5' Nucleotide Sequence of Sindbis Viral RNA

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The 5' sequence of Sindbis viral RNA is $m^7G^{5'}pppApUpGp\ldots$

Recent analyses have shown that the messenger polarity RNA plus strands of cytoplasmic polyhedrosis virus or human reovirus possess modified 5' nucleotide sequences (11, 12). The complementary minus strands of the double-stranded genomes of these viruses do not have modified nucleotides (11, 12). The 5' sequence of reovirus plus strand RNA (8) is believed to be $m^7G^{5'}pppGmpCp\ldots$, with a methyl residue present on the 7-position of the capping guanosine (m^7G) and a second 2'-O-methyl group present on the ribose of the next guanosine nucleotide (Gmp). The presence of capping m^7G on the messenger or putative mRNA species of vesicular stomatitis or vaccinia viruses has also been demonstrated (13, 24).

In this communication we present evidence indicating that the 5' terminal nucleotide of the alphavirus Sindbis is modified and that the sequence is $m^7G^{5'}pppApUpGp\ldots$

MATERIALS AND METHODS

Reagents. [³H]nucleosides, [¹⁴C]guanosine, and [³²P]phosphoric acid were obtained from International Chemical and Nuclear Corp., Irvine, Calif. [¹⁴C]-uridine and [methyl-³H]methionine were obtained from New England Nuclear Corp., Boston, Mass. Nucleoside and nucleotide standards for chromatography were obtained from P. L. Biochemicals Inc., Milwaukee, Wis. Alkaline phosphatase and venom phosphodiesterase were obtained from Boehringer Mannheim Corp., New York, N.Y.

Preparation and purification of ³H-, ¹⁴C-, or ³²P-labeled virus. The procedures used to obtain labeled virus grown in the presence of [5-³H]uridine (25 µCi/ml, 8 Ci/mmol) and [5-³H]cytidine (25 µCi/ml, 16.3 Ci/mmol), [8-³H]guanosine (25 µCi/ml, 20 Ci/mmol), [8-¹⁴C]guanosine (0.5 µCi/ml, 55 mCi/mmol), [8-³H]adenosine (25 µCi/ml, 21 Ci/mmol), [³²P]phosphate (200 µCi/ml), or [2-¹⁴C]uridine (0.04 µCi/ml, 0.4 mCi/mmol) have been described (1, 15,

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21). The purification of these labeled viruses used a procedure described previously (15) for vesicular stomatitis virus (Indiana serotype).

Sindbis virus serotype was authenticated by plaque neutralization tests using a Sindbis antiserum.

For the preparation of [methyl-³H]methionine-labeled Sindbis virus, confluent monolayers of BHK-21 cells in two roller bottles (containing approximately 4×10^8 to 5×10^8 cells) were infected with virus (10 ml/bottle) at an input multiplicity of approximately 1 PFU/cell. After an adsorption period of 60 min at 37 C, 90 ml of medium was added to each roller bottle. The medium (Eagle minimal medium [5] plus non-essential amino acids) contained 3 µg of methionine per ml (20% of the normal level) and 10 mCi of [methyl-³H]methionine (11.5 Ci/mmol; final methionine specific activity, 2.5 Ci/mmol) and was supplemented with unlabeled adenosine and guanosine to diminish labeling of the purine rings (4). The medium also contained actinomycin D (0.5 µg/ml). The roller cultures were incubated at 37 C, and virus was harvested 24 h later and purified as described elsewhere (21, 23).

Extraction and purification of labeled RNA; nuclease, phosphatase, and alkali digestion conditions; base ratio analyses; resolution of oligonucleotides by DEAE-cellulose column chromatography. The procedures used for extraction of labeled viral RNA and its subsequent purification through 4% agarose column chromatography have been described (9). Pancreatic RNase and/or RNase T₁ or alkali digestion of RNA and resolution of the derived nucleotides by DEAE-cellulose column chromatography at pH 8.0 in 7 M urea have also been described (9, 17). Nucleotides were recovered from column eluants by barium precipitation as described previously (9). Base ratios of RNA were determined after paper electrophoresis of alkali-treated RNA samples (9, 18). RNase T₂ digestion involved treating an RNA sample in 1 ml of 0.05 M sodium acetate buffer, pH 4.5, containing 2 mM EDTA with 2 U of RNase T₂ and incubating the mixture at 37 C for 60 min. Alkaline phosphatase digestion of nucleotides involved incubating a sample in 1 ml of 0.01 M Tris-hydrochloride buffer, pH 8.0, 0.001 M MgCl₂ with 3.5 U of alkaline phosphatase at 37 C for 30 min. The venom phosphodiesterase digestion involved incubation at 37 C for 4 h in 100 µl of 0.02 M Tris-hydrochloride buffer, pH 7.4, containing 0.005 M NaH₂PO₄ and 1 U of enzyme.

Acid hydrolysis and paper electrophoresis or chromatography of nucleosides and nucleotides. RNA was subjected to acid hydrolysis (1 N HCl, 100 C for 45 min), the HCl was removed by lyophilization, and the residue was dissolved in 25 μl of 0.05 M ammonium formate buffer (pH 3.5) and subjected to paper electrophoresis at 3,000 V for 3 h using a 110-cm strip of Whatman no. 3 MM filter paper. Paper chromatography employed alkaline system 4 of Littlefield and Dunn (10).

RESULTS

Base ratio and molecular weight estimates of Sindbis viral RNA. A preparation of ^{32}P-labeled Sindbis viral RNA was subjected to alkali digestion, and the products were resolved by paper electrophoresis at pH 3.5 to determine the nucleotide composition (9, 18). A base ratio of 27.7% AMP, 24.6% CMP, 25.7% GMP, and 22.0% UMP was obtained.

To estimate the molecular weight of Sindbis viral RNA, a sample of [^{3}H]cytidine- and [^{3}H]uridine-labeled RNA was co-electrophoresed on a 1.8% polyacrylamide gel together with a sample of ^{32}P-labeled vesicular stomatitis virus Indiana viral RNA (Fig. 1A). No detectable difference was observed in their electrophoretic mobilities, suggesting that the size of Sindbis viral RNA is about 3.8 × 10^6 daltons (15).

