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Determination of the Molecular Weight of Animal RNA Viral Genomes by Nuclease Digestions

I. Vesicular Stomatitis Virus and Its Defective T Particle

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A procedure has been developed for the determination of the weight of animal RNA virus genomes using controlled nuclease digestions and computation of the moles of oligonucleotides obtained from 1 mol of RNA. Using both pancreatic RNase and RNase T_1 to digest viral RNA labeled by ^{3}H-uridine, ^{3}H-cytidine, or ^{3}H-guanosine, the weight of the virion RNA of vesicular stomatitis virus (VSV) is estimated as $3.82 \pm 0.14 \times 10^6$ whereas that of the VSV-defective T particle is estimated as $1.23 \pm 0.04 \times 10^6$.

A variety of procedures are available for determining the molecular weight of an RNA, including sedimentation, electrophoresis, end group to total nucleotide analyses, as well as complete sequencing of the molecule (2, 8, 11, 12, 18). Apart from sequencing, the other available methods suffer from technical or inherent difficulties which give a degree of inaccuracy to any one determination. For example, sedimentation measures the average *S* value of the population of molecules so that if the RNA preparation contains a significant proportion of nicked strands, then the true *S* value is underestimated. Polyacrylamide gel electrophoresis is a valuable tool for estimating the apparent molecular weight of an RNA molecule, and a decision on the molecular weight of an unbroken molecule is not influenced by the presence of nicked molecules in the population, provided that not all the molecules are degraded (7, 11, 12). However, it has been shown that certain RNA molecules do not exhibit the expected electrophoretic mobility (for instance rRNA), presumably due to secondary structure configurations (2, 11). Secondary structure also influences sedimentation constants, and the effect of monovalent or divalent cation concentrations on both sedimentation and electrophoretic mobilities complicate the reliability of interpreting the results obtained.

End group to total nucleotide analyses involve labeling either the 3′ terminal nucleoside after oxidation, and then reduction with ^{3}H-borohydride, or enzymatically phosphorylating the 5′ terminal nucleotide with ^{32}P-γ-ATP of known specific activity (8). For dependable results, both methods need to be executed quantitatively and specifically and can be confounded by the presence of significant quantities (on a molar basis) of smaller molecules (whether contaminants or degradation products initially present or derived during the procedures).

Although entire sequencing of a molecule will give the molecular weight of an RNA, such sequencing with most RNA species, without enormous effort, is relatively unrealistic except (8, 18) for RNA molecules of small size (tRNA, 5*S* RNA), or where specific labeling techniques can be used (e.g., with the in vitro synthesis of Qβ RNA by Qβ replicase).

To obtain reliable molecular weight estimates of large RNA molecules, such as those of animal viruses, we have adapted simplified techniques of RNA sequencing to determining molecular weights. The procedure involves labeling in vivo the viral RNA by a specific nucleoside, isolating the intact RNA, and performing controlled nuclease digestions to obtain an essentially complete digest. After separation of the resulting oligonucleotides by DEAE chromatography in 7 M urea, the molecular weight of the original RNA can be calculated on the assumption that 1 mol of RNA after digestion yields 1 mol of any particular oligonucleotide. The results for the molecular weight of the RNA isolated from the

complete B particle of vesicular stomatitis virus (VSV-1) and that of the VSV-defective T particle (VSV-111) are presented and are in excellent agreement with analyses obtained by other techniques. The application of these techniques to other viral RNA molecules to determine molecular weights and homology is discussed.

MATERIALS AND METHODS

Preparation of ^{3}H-labeled virus, purification, and extraction of viral RNA. The purification of ^{3}H-labeled virus through polyethylene glycol (PEG) precipitation and equilibrium and velocity centrifugation has been described (1, 16). The preparation of B particles was performed using a cloned stock of wild-type virus and gave no detectable T particles. Preparation of T particles was performed with a stock of virus which had been passaged twice at high multiplicity of infection and gave approximately equal proportions of B and T particles. Virus, harvested from equilibrium or velocity gradients, was dialyzed against 0.15 M NaCl, 0.01 M Tris-hydrochloride, pH 7.4, to remove sucrose, and extracted for RNA. The extraction procedure involved addition of sodium dodecyl sulfate (SDS) (to 1% wt/vol), NaCl (to 0.4 M) and 1 vol of phenol-*m*-cresol-8-hydroxyquinoline mixture (500 g:70 g:0.5 g; previously saturated with 0.15 M NaCl, 0.01 M Tris-hydrochloride, pH 7.4). After shaking at room temperature for 2 min, the phases were separated by centrifugation and the aqueous phase was re-extracted by the phenol mixture. The second aqueous phase was adjusted to 70% (vol/vol) ethanol and stored at −20 C overnight. RNA was collected by centrifugation at 10,000 rpm by use of a Sorvall HB4 swinging-bucket rotor and siliconized Corex centrifuge tubes, and then dissolved in 0.5 ml of 0.4 M NaCl, 0.01 M Tris-hydrochloride buffer, pH 7.4, 0.1% (wt/vol) SDS, and chromatographed through a column (90 by 1 cm) of 4% agarose and eluted in the same buffer. The void fractions, containing VSV-1 viral RNA, or including fractions containing VSV-111 RNA, were pooled and precipitated for 4 h at −20 C with ethanol. The viral RNA was collected by centrifugation as before, and the pellet was drained, dried, and then dissolved in 0.001 M EDTA, pH 7.4. Samples containing between 10^6 and 6×10^6 counts/min were stored frozen at −20 C until required. The specific activity, distribution of label among the nucleotides, and homogeneity—as monitored by polyacrylamide gel electrophoresis—were determined for each RNA preparation (see Results).

