaturing conditions corraborated the mean value (1). The weights determined in this study by electrophoresis in 2.4 and 2.6% gels were in the range of 4.1×10^6 to $4.5 \quad 10^6$ (Table 1, p. 84). It thus appears that more accurate estimates of MW by PAGE are obtained in gels which provide linear calibration curves throughout the desired range of MW. It should be noted, however, that the most accurate determinations probably are obtained by methods in which secondary structure of the RNA is not a factor; e.g., electron

microscopy (8) or electrophoresis under denaturing conditions (1, 15). It would be of interest to obtain estimates of the MW of Sindbis virus messenger RNA by such methods.

As mentioned previously, the mobility of 45S HeLa cell precursor RNA created a break in the calibration curves for 2.0 and 2.2% gels but not in those for 2.4 and 2.6% (Fig. 2, p. 80). It thus appears that either the 45S RNA migrates slower than would be expected for the lower percentage gels or that the other reference RNA's migrate faster than would be expected. The former possibility seems doubtful since it would not be expected that a decrease in pore size (provided by the higher percentage gels) would enable the 45S RNA to migrate faster with respect to other RNA's and thereby generate the observed linear calibration curve. It appears that the latter alternative is the better interpretation. This interpretation is consistent with the observation if it is assumed that these RNA molecules are migrating as rods, end-on, through the gels and, of course, that the diameters of the rods are smaller than those of the gel pores.

Because the apparent molecular weight (and mobility) of Sindbis virion RNA varied considerably with gel concentration (Table 1, p. 84), its behavior during PAGE was compared to that of the reference RNA's by a method which is independent of the measured MW's. In effect, the change in mobility with gel concentration for the Sindbis RNA was compared to the changes for HeLa cell RNA's (Fig. 4, p. 86). Electrophoretic mobility (M) may be defined as follows (7):

[Eq. 1] $$M = (d)(K)/(i)(t),$$

where d is distance migrated (in cm), K is the specific conductance of the buffer at the temperature of the gel, i is the current density (in A/cm^2), and t is the time (in sec). For the purposes in this study, it was sufficient to use d as a measure of mobility, since all other factors were constant. The lines shown in Fig. 4, page 86, are defined by the following equation (6, 17):

[Eq. 2] $$\log M = \log M_0 - K_R T,$$

where M_0 is the mobility at zero gel concentration, K_R is the retardation coefficient, and T is the total gel concentration (acrylamide + bis-acrylamide). The parameter of interest is the slope, K_R, of the line. It is a parameter which depends on the properties of the gel and on the shape and size of the RNA molecule. K_R can be related to the molecular geometry of the molecule by the expression (17)

[Eq. 3] $$(K_R)^{1/2} = c(\bar{R} + r)$$

where c is an empirical constant for each gel system, $\bar{R}$ is the effective radius of the RNA molecule with respect to its electrophoretic behavior in polyacrylamide gels (16), and r is the effective radius of the gel fiber.

From Equations 1 and 2 above, it can be shown that the slopes of the curves in Fig. 4 are in fact the negative of the K_R values for each RNA. (The slopes are given in Table 2, p. 87.) The K_R value for Sindbis virion RNA (0.862) is considerably different from those of 45S, 32S, and 28S RNA's (K_R's of 0.362, 0.430, and 0.366, respectively), from that of 18S RNA

(K_R = 0.182), and from that of Sindbis messenger RNA (K_R = 0.384). Thus there is some anomalous property of Sindbis virion RNA which affects its change in mobility with gel concentration in a way that is not observed with the other RNA species. Equation 3 suggests that this property is a function of effective molecular radius ($\bar{R}$). The effective radii of 45S HeLa RNA and Sindbis virion RNA are quite different, yet they have similar MW's [4.5×10^6 (ref. 12) and 4.6×10^6 (ref. 8), respectively]. One may therefore speculate that Sindbis virion RNA migrates during PAGE in a configuration different from that of 45S HeLa RNA (and perhaps from that of the other RNA's, as well). Such speculation is supported to some extent by the observations that the viral RNA may be circular with cohesive ends (8) and that the precursor RNA probably has considerable secondary structure (19). As mentioned above, the HeLa cell RNA molecules may migrate end-on through the gels. The larger value of K_R for Sindbis virion RNA (and, by Eq. 3, its larger effective radius) may indicate that some feature of secondary structure complicates end-on migration of the viral RNA.

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APPENDIX

TABLE A-1. Values of RDM at different gel concentrations for HeLa cell reference RNA's.

GEL CONC.[a]	RNA	N[b]	MEAN RDM[c]	MEAN CC[d]
2.0	45S	2	0.212	
	32S	2	0.550	
	28S	2	0.606	
	18S	2	0.825	
				0.9682 ± 0.0124
2.2	45S	9	0.180 ± 0.017	
	32S	9	0.479 ± 0.027	
	28S	9	0.532 ± 0.026	
	18S	9	0.749 ± 0.028	
2.4	45S	5	0.140 ± 0.010	
	32S	5	0.362 ± 0.014	
	28S	5	0.419 ± 0.007	
	18S	5	0.685 ± 0.014	
				0.9971 ± 0.0024
2.4a	45S	3	0.154 ± 0.007	
	32S	3	0.308 ± 0.014	
	28S	3	0.389 ± 0.025	
	18S	3	0.650 ± 0.025	
2.6	45S	2	0.132	
	32S	2	0.312	
	28S	2	0.375	
	18S	2	0.644	

[a]Percent acrylamide. Gels designated 2.0, 2.2, and 2.4a were composite with 0.5% agarose.

[b]Number of determinations.

[c]Relative distance migrated, a fraction of gel length (8 cm).

[d]The curve fitting log (MW) vs. RDM and the coefficient of correlation (CC) of the measured values were computed by linear least squares analysis. The mean values of CC for the data grouped as shown were significant at $p = 0.005$ by student's t test.