From the base ratio analysis and the estimated molecular weight of the viral RNA, it was concluded that Sindbis viral RNA is composed of approximately 11,000 nucleosides, consisting of 3,050 adenosine, 2,700 cytidine, 2,830 guanosine, and 2,420 uridine residues per strand of RNA.

Demonstration that Sindbis viral RNA can be labeled by [methyl-^{3}H]-methionine. A preparation of Sindbis viral RNA extracted from virus grown in the presence of [methyl-^{3}H]methionine was centrifuged in a gradient of sucrose (Fig. 1B). A labeled 42S viral RNA was obtained.

Alkali digestion of Sindbis viral RNA. Preparations of [^{3}H]adenosine-, [^{3}H]uridine- and [^{3}H]cytidine-, or [^{3}H]guanosine-labeled Sindbis viral RNA were mixed with [^{32}P]phosphate-labeled Sindbis viral RNA, and each mixture was subjected to alkali digestion. The tritium exchange with water was determined (9), and the digests were chromatographed on DEAE-cellulose. After the mononucleotides had been eluted, the columns were stripped with 0.7 M LiCl in 7 M urea (9), and the eluant fractions containing labeled material were pooled, 0.1 μmol each of 2′,3′ mixed AMP, CMP, GMP, and UMP nucleotides were added, and the mixture was quantitatively precipitated by ad-
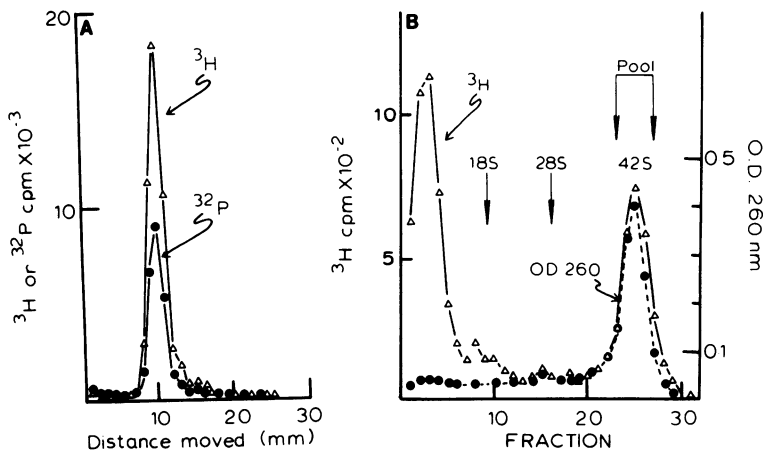

FIG. 1. *Gel electrophoresis and sucrose gradient centrifugation of Sindbis viral RNA. (A) Sample of [^{3}H]uridine- and [^{3}H]cytidine-labeled Sindbis viral RNA, purified as described in Materials and Methods, was mixed with a sample of ^{32}P-labeled vesicular stomatitis virus Indiana viral RNA. Sodium dodecyl sulfate was added to give a final concentration of 0.1%, and the mixture was loaded onto a 1.8% polyacrylamide gel (2) and subjected to electrophoresis at 10 mA for 1 h. After electrophoresis, the gel was frozen on dry ice and sliced with a CO$_2$-freezing microtome. The 1-mm slices were dissolved in 0.5 ml of 30% (vol/vol) H$_2$O$_2$ at 60 C overnight. The solubilized material was counted in a liquid scintillation cocktail consisting of Triton and toluene (7). (B) [methyl-^{3}H]methionine-labeled Sindbis virus was subjected to phenol extraction, and the RNA was centrifuged on a sucrose density gradient (see Materials and Methods). Thirty 0.4-ml fractions were collected, and the absorption at 260 nm and content of label were determined. The label observed at the top of the gradient probably represents high-specific-activity tRNA species. It has not been examined further. The arrows indicate the positions of 18S and 28S rRNA species run in a parallel gradient.*

dition of 0.1 ml of saturated BaCl₂ and 5 volumes of alcohol (9, 17). After recovery by centrifugation, the nucleotides were converted to their hydrogen form using Dowex 50 (H⁺) ion-exchange resin and redigested with alkali to hydrolyze residual oligonucleotides, which had survived the initial digestion (9). Each hydrolysate was again monitored for tritium exchange (9) and chromatographed at pH 8.0 on DEAE-

cellulose together with a pancreatic RNase digest of 1.5 mg of unlabeled chicken embryo fibroblast RNA to provide marker mono-, di-, tri- (etc.) oligonucleotides. The results obtained indicated that for RNA labeled by [³H]adenosine and [³²P]phosphate an alkali-resistant, labeled nucleotide survived the digestion. This fragment eluted from the DEAE-cellulose column between the trinucleotides and tetranu-
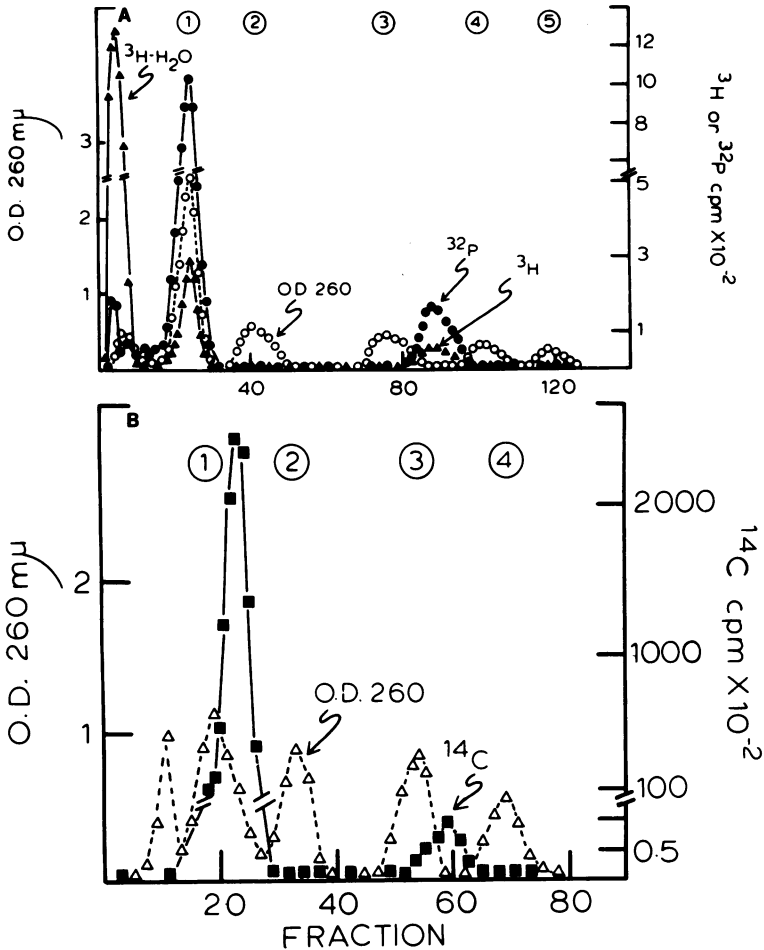