Nuclease digestions, separation of oligonucleotides by DEAE column chromatography in 7 M urea. Pancreatic RNase (RNase A, 3,300 U/mg, Worthington Biochemical Corp., Freehold, N. J.) was suspended in 0.001 M EDTA, pH 7.4, at a concentration of 1 mg/ml (assuming an $E_{1\,cm}^{1\%}$ at 280 nm of 7.3), and stored in samples at −20 C. RNase T_1 (Calbiochem, San Diego, Calif.) was suspended in 0.001 M EDTA, pH 7.4, at a concentration of 5,000 U/ml and stored at −20 C. Samples of VSV-labeled RNA were mixed with *Escherichia coli* bulk RNA, and nuclease digestion was performed at RNA-to-nuclease ratios of 400 μg of RNA to 10 or 20 μg of pancreatic RNase A and 200 U of RNase T_1 in a solution of 0.3 ml of 0.10 M NaCl, 0.005 M Tris-hydrochloride, 0.001 M EDTA, pH 7.4, for 30 min at 37 C. Under these conditions, greater than 99% of the RNA was rendered trichloroacetic acid soluble. To reduce the number of cyclic nucleotides (18), the digest was then mixed with one-tenth vol of 1 M HCl, incubated at 37 C for 60 min, and readjusted to pH 8.0 by the addition of one-tenth vol of 1 M NaOH and 3 ml of 7 M urea, 0.01 M Tris-hydrochloride, 0.003 M EDTA, pH 8.0. The digest was then loaded on a 45-cm column of DEAE-cellulose (Schleicher & Schuell, type 20, lot number 1773) prepared in a 10-ml burette as described previously (3, 17, 20). Nucleotides were eluted by a gradient of 0 to 0.22 M LiCl in urea buffer (7 M urea, 0.01 M Tris-hydrochloride, 0.003 M EDTA, pH 8.0). The gradient was formed by use of three chambers of a Buchler Varigrad (Buchler Instrument Inc., Fort Lee, N.J.), in which the outer and middle chambers each contained 100 ml of 0.22 M LiCl in urea buffer, and the inner, feeder chamber contained 100 ml of urea buffer lacking added LiCl. Since consecutive oligonucleotide isopleths, after the dinucleotides, elute from the DEAE at decreasingly smaller increments of salt (20), this gradient was designed to maximize the resolution between the larger oligonucleotides. After the gradient was exhausted, the column was finally eluted with a solution of 1 M LiCl in urea buffer to recover any large, uneluted oligonucleotides or incompletely digested sequences. Fractions (36 drops, approximately 0.8 ml) from the column were collected directly into scintillation vials to avoid transfer losses. Vials were filled with scintillation cocktail (15 to 20 ml of 25% [vol/vol] Triton X-100 in toluene containing 0.13% BBOT [2,5-bis-2-(5-*tert*-butylbenzoxazolyl)-thiophene]), and the mixture was shaken until completely in solution (17). Using a ^{3}H standard and internal standardization techniques, there was approximately 10% quenching of the ^{3}H label under these conditions, provided the sample was completely in solution, but no detectable difference in quenching for nucleotides which eluted in the lower or higher portions of the gradient.

Preparation of ^{32}P-labeled VSV RNA, determination of RNA base ratios. Virus was grown in BHK monolayers in the presence of 20 μCi of ^{32}P-phosphoric acid per ml of Eagle medium. After 36 h of growth at 37 C, the virus was purified through PEG and equilibrium gradient centrifugations and the RNA was extracted as described above. Base ratios of ^{3}H- or ^{32}P-labeled VSV RNA were determined after digestion in 0.3 M KOH at 37 C for 18 h by subsequent paper electrophoresis at pH 3.5, as described previously (3, 17, 18).

RESULTS

RNA specific activity and the efficiency of labeling viral RNA by ^{3}H-nucleosides. Vesicular stomatitis virus was grown in BHK monolayers for 48 h after infection at an added multiplicity of infection of approximately 0.1

PFU per cell. When ^{3}H-guanosine (20 μCi/ml, 23 Ci/mmol) was included in the growth media, the RNA extracted from purified virions was found to possess a specific activity of 3×10^8 counts per min per mg of RNA. After alkali hydrolysis, 99.0% of the label was recovered in ^{3}H-GMP, 1% was recovered in ^{3}H-AMP, and none was recovered in CMP or UMP.

When VSV was similarly grown for 48 h in the presence of ^{3}H-cytidine (20 μCi/ml, 22 Ci/mmol) and the virion RNA was similarly extracted and analyzed, the viral RNA was found to possess a specific activity of 8×10^8 counts per min per mg of RNA and to contain 13% of its label in ^{3}H-UMP, 87% in ^{3}H-CMP, and none in AMP or GMP. It was found that, if the virus was grown for 24 h in the presence of ^{3}H-cytidine (20 μCi/ml) and the virion RNA was extracted and analyzed, 7% of the label was recovered in ^{3}H-UMP, 93% was recovered in ^{3}H-CMP, none was recovered in AMP or GMP, and the RNA had a specific activity of 6×10^8 counts per min per mg of RNA.