Fig. 2. *DEAE-cellulose column chromatography at pH 8.0 of nucleotides obtained after alkali digestion of labeled Sindbis vRNA. A mixture of (A) 4.8 × 10⁶ counts/min of [³H]adenosine and 5.2 × 10⁶ counts/min of ³²P-labeled Sindbis viral RNA was treated with alkali and resolved on DEAE-cellulose. After the mononucleotides had eluted, the column was stripped with high salt, and the eluted nucleotides were recovered and redigested with alkali (9). In (B) a single alkali digest of 1.1 × 10⁶ counts/min of [¹⁴C]guanosine-labeled Sindbis viral RNA was performed. Each digest was resolved by DEAE-cellulose column chromatography. Columns (5 ml, 0.5 by 25 cm) of DEAE-cellulose equilibrated in 7 M urea, 0.01 M Tris-hydrochloride, and 0.003 M EDTA (pH 8.0) were loaded with nucleotide mixtures (see below) and eluted with a 160-ml linear gradient of LiCl (0.03 to 0.35 M) in 7 M urea, 0.01 M Tris-hydrochloride, and 0.003 M EDTA (pH 8.0). Fractions (about 0.8 ml) were collected, and their content of unlabeled nucleotides (optical density at 260 nm) or labeled nucleotides was determined by counting in a liquid scintillation spectrometer after mixing with 15 ml of the Triton-toluene scintillation cocktail (17). Nucleotide mixtures consisted of a pancreatic RNase digest of unlabeled chicken embryo fibroblast RNA (1.5 mg of RNA, 0.15 mg of RNase in 0.05 M NaCl, pH 7.0, incubated at 37 C for 60 min) together with the labeled sample. The elution positions of the mono-, di-, (etc.) oligonucleotides from the pancreatic RNase digest are indicated.*

cleotides (Fig. 2A).

A preparation of [^{14}C]guanosine-labeled Sindbis viral RNA was similarly subjected to alkali treatment, and the digest was chromatographed on DEAE-cellulose (Fig. 2B). A peak of labeled material was observed eluting between the trinucleotides and tetranucleotides.

The recovery, in moles, of labeled phosphate or nucleoside in the alkali-resistant fragment per mole of original RNA was determined from these chromatograms after taking into account the tritium exchange with water during the alkali digestions and the base composition of the labeled RNA (9). The results are presented in Table 1. It was found that for the three ^{32}P-labeled digests an average of about 3.5 mol of phosphate per mol of viral RNA was recovered in the alkali-resistant nucleotides. No ^{3}H-labeled cytidine or uridine residues were recovered. However, 1.3 mol of [^{3}H]adenosine and 0.3 mol of [^{3}H]guanosine were recovered in the nucleotide. The amount of radioactivity found in the [^{14}C]guanosine-labeled structure represented 1.3 mol of guanosine residues per original RNA strand (containing 2,830 guanosine nucleotides). The low [^{3}H]guanosine labeling of the terminal fragment by comparison to the [^{14}C]guanosine results may be explained by the fact (see below) that 7-methylguanosine is present in the terminal structure. It has been suggested that an electrophilic attack on the N^{7} of guanine, such as occurs upon methylation, results in exchange of the ^{3}H at the 8-position of the precursor guanosine (20). Such an exchange could be the reason why the terminal fragment contained little radioactivity upon [^{3}H]guanosine labeling by comparison to the results observed using [^{14}C]guanosine.

A preparation of [^{14}C]uridine- and [methyl-^{3}H]methionine-labeled 42S viral RNA (purified as described for the [methyl-^{3}H]methionine 42S RNA shown in Fig. 1) was subjected to alkali digestion, and the proceeds were chromatographed as described in Fig. 2. After a second alkali digestion, the products, after resolution on DEAE-cellulose column chromatography, revealed a peak of ^{3}H-labeled material eluting between the tri- and tetranucleotides (see Fig. 2).

It was concluded from these results that an alkali-resistant nucleotide could be obtained from Sindbis viral RNA. This nucleotide probably contained adenosine and guanosine nucleotides, three to four phosphates, and a methylated component.

Identification of m^{7}G as part of the end sequence of Sindbis viral RNA. In identifying the methylated residue(s) of the viral RNA, we relied heavily on hydrolysis of methyl-labeled

TABLE 1. *Recovery of 5' nucleotides from alkali digests of labeled Sindbis RNA^{a}*

Labeled RNA	Counts/min recovered between tri- and tetra- nucleotides	Mole equivalent per mole of RNA
(1) [^{3}H]adenosine	360	1.3
[^{32}P]phosphate	1,618	3.3
(2) [^{3}H]uridine	23	0
[^{3}H]cytidine		
[^{32}P]phosphate	1,968	3.9
(3) [^{3}H]guanosine	60	0.3
[^{32}P]phosphate	1,100	3.4
(4) [^{14}C]guanosine	481	1.3

^{a} Mixtures of (1) 4.8 × 10^{6} counts/min of [^{3}H]-adenosine and 5.2 × 10^{6} counts/min of ^{32}P-labeled Sindbis viral RNA, (2) 1.3 × 10^{7} counts/min of [^{3}H]cytidine and [^{3}H]uridine plus 6.0 × 10^{6} counts/min of ^{32}P-labeled Sindbis viral RNA, (3) 1.8 × 10^{6} counts/min of [^{3}H]guanosine plus 4.6 × 10^{6} counts/min of ^{32}P-labeled Sindbis viral RNA, or (4) 1.1 × 10^{6} counts/min of [^{14}C]guanosine-labeled viral RNA were treated with two cycles of alkali digestion (Fig. 2), and the ^{3}H, ^{14}C, or ^{32}P counts per minute recovered between the trinucleotides and tetranucleotides were computed. The total nucleotides present in Sindbis RNA have been estimated by comparison to vesicular stomatitis virus RNA (Fig. 1) to be approximately 11,000, so that the count equivalent for 1 mol of phosphate obtainable from 1 mol of RNA could be calculated for each digest (taking into consideration the ^{32}P decay during the analysis time course). Likewise, on the basis that Sindbis RNA contains 3,050 adenosine, 2,420 uridine, 2,700 cytidine, and 2,830 guanosine nucleotides (see text), the count equivalent from 1 mol of adenosine, guanosine, cytidine, and uridine could be calculated after correction for the tritium exchange (48% for each alkali digest for adenosine; 40% for each alkali digest for guanosine). The recovery of each label is given in terms of the mole equivalent obtained from 1 mol of RNA, considering the percentage of label present in the various nucleotides (9). The distribution of ^{3}H label among the mononucleotides was: (1) 69.5% in adenylic acid, 28.2% in guanylic acid, 2.3% in cytidylic acid, and none in uridylic acid; (2) 49.2% in cytidylic acid, 45.1% in uridylic acid, 5.1% in guanylic acid, and 0.6% in adenylic acid; and (3) 97.8% in guanylic acid and 2.2% in adenylic acid.

RNA, or of isolated end groups therefrom, with 1 N HCl at 100 C (see Materials and Methods). This procedure releases mainly free purines, pyrimidine nucleotides, ribose, and inorganic phosphate (19) and is especially useful for detecting 7-methylguanine residues, since the degradation that tends to occur at alkaline or even neutral pH is precluded. There is little published information on the behavior of 2'-O-methyl sequences under these conditions. However, we have performed extensive studies using

as models methyl-labeled NmNp residues from BHK 28S rRNA (in which these are the predominant methylated constituents) and have found that our acid hydrolysis procedure releases >90% of the label as a mixture of methylated nucleotides plus ribose phosphate. These compounds are negatively charged at pH 3.5 and run in the region of Ap through pGp; on chromatography in an alkaline system ("4" of Littlefield and Dunn [10]), they remain at the origin (D. T. Dubin, R. H. Taylor, and R. Baer, manuscript in preparation).

A sample of [methyl-³H]methionine-labeled Sindbis 42S viral RNA (Fig. 1B) was mixed with 7-methylguanosine (50 nmol) unlabeled RNA (100 µg) and then subjected to acid hydrolysis, followed by paper electrophoresis at pH 3.5 (Fig. 3). By comparison to the markers derived from the hydrolysis (adenine and guanine or the pyrimidine nucleotides, cytidylic and uridylic acids) or the added pGp, the majority (71%) of the ³H labeled was recovered in a spot comigrating with 7-methylguanine (Fig. 3).

Minor amounts of label that migrated with adenine (9%), guanine (5%), and the nucleotide residues Cp (3%) or Up (5%) may represent the intracellular conversion of the methyl group of methionine into bases or other residues. They were not investigated further. A sample of [methyl-³H]methionine-labeled Sindbis viral

RNA was similarly digested with acid and then subjected to paper chromatography in system no. 4. In this system, as noted above, nucleotides and ribose phosphate remain exactly at the origin; guanine and methylguanine derivatives (such as 7-methylguanine) move as a group slightly off the origin, whereas adenine and methylated adenine derivatives move considerably further and are reasonably well resolved (3). About 84% of the label moved with the guanine derivatives and 10% with the adenine derivatives, and the rest of the label stayed at the origin. These results support the conclusion that most of the methyl residue in the 42S viral RNA is present in 7-methylguanine, little is present in adenine derivatives, and little, if any, resides in 2'-O-methyl ribose phosphate derivatives.

A sample of intact 42S [methyl-³H]methionine-labeled Sindbis viral RNA was subjected to periodate oxidation to disrupt the 2'-3' bonds of the terminal nucleosides and then treated with aniline (7) to eliminate these nucleosides (β elimination). It was found that 70% of the methionine-labeled material was released. In an equivalent experiment using [methyl-³H]-methionine-labeled 28S rRNA (which is known to contain internal methyl residues [D. Dubin, unpublished data]), less than 1% of the label was removed.
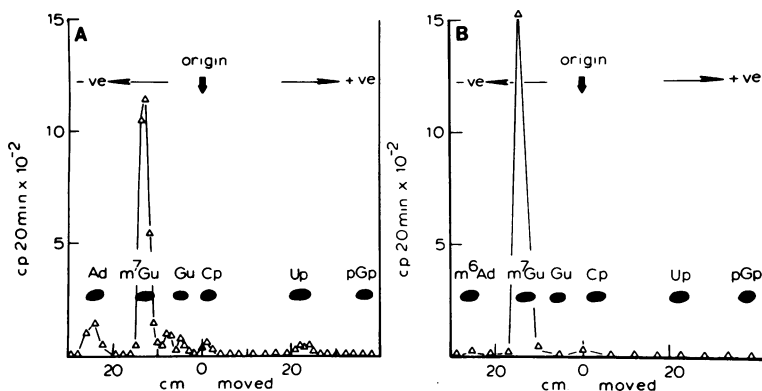
Finally, a sample of the [³H]methionine-