Growth of VSV for 24 h in the presence of ^{3}H-uridine (2 μCi/ml, 21 Ci/mmol) gave a viral RNA possessing a specific activity of 4.4×10^7 counts per min per mg of RNA having 79% of its label in ^{3}H-UMP, 21% in ^{3}H-CMP, but none in GMP or AMP. When the virus was grown for a longer period, the distribution of label was approximately the same. To reduce the amount of label in ^{3}H-CMP in the viral RNA, two methods of labeling with ^{3}H-uridine were investigated: addition of label at different times after the initiation of virus infection, or inclusion of unlabeled cytidine in the growth medium. As shown in Fig. 1, when ^{3}H-uridine was added to cells infected at a multiplicity of infection of approximately 0.1 PFU/cell and virus was harvested 24 h after the initiation of infection, not only was the recovery of ^{3}H-CMP reduced but also the specific activity of the viral RNA decreased fivefold. Addition of various concentrations of unlabeled cytidine together with the ^{3}H-uridine at the time of cell infection also caused a reduction in both the recovery of ^{3}H-CMP and VSV RNA specific activity (Fig. 1). Comparison of the two experiments revealed that, in the latter experiment, a twofold-higher, RNA specific activity was obtained under conditions in which the viral RNA in either case contained 4% of its label in ^{3}H-CMP. Although it was desirable to have no other labeled nucleotides than UMP in the viral RNA, we observed that to obtain this result would require using 0.1 mM cytidine in the growth media, with the result that the RNA would have had a lower specific activity. This, in turn, meant that the

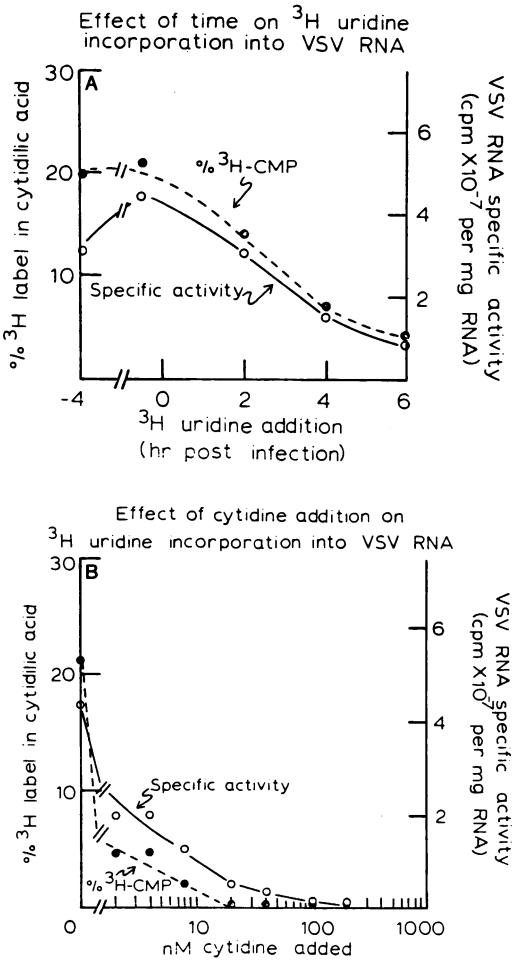

FIG. 1. *Specific activity of VSV RNA. Confluent monolayers of BHK/21 cells in 32-ounce prescription bottles were infected at a multiplicity of infection of 0.1 PFU/cell and virus was harvested from the supernatant fluids 24 h later. A, The precursor nucleoside (^{3}H-uridine, 2 μCi/ml, 50 ml of medium per bottle) was added before or at various times postinfection. B, The ^{3}H-uridine was added at the time of infection but in the presence of various amounts of unlabeled cytidine. Purified virus was extracted for RNA, and the RNA specific activity and percentage of label in ^{3}H-CMP were determined as described in the text.*

nuclease digestions and column analyses would utilize greater masses of viral RNA. It was decided that a 2 to 4% recovery of ^{3}H-uridine label in ^{3}H-CMP was acceptable, and consequently 0.008 mM cytidine was incorporated in the growth media.

To obtain higher VSV RNA specific activities, the amount of ^{3}H-uridine per ml of growth medium was increased from 2 to 20 μCi per ml (i.e., to 0.001 mM ^{3}H-uridine) in the presence of

0.008 mM or 0.08 mM unlabeled cytidine. The viral RNA specific activities obtained were 1.3 $\times 10^8$ and 7 $\times 10^7$ counts per min per mg of RNA, respectively, and the percentage of ^{3}H-CMP was 2 and 0%, respectively. When the growth period was then increased from 24 to 48 h in the presence of 0.008 mM unlabeled cytidine, the viral RNA specific activity was found to be 6 $\times 10^8$ counts per min per mg of RNA with 4% of the label in ^{3}H-CMP.

It was noted that, for the 24-h growth periods with a 10-fold increase in ^{3}H precursor, the RNA specific activity also increased 10-fold. When the uridine specific activity in the viral RNA was compared to that of the nucleoside precursor (in the conditions in which ^{3}H-uridine was present at 2 μCi/ml), it was calculated that the uridine in the RNA prossessed approximately $\frac{1}{2,000}$ the specific activity of the precursor nucleoside (assuming that there are about 3,600 uridine residues per 1 g mol of RNA [weighing approximately 4 $\times 10^6$ g], and that ^{3}H was counted in a scintillation counter at about 65% efficiency). Therefore, when the amount of added precursor was increased from 2 to 20 μCi/ml, it was not unreasonable to expect that the specific activity of the viral RNA could increase, reflecting a greater uptake of precursor by the cells.

RNase digestion conditions. The conditions for RNA digestion by RNases were determined so that, as far as possible, the RNA was neither underdigested nor overdigested. Underdigestion is defined as the incomplete release of a particular oligonucleotide from a population of RNA molecules. Overdigestion is defined as the secondary hydrolysis of a particular oligonucleotide at nucleotide residues which are not the prime target of the particular nuclease. For example if an RNA contains the sequence ... ApCpApApApApUpGp ... and is digested by pancreatic RNase (hydrolyzing at Cp and Up residues) and RNase T_1 (hydrolyzing at Gp residues), one should obtain 1 mol of the oligonucleotide ApApApApApUp from 1 mol of RNA, assuming that there is only one ApApApApApUp sequence in the RNA molecule. Overdigestion would result in the hydrolysis of the ApApApApApUp sequence (for instance to ApApAp and ApApUp, or other nucleotides). If this happened to half of the excised ApApApApApUp oligonucleotides, then the estimation of the molecular weight of the viral RNA on the basis of the observed hexanucleotide recovery would be twice the actual molecular weight. Clearly for the purposes of this investigation, the possibility of overdigestion was a prime concern to the experimental procedure and validity of the computations.

Minor amounts of underdigestion of RNA were not necessarily a cause for concern and it was found that a certain degree of underdigestion was tolerable (see Discussion). Gross underdigestion was not, however, acceptable. Two criteria of gross underdigestion were investigated—the rendition of RNA into acid-soluble nucleotides and the elution of underdigested sequences from the DEAE chromatograms by a 1 M LiCl wash. In all the experiments reported here, greater than 99% of the initial RNA was rendered acid soluble by the nuclease digestion conditions employed, and less than 0.01% of the original label was eluted from the DEAE chromatograms by the high-salt wash. Greater than 90% of the initial labeled RNA was recovered from the DEAE chromatograms in acid-soluble nucleotides—as calculated from the sum of the counts per minute of the eluted nucleotides. However, the counting efficiency of ^{3}H in the scintillation cocktail, containing Triton-BBOT-toluene and the 0.8 ml of 7 M urea buffer, was approximately 90% that of ^{3}H-labeled RNA when it is acid precipitated on a membrane filter and counted in 0.13% BBOT-toluene. Therefore, we concluded that essentially all the initial RNA was eluted from the columns as oligonucleotides and that gross underdigestion was not a problem under the conditions employed.

The nuclease digestion conditions which would lead to overdigestion were investigated in the following manner. A preparation of ^{3}H-cytidine-labeled VSV RNA (8 $\times 10^6$ counts/min) was hydrolyzed by pancreatic RNase (400 μg of RNA to 10 μg of RNase) and RNase T_1 (200 U, see Materials and Methods), and the oligonucleotides were resolved by DEAE chromatography. The fractions containing labeled pentanucleotides were pooled and precipitated as their barium salts from 70% (vol/vol) ethanol, and the nucleotides were converted to their sodium salts as described previously (17). Samples of the purified labeled pentanucleotides (^{3}H-ApApApApCp) were mixed with 400 μg of *E. coli* carrier RNA and incubated with 200 U of RNase T_1 and various amounts of pancreatic RNase for 30 min at 37 C, and then again resolved by DEAE column chromatography. The ratios of RNA to pancreatic RNase which were investigated were 400 to 10, 400 to 20, 400 to 40, and 400 to 54. A control experiment of ^{3}H-pentanucleotides, which were mixed with a digest of *E. coli* RNA and immediately subjected to chromatography, was also performed. The results of this latter control column (Fig. 2) indicated that the ^{3}H-pentanucleotides were essentially undegraded by the prior purification procedure or subsequent column chromatogra-