FIG. 3. *Electrophoretic analysis of acid hydrolysates of [methyl-³H]-methionine-labeled 42S Sindbis viral RNA and its 5' terminal nucleotide. In (A), a portion of the [methyl-³H]methionine-labeled 42S viral RNA (Fig. 1B) was subjected to acid hydrolysis (1 N HCl, 100 C, 45 min). After removal of the HCl by lyophilization, the residue was dissolved in 25 µl of 0.05 M ammonium formate buffer, pH 3.5, spotted at the center of a 110-cm length of Whatman no. 3 MM paper, and subjected to electrophoresis at 3,000 V for 3 h. For standards, 100 µg of unlabeled RNA and 50 nmol of 7-methylguanosine were added prior to hydrolysis, and 100 nmol of pGp was added after hydrolysis. The positions of the 7-methylguanine, guanine, adenine, cytidylic acid, uridylic acid, and pGp were identified by their UV absorption. The distribution of ³H label was determined. The recovery of label shown equalled that applied to the paper. In (B), a 5' nucleotide fragment obtained, as described in Fig. 4, by RNase T₂ digestion of [methyl-³H]methionine-labeled purified 42S Sindbis viral RNA was subjected to acid hydrolysis and similarly separated by electrophoresis. The positions of N⁶ adenine (N⁶ Ade) and other markers were identified by their UV absorption.*

labeled Sindbis viral RNA was treated with RNase T$_2$ and subjected to DEAE-cellulose column chromatography. Seventy-seven percent of the label was recovered with the trinucleotide isoplith, whereas 23% eluted with the mononucleotides (as expected from the results described above). The material eluting with the trinucleotides was recovered, and an aliquot was subjected to acid hydrolysis and resolved by electrophoresis at pH 3.5 (Fig. 3B). Ninety-two percent of the label was recovered as 7-methyl-guanine, less than 1% ran with adenine (or N^6-methyladenine), 5% of the label remained on the origin, and less than 2% migrated towards the anode. Since the recovered radioactivity accounted for essentially all of the label applied to the paper, these results indicate that there was only one methylated component in the terminus, namely, 7-methylguanosine. Treatment of another aliquot of the methyl-labeled end group with a mixture of alkaline phosphatase and venom phosphodiesterase (24) provided confirmatory results: 93% of the counts were released as 7-methylguanosine and <7% were associated with Nm residues.

We conclude that, when ^{3}H is incorporated into Sindbis 42S viral RNA from [methyl-^{3}H]methionine, the label is mostly present in a single methylated component (7-methylguanosine), possessing exposed 2' and 3' hydroxyl groups, and that in the terminus essentially all the label resides in 7-methylguanosine. Since Sindbis is believed to possess a 3'-polyadenosine sequence (6), and the alkali-resistant residue containing 7-methylguanosine possesses more phosphate groups than nucleotides, it seems reasonable to conclude that the methylated nucleotide resides at the 5' end of the viral RNA and that the structure is m^{7}G^5'p..., with no other methylated components in the 5' terminus.

Demonstration that the Sindbis terminus consists of m^{7}GpppAp. A preparation of [^{3}H]adenosine-labeled Sindbis viral RNA was mixed with ^{32}P-labeled Sindbis RNA and subjected to digestion by pancreatic RNase (to hydrolyze the RNA at Cp and Up residues) and RNase T$_1$ (to hydrolyze the RNA at Gp residues). The tetranucleotides were recovered (see Fig. 6 and 7). After RNase T$_2$ (used to hydrolyze the tetranucleotides at Ap residues), the material was chromatographed on DEAE-cellulose with an elution pattern similar to the one shown in Fig. 4. It was found that, other than the mononucleotides, the only labeled nucleotide recovered from the column eluted with the trinucleotides. The recoveries of moles of

[^{3}H]adenosine and [^{32}P]phosphate per mole of orginal RNA were 0.83 and 3.8, respectively. When this material was recovered and treated with alkali it eluted between the trinucleotides and tetranucleotides (Fig. 4, inset).

It is known that 7-methylguanosine is positively charged at the 7-position. Upon alkali treatment there can be a scission of the imidazole ring to give some 2-amino-4-hydroxy-5 (N - methyl)formamido - 6 - ribosyl - amine - aminopyrimidine (>m^{7}G), which lacks the positive charge at the N-methyl position. Consequently, with the removal of a positive charge by alkali treatment, a nucleotide that originally contained 7-methylguanosine would be expected to yield a structure with greater net negative charge. This concurs with the observations made in these experiments (Fig. 4).

The terminal [^{3}H]adenosine-, [^{32}P]phosphate-labeled nucleotide isolated by T$_2$ digestion, described above, was rechromatographed, and the indicated fractions were collected, treated with alkaline phosphatase, and then rechromatographed (Fig. 5). After the phosphatase treatment, three-quarters of the phosphate and all of the ^{3}H label were recovered in a single peak eluting just after the mononucleotides. The rest of the ^{32}P was recovered in a broad peak eluting just before the mononucleotides, as expected for free phosphate released by the enzyme treatment.

Taken together, all these results suggest that the terminal sequence of Sindbis viral RNA is m^{7}G^5'pppAp which, after isolation by RNase T$_2$ digestion, elutes with the trinucleotides (Fig. 4), but upon alkali treatment (giving >m^{7}G^5'pppAp, where >m^{7}G represents the broken guanine imidazole ring) the fragment elutes between the tri- and tetranucleotides (Fig. 2). The results are also consistent with the removal of only one phosphate by phosphatase treatment (to give m^{7}G^5'pppA or >m^{7}G^5'pppA, respectively).

Identification of the penultimate nucleotides. A preparation of ^{32}P-labeled Sindbis viral RNA was treated with pancreatic RNase, the digest was resolved by DEAE-cellulose chromatography (Fig. 6), and each indicated area of the chromatogram was recovered, treated with alkali, and rechromatographed to see which contained the 5' sequence (Fig. 6). It was found that only the tetranucleotides contained the sequence and, since it contained one charge greater than the T$_2$ nucleotide (Fig. 4 and 5), it was concluded that the pancreatic RNase-derived sequence was m^{7}G^5'pppApPyp.