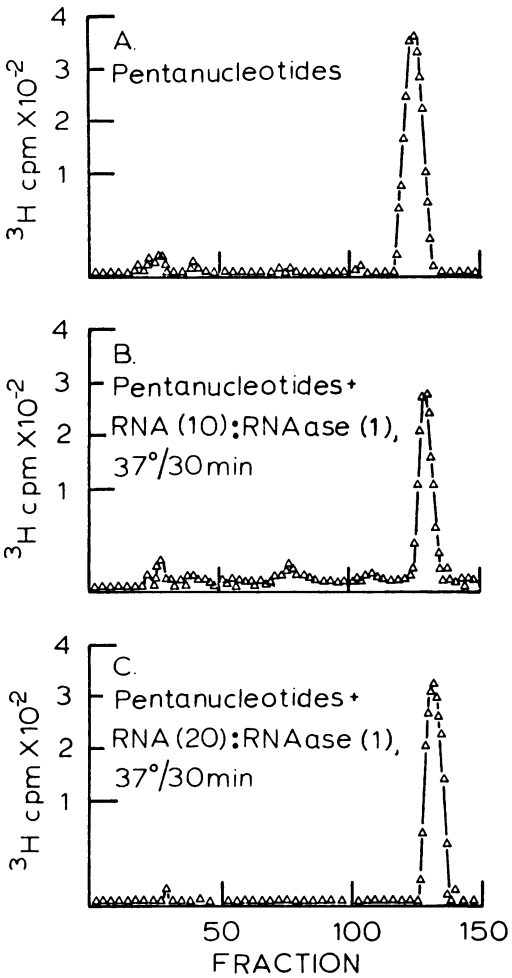

FIG. 2. *DEAE chromatography of ^{3}H-labeled pentanucleotides before or after RNase digestion. A nuclease digest of ^{3}H-cytidine-labeled VSV-1 RNA was chromatographed as described in Fig. 4, and the labeled pentanucleotides were collected, precipitated from alcohol as their barium salts, and converted to their sodium form as described previously (17). A sample of these labeled nucleotides was mixed with a pancreatic RNase and RNase T_1 digest of E. coli RNA and rechromatographed (A). Or the ^{3}H-pentanucleotides were mixed with E. coli RNA and digested at an RNA to RNase A ratio of 400 μg to 40 μg (B), respectively, or 400 μg to 20 μg (C), respectively, each in the presence of 200 U of RNase T_1. After incubation at 37 C for 30 min the digests were chromatographed on a 5-ml column DEAE as described in Materials and Methods except that a 200-ml linear gradient of 0 to 0.22 M LiCl in urea buffer was used. Column fractions were collected and counted for content of radioactivity.*

phy. Furthermore, the ^{3}H-pentanucleotides were recovered as a single peak from the column, indicating that other (unlabeled) nucleotides did not cause the premature elution of pentanucleotides into the mono-, di-, tri-, or tetranucleotide isopleths. A comparison of the effect of incubating ^{3}H-pentanucleotides with a constant amount of RNase T_1 and various amounts of pancreatic RNase indicated that, at the higher concentrations of pancreatic RNase (i.e., RNA-to-RNase A ratios of 400 to 40 or 400 to 54), there was hydrolysis of the labeled pentanucleotides (Fig. 2). It was noted from the distribution of ^{3}H label that the hydrolysis occurred principally between adenosine residues and not just between the adenosine and cytidine residues (to give ^{3}H-CMP). No detectable hydrolysis was observed at the lower RNase concentrations, indicating that either a 20 to 1 or 40 to 1 ratio of RNA to pancreatic RNase was suitable for avoiding overdigestion. These results also indicated that the pancreatic RNase preparation was the cause of hydrolysis of nucleotides at adenosine residues, although whether this activity was inherent to the enzyme preparation or due to a contaminating activity is not known.

Wherever possible, therefore, a 40 to 1 ratio of RNA to pancreatic RNase was employed for the subsequent analyses.

Nuclease analyses of ^{3}H-nucleoside-labeled VSV-1 RNA. Preparations of VSV-1 RNA, labeled by ^{3}H-guanosine, ^{3}H-uridine, or ^{3}H-cytidine were obtained as described in Materials and Methods and analyzed to determine the distribution of label between the nucleotides of the RNA and RNA homogeneity as determined by polyacrylamide gel electrophoresis. For all three preparations, greater than 95% of the label was recovered in a single RNA band on gel electrophoresis (2, 4). Each RNA was subjected to nuclease digestion (see figure legends), and the oligonucleotides were resolved by DEAE column chromatography (Fig. 3–5).

^{3}H-uridine-labeled VSV-1 RNA. The preparation of ^{3}H-uridine-labeled VSV-1 RNA was subjected to combined RNase T_1 and pancreatic RNase digestion at two different concentrations of pancreatic RNase (RNA to pancreatic RNase ratios of 400 to 20, Fig. 3A; or 400 to 10, Fig. 3B). For either concentration, the total radioactivity recovered from the columns and the amount of label eluted by 1 M LiCl was the same. At the higher pancreatic RNase concentration (Fig. 3A), seven major peaks of nucleotides were identified corresponding to the mononucleotide Up, dinucleotide ApUp, etc., to the septanucleotide peak, ApApApApApApUp. A peak of label was recovered eluting before the mononucleotides and corresponded to either uridine or, more probably, residual 2′,3′ cyclic UMP (Up!). Another peak of nucleotides eluted

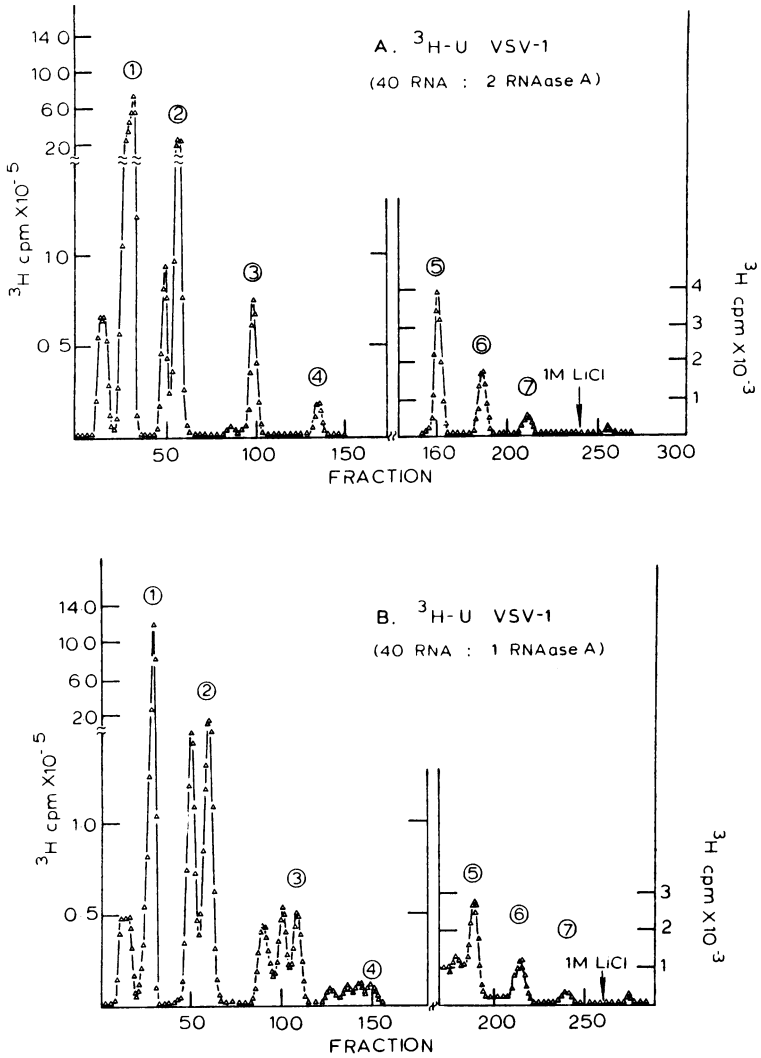

FIG. 3. *DEAE chromatography of RNase A and T_1 digests of ^{3}H-uridine-labeled VSV-1 RNA. VSV B particles (VSV-1) were obtained from cells grown for 48 h at 37 C in the presence of ^{3}H-uridine and 0.008 mM unlabeled cytidine. The viral RNA was extracted and chromatographed through 4% agarose. The void fractions containing VSV-1 RNA were precipitated with alcohol, and the base ratio of label in the RNA was determined (96% ^{3}H-UMP, 4% ^{3}H-CMP), as well as the gel electrophoretic pattern (reference 4, 95% in VSV-1 RNA). The specific activity of the viral RNA was 6 × 10^8 counts per min per mg RNA. Samples of RNA, 6.1 × 10^6 counts/min, were mixed with 400 μg of E. coli RNA, 200 U of RNase T_1 and either 20 μg (A) or 10 μg (B) of pancreatic RNase A in a total volume of 0.3 ml of 0.10 M NaCl, 0.005 M Tris-hydrochloride buffer, 0.001 M EDTA, pH 7.4, and incubated at 37 C for 30 min. The digests were loaded directly on DEAE columns and eluted with a LiCl gradient in urea buffer as described in Materials and Methods. At the end of the gradient, a high-salt (1 M LiCl) wash of the column was performed to elute residual large oligonucleotides and undigested sequences. The principal nucleotide isopleths, mono, ① through septanucleotides, ⑦ are labeled.*

prior to the dinucleotide ApUp. It was concluded that this peak may have contained some 2',3' cyclic ApUp (ApUp!) and undigested pyrimidine dinucleotides (UpUp, UpCp and CpUp, see later), but not guanosine and uridine dinucleotides (UpGp and GpUp), since not only do purine-containing nucleotides elute after pyrimidine nucleotides but also guanosine nucleotides elute after corresponding nucleotides containing adenosine.

At the lower pancreatic RNase concentration (Fig. 3B), seven major families of oligonucleotides were eluted from the chromatogram. It has been shown that DEAE chromatography in 7 M

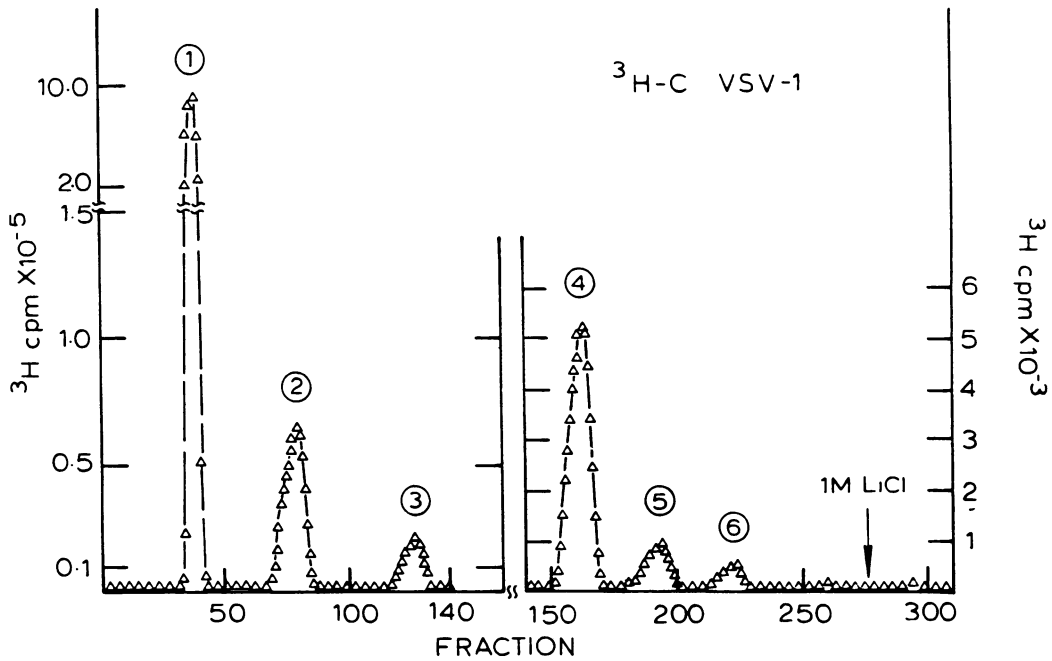

FIG. 4. *DEAE chromatography on RNase A and T_1 digests of ^{3}H-cytidine-labeled VSV-1 RNA. ^{3}H-cytidine-labeled VSV-1 RNA was prepared as described in the text and Fig. 3. The viral RNA (containing 93% ^{3}H-CMP, 7% ^{3}H-UMP: 95% of VSV-1 RNA) had a specific activity of 6 × 10^8 counts per min per mg of RNA. Samples of 4.4 × 10^6 counts/min were subjected to RNase T_1 and pancreatic RNase digestion (RNA to pancreatic RNase A ratio of 400 to 10) and resolved by DEAE column chromatography as described in Fig. 3, text, and Materials and Methods.*