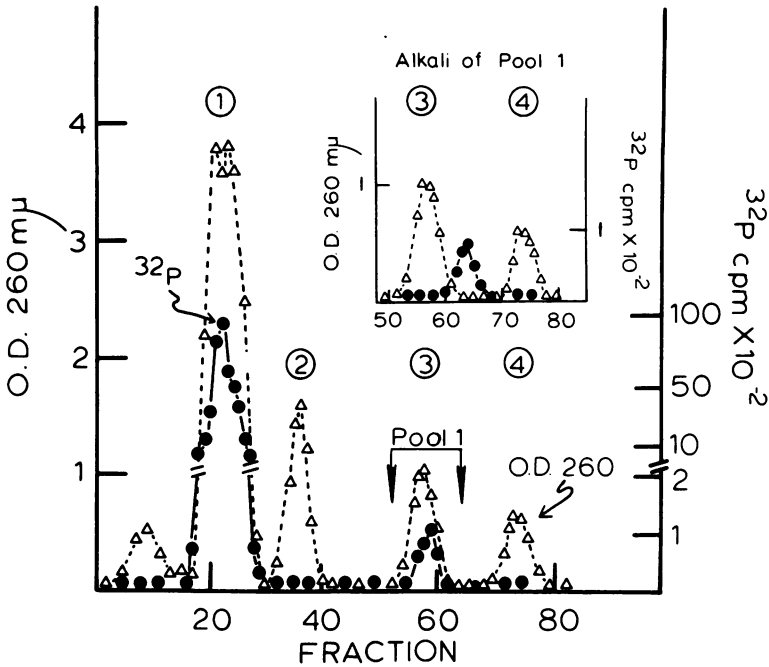Another preparation of ^{32}P-labeled Sindbis

FIG. 4. *DEAE-cellulose column chromatography of an RNase T_2 digest of tetranucleotides obtained from a pancreatic RNase and RNase T_2 digest of ^{32}P-labeled Sindbis RNA. ^{32}P-labeled Sindbis viral RNA was digested with pancreatic RNase as well as with RNase T_2, and the resulting nucleotides were resolved on DEAE-cellulose (15) at pH 8 (results not shown). The tetranucleotide fractions, which contained the 5' terminus (see Fig. 6), were pooled and subsequently digested by RNase T_2. This digest was chromatographed on a DEAE-cellulose column at pH 8.0 with a pancreatic RNase digest of chicken embryo fibroblast RNA. The 5' terminal nucleotide fragment was recovered, digested with alkali, and rechromatographed on DEAE-cellulose at pH 8.0 with another pancreatic RNase digest of chicken embryo fibroblast RNA (inset).*
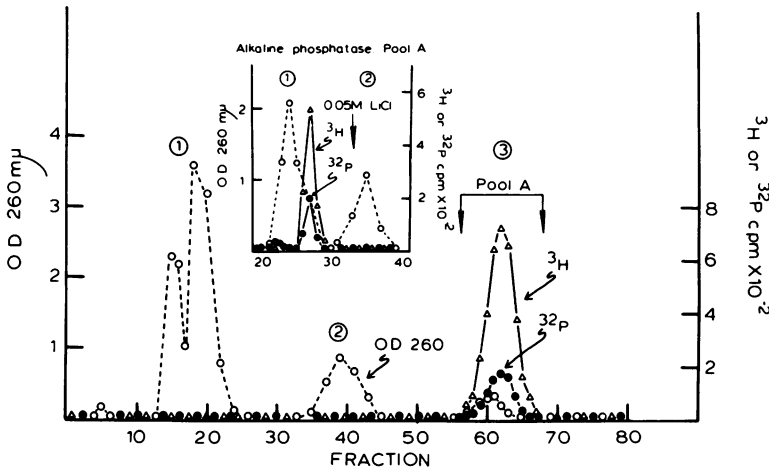
FIG. 5. *DEAE-cellulose column chromatography of the 5' terminal nucleotide before and after alkaline phosphatase treatment. An aliquot of the [^{32}P]phosphate- and [^3H]adenosine-labeled purified terminal fragment, derived by RNase T_2 digestion, was rechromatographed on DEAE-cellulose at pH 8.0. The indicated fractions, containing only the terminus, were pooled and treated with alkaline phosphatase as described in Materials and Methods and rechromatographed (inset).*
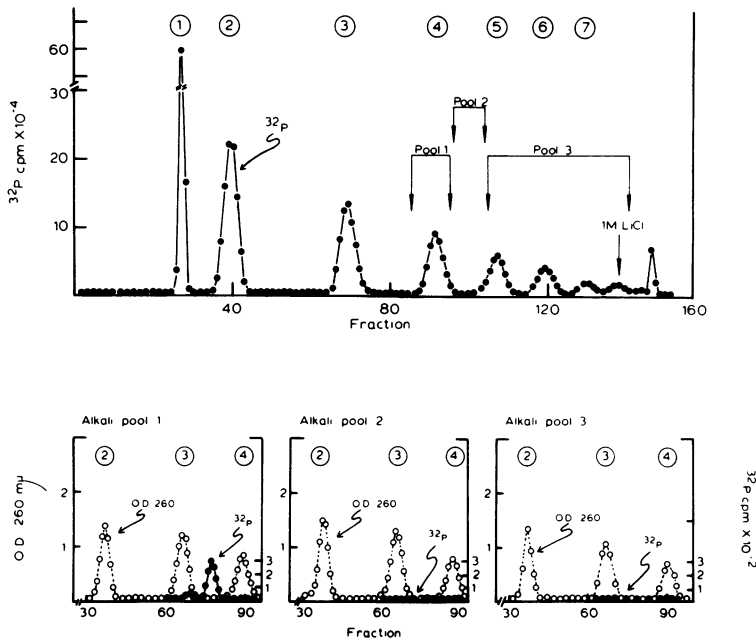
FIG. 6. Cellulose column chromatography of a pancreatic RNase digest of ^{32}P-labeled Sindbis RNA. ^{32}P-labeled Sindbis RNA (10^7 counts/min) plus 3 mg of chicken embryo fibroblast RNA were digested with pancreatic RNase and resolved by DEAE-cellulose column chromatography (upper). The indicated fractions were pooled, and the nucleotides were recovered and digested by alkali. These digests were then chromatographed at pH 8.0 with marker nucleotides derived from a pancreatic RNase digest of 1.5 mg of chicken embryo fibroblast RNA (lower). The recovery of ^{32}P in the trinucleotides resulting from the alkali digests of pool 1 was equivalent to 4.0 mol of phosphate per mol of original RNA.