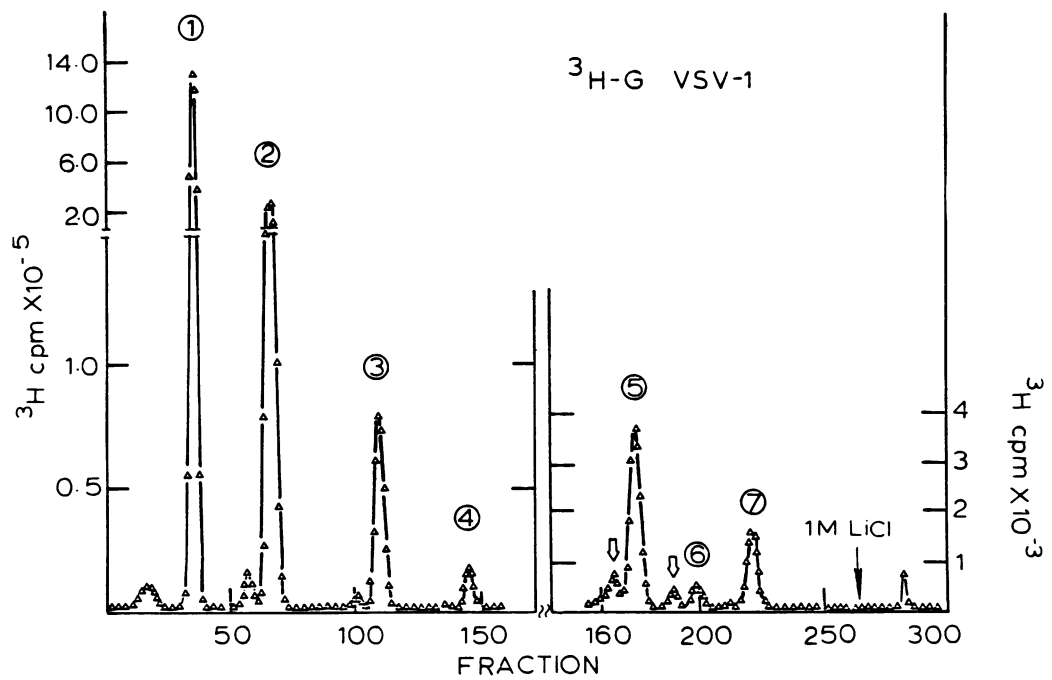

FIG. 5. *DEAE chromatography of RNase A and T_1 digests of ^{3}H-guanosine-labeled VSV-1 RNA. ^{3}H-guanosine-labeled VSV-1 RNA was prepared as described in the text and Fig. 3. The viral RNA (containing 99% ^{3}H-GMP, 1% ^{3}H-AMP: 95% of VSV-1 RNA) had a specific activity of 3 × 10^8 counts per min per mg of RNA. Samples of 5.5 × 10^6 counts per min were subjected to RNase T_1 and pancreatic RNase A digestion (RNA to pancreatic RNase A ratio of 400 to 10) and resolved by DEAE column chromatography as described in Fig. 3 and the text.*

urea elutes oligonucleotides primarily by their net phosphate charge and secondarily according to differences in base charges at pH 8.0 (20). Consequently, it was presumed that each family of nucleotides possessed similar phosphate charges but distinct base charges which indicated that there had been underdigestion of the viral RNA. It was also noted that whereas the dinucleotides were resolved into two peaks, the trinucleotides were recovered in three peaks, the tetranucleotides in four peaks, and the pentanucleotides had a broad shoulder of nucleotides. However, both the hexanucleotides and heptanucleotides were recovered in single peaks, and the percentage of the total label recovered in these peaks was the same as that obtained from the higher RNase digestion (Fig. 3A). Moreover, the percentage of the total label recovered in the slowest eluting peak of the tetranucleotides and pentanucleotides was within 5% that obtained for the corresponding oligonucleotides in Fig. 3A. Clearly, therefore, the other peaks in each family of nucleotides represented underdigested sequences. No attempt was made to identify these underdigested sequences although one obvious possibility was that the number of peaks in each family could be related to the number of adenosines present. For example, with the dinucleotides, the faster eluting peak probably contained dipyrimidine nucleotides (CpUp, UpUp, and UpCp), whereas the slower peak corresponded to ApUp. For the trinucleotides, the fastest eluting peak probably contained underdigested tripyrimidine nucleotides (i.e., CpUpCp, CpCpUp, UpCpCp, CpUpUp, UpUpCp, UpCpUp and UpUpUp), the middle peak to underdigested dipyrimidine, monoadenosine nucleotides (i.e., ApUpUp, UpApUp, ApCpUp, CpApUp, ApUpCp and UpApCp), and the slowest eluting peak to ApApUp, etc. These conclusions assumed that the RNase T_1 digestion was equally efficient irrespective of the pancreatic RNase digestion.