viral RNA was treated with RNase T_1 and the digest was resolved by DEAE-cellulose column chromatography. As before, the designated nucleotide pools were recovered, and an aliquot of each was treated with alkali and rechromatographed to identify which contained the 5' fragment (Fig. 7). Only the pool 3 nucleotides were found to contain the terminus. When the rest of the pool 3 nucleotides was rechromatographed on DEAE-cellulose at pH 8.0 with a pancreatic RNase digest of chicken embryo fibroblast RNA for marker nucleotides, it was found that the labeled material eluted in a position corresponding in charge to the pentanucleotides of the pancreatic digest of chicken embryo fibroblast RNA. Since this was one charge greater than the pancreatic RNase-derived sequence (Fig. 6), it was concluded that the sequence of the fragment was m^{7}G$^{5'}$pppAp-PypGp.

To identify the pyrimidine nucleotide and confirm the presence of 7-methylguanosine, a sample of purified ^{32}P-labeled Sindbis viral RNA was digested with pancreatic RNase, the tetranucleotides were recovered and redigested with RNase T_1, and the residual tetranucleo-

tides were again recovered. It was expected that these tetranucleotides would contain the sequences ApApApCp, ApApApUp, as well as the Sindbis 5' nucleotide (m^{7}G$^{5'}$pppApPyp). This was confirmed by both alkali digestion (see Fig. 2) and by DEAE-cellulose column chromatography at pH 3.5 (which separated ApApApCp from ApApApUp; data not shown). Since 7-methylguanosine possesses a pK of 7.1, the tetranucleotides were resolved (together with optical quantities of a pancreatic RNase digest of chicken embryo fibroblast RNA), using DEAE-cellulose and a gradient of 0.05 to 0.30 M LiCl in 7 M urea, 0.01 M Tris-hydrochloride buffer (pH 6.0). A small peak of radioactivity was eluted after the trinucleotides but before the tetranucleotides (Fig. 8). Alkali analysis indicated that this material contained only the 5' terminal fragment. The purified terminal nucleotide was then subjected to the following analyses.

An aliquot of 900 counts/min of terminus was digested with alkali to release the pyrimidine nucleotide. After chromatography on Dowex-1-formate, together with optical quantities of marker mononucleotides, 164, 0, 39, and 4
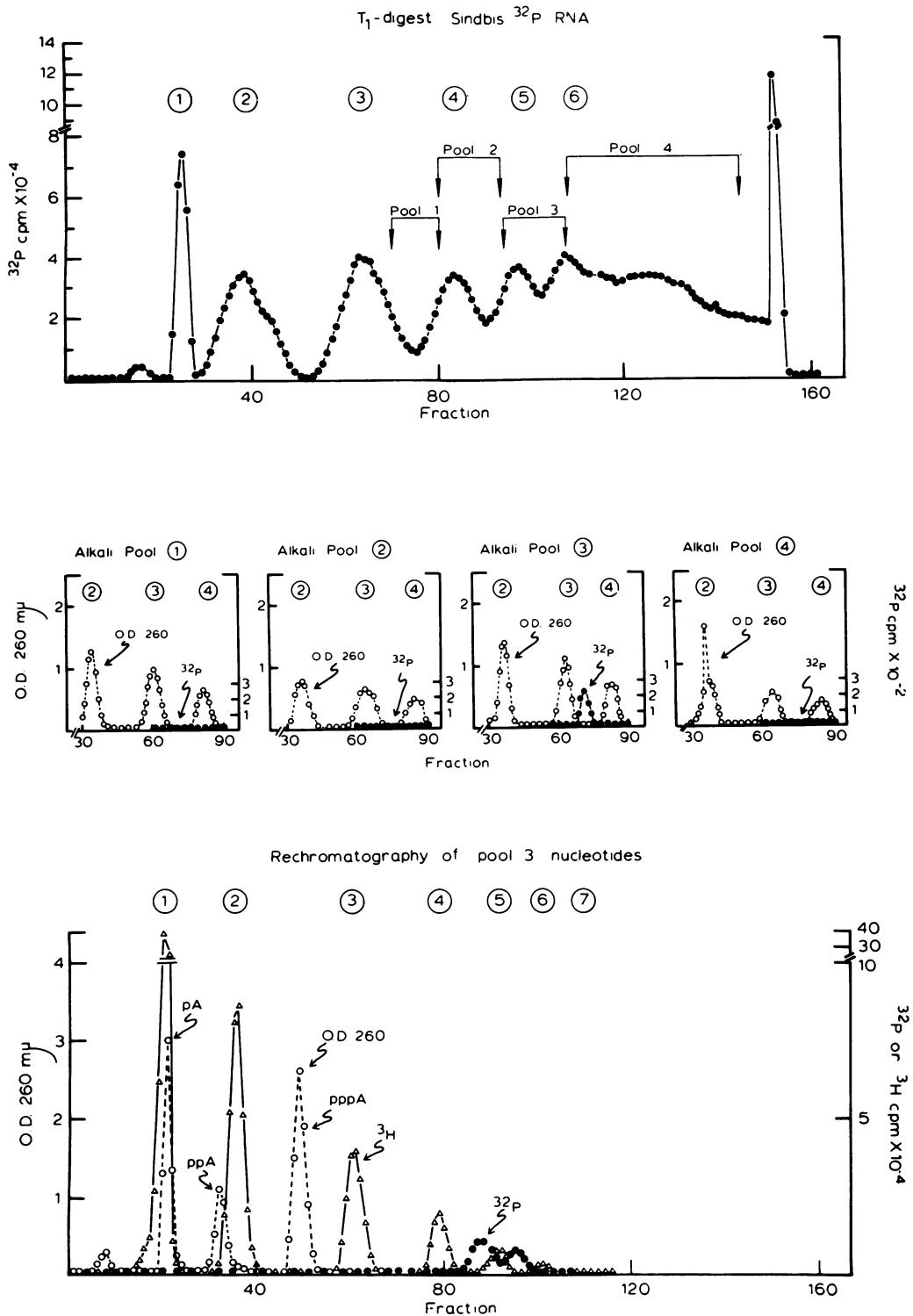
FIG. 7. *DEAE-cellulose column chromatography of an RNase T_1 digest of ^{32}P-labeled Sindbis RNA. A sample of ^{32}P-labeled Sindbis RNA (3.2×10^6 counts/min) was digested by RNase T_2, and the products were resolved by DEAE-cellulose column chromatography at pH 8.0 (upper). The indicated fractions were pooled, and a portion was digested with alkali and chromatographed with marker nucleotides derived from a pancreatic RNase digestion of chicken embryo fibroblast RNA (middle). An aliquot of the pool 3 nucleotides (containing the terminal nucleotide and other nucleotides) was rechromatographed on DEAE at pH 8.0 with a pancreatic RNase digest of chicken embryo fibroblast RNA, as well as with adenosine mono- di-, and triphosphates as optical markers (bottom).*
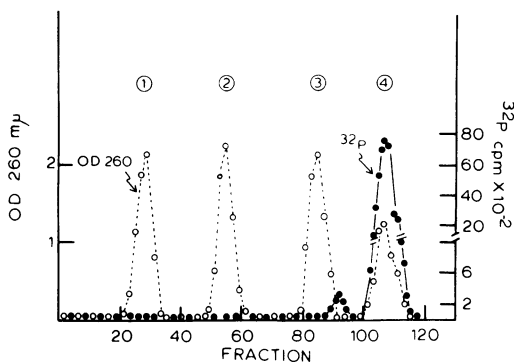
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FIG. 8. *DEAE-cellulose column chromatography at pH 6.0 of tetranucleotides obtained from a pancreatic RNase digest and RNase T_1 digest of ^{32}P-labeled Sindbis RNA. A sample of ^{32}P-labeled Sindbis viral RNA was digested with pancreatic RNase, the tetranucleotides were recovered and redigested with RNase T_1, and the residual tetranucleotides were again recovered. These tetranucleotides, containing the sequences ApApApCp, ApApApUp as well as the Sindbis 5' nucleotide, were rechromatographed on DEAE-cellulose columns equilibrated in 7 M urea buffer, adjusted to pH 6.0. The nucleotides were eluted with a gradient of 0.05 to 0.30 M LiCl in the same buffer.*

counts/min were recovered in Up, Cp, Ap, and Gp, respectively. The rest of the label (presumably m⁷G⁵'pppAp) remained bound to the column and was not analyzed further.

A second aliquot was treated with venom phosphodiesterase, and the proceeds were resolved by paper electrophoresis at pH 3.5 with 7-methylguanosine 5'-monophosphate (pm⁷G) and other markers. Thirty-two counts/min were recovered in the position of pm⁷G, 193 counts/min were recovered in a spot possessing an electrophoretic mobility between that of Gp and Up (presumably ppApUp), and 97 counts/min were recovered as free phosphate, indicating that some phosphomonoesterase activity was present in the diesterase preparation. Although this result did positively identify 7-methyl-guanosine 5'-monophosphate, the monoesterase activity precluded a determination of the molar yield of guanosine nucleotide from the terminus.

A third aliquot of the terminus was treated with venom phosphodiesterase and alkaline phosphatase and then resolved by paper electrophoresis. All the label was recovered as free phosphate, indicating that the cooperative action of both enzymes solubilized all label from the nucleotide.

These results are consistent with the interpretation that the terminus of Sindbis viral RNA is m⁷G⁵'pppApUp . . .

DISCUSSION

The base ratio we have obtained for Sindbis viral RNA, as well as the observed molecular weight as determined by polyacrylamide gel electrophoresis, are in reasonable agreement with results reported by others (14, 21, 22).

Evidence has been presented indicating that the 42S viral RNA of the togavirus Sindbis is capped, methylated, and possesses the sequence m⁷G⁵'pppApUpGp . . . In consideration of the possibility that the sequence could be m⁷GppAmpApUpGp . . ., the following observations can be made. First, in the alkali hydrolyses of the [³H]nucleoside-labeled viruses, there was greater than 1 mol of adenosine in the nucleotide per 4 mol of phosphate. Although this would tend to support the idea of two adenosine nucleotides per terminus (as in m⁷GppAmpAp), the calculation of ³H recovery is jeopardized by the sizable correction factors of tritium exchange with water during the hydrolyses (Table 1). Second, neither the data on the recovery of phosphate (3 to 4 mol/terminus), nor the phosphatase release of one-quarter of that phosphate, argue for or against the two possible sequences (after phosphatase, m⁷GpppAp would give m⁷GpppA + P, whereas m⁷GppAmpAp would give m⁷GppAmpA + P). Third, in the RNase T_2 digest of the tetranucleotides obtained from an RNase ribonuclease T_1 and pancreatic RNase digest of [³H]adenosine- and [³²P]phosphate-labeled Sindbis viral RNA, it was calculated that there was, per mole of original RNA, 0.83 mol of adenosine per 3.8 mol of phosphate recovered in the terminus. Since no tritium exchange with water was detected in these digests (unlike the alkali treatment), we consider these molar ratios more reliable than those of the alkali digests reported in Fig. 2 and Table 1. Fourth, all the evidence obtained with [*methyl*-³H]methionine-labeled RNA and its 5' terminus supports the contention that there is only one methyl residue in the 5' terminus of Sindbis viral RNA and that this methyl group resides in a 7-methylguanosine residue. Fifth, after acid hydrolysis of the [³H]adenosine-labeled fragment derived by RNase T_2 digestion (see Fig. 5), when the hydrolysate was chromatographed in the acid or alkaline systems of Littlefield and Dunn (10), the label chromatographed as adenine and not as any known methyl adenine derivatives (data not shown). Consequently, we propose that the 5' sequence of Sindbis viral RNA is m⁷G⁵'pppApUpGp . . .

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