The salient features of the digestion at low pancreatic RNase concentration were that the total recovery of ^{3}H-uridine-labeled nucleotides was the same as that obtained by higher RNase concentrations and that the percentage of label recovered in the hexanucleotides and heptanucleotides was the same for the two experiments. Although such digests could be used to determine the molecular weight of an RNA by computation based on the hexanucleotide and heptanucleotide recovery, it was considered undersirable to have so much underdigestion of the viral RNA on the grounds that integration of the counts per oligonucleotide would be an inaccurate representation of the molar number of that sequence derived from 1 mol of RNA (see later).

^{3}H-cytidine-labeled VSV-1 RNA. The preparation of ^{3}H-cytidine-labeled VSV-1 RNA was subjected to combined nuclease digestion at an RNA to pancreatic RNase ratio of 40 to 1. The profile of recovered oligonucleotides is shown in Fig. 4.

^{3}H-guanosine-labeled VSV-1 RNA. The preparation of ^{3}H-guanosine-labeled VSV-1 RNA was also subjected to combined nuclease digestion at an RNA to pancreatic RNase ratio of 40 to 1. From the profile of resulting oligonucleotides (Fig. 5), 14 peaks of radioactivity were counted, excluding the high-salt eluant. An essentially identical profile was obtained when the digestion was performed at an RNA to pancreatic RNase ratio of 20 to 1. From the position of elution, it was concluded that the first peak corresponded to either free guanosine or, more probably, 2,3′ cyclic GMP, whereas the second peak, containing the largest amount of label eluted in one peak, corresponded to GMP. It was observed that the other 12 peaks occurred in pairs with the second peak the larger in each case, except for the hexanucleotides in which the two peaks were almost identical. Base ratio analysis had demonstrated that, in the initial ^{3}H-guanosine-labeled RNA, 1% of the label was present in adenylic acid residues. Consequently, all adenosine-containing sequences should contain a porportion of the total label (i.e., both those terminating in cytidine and uridine as well as the guanosine nucleotides). Since the number of adenosines in dinucleotides, trinucleotides, tetranucleotides, etc., increased with the size of the oligonucleotide, the percentage of ^{3}H-adenosine in successive guanosine-containing nucleotides also should increase and this was taken into consideration in computing the actual guanosine label in each oligonucleotide (Table 1). It was concluded, therefore, that the adenosine-containing pyrimidine sequences might have a significant quantity of ^{3}H-adenosine and this could be responsible for the smaller peak preceding each large peak of the chromatogram. When the computations were performed for the number of adenosine residues present in the various pyrimidine oligonucleotides recovered from a mole of RNA (see Table 1), these predictions were in excellent agreement with the amount of label recovered in the peaks preceding the principle tetra-, penta-, hexa-, and septanucleotides. Furthermore, these nucleotides, when recovered by barium precipitation and subjected to alkali digestion, contained mostly ^{3}H-adenylic acid. The amounts of label in the peaks preceding the

TABLE 1. *Oligonucleotides obtained by nuclease digestion of ^{3}H-uridine-, ^{3}H-cytidine-, or ^{3}H-guanosine-labeled VSV-1 RNA*[a]

Oligonucleotide	^{3}H-uridine RNA[b]	^{3}H-cytidine RNA[c]	^{3}H-guanosine RNA[d]
1 + 1!	1,140 (±38)	567 (±7)	1,738 (±40)
2 (−2!)*	483 (−116)* ± 40	102 (±6)	548 (±7)
3	108 (±4)	33 (±0.5)	173 (±3)
4	24.9 (±1.1)	8.3 (±0.1)	39 (±0.7)
5	5.8 (±0.1)	1.9 (±0.02)	8.0 (±0.2)
6	2.9 (±0.05)	1.0 (±0.02)	1.0 (±0.04)
7	1.0 (±0.02)	0	4.0 (±0.01)
Minimum sum oligonucleotides	1,766 (±83)	713 (±14)	2,511 (±51)
Probable number oligonucleotides	3,532 (±166)	2,139 (±42)	2,511 (±51)
Minimum number adenylic acids	703 (±43)	206 (±7)	1,072 (±16)
Probable number adenylic acids	1,406 (±86)	618 (±21)	1,072 (±16)

[a] Preparations of ^{3}H-uridine-, ^{3}H-cytidine-, or ^{3}H-guanosine-labeled VSV-1 RNA were subjected to combined RNase T_1 and pancreatic RNase digestion as described in Fig. 3, 4, and 5. The distribution of label recovered from the ^{3}H-uridine RNA digest was computed for each peak and totaled. Since the viral RNA contained 4% of its label in ^{3}H-cytidine residues (see text), the total recovery of counts per minute was corrected for this cytidine content and then divided by the counts recovered in the septanucleotide isopleth to obtain the minimum number of oligonucleotides labeled by ^{3}H-uridine which were recovered from 1 mol of viral RNA. Note that the ^{3}H-cytidine digest (Fig. 4) contained no labeled septanucleotides, so that it was presumed that the ^{3}H-uridine-labeled septanucleotides (Fig. 3) contained no ^{3}H-cytidine residues. The fractional content of the 4% of label in ^{3}H-cytidine residues present in each of the other peaks of Fig. 3 was calculated from the distribution of C label in Fig. 4 and subtracted from the Fig. 3 isopleth sums. The net counts per minute representing only ^{3}H-uridine-labeled nucleotides, was then divided by the label in the septanucleotide isopleth to give the number of oligonucleotides in each peak. The average of four experiments (and standard deviations) are given. For the purposes of these calculations the counts in the cyclic UMP (1!) and free UMP were combined, as were the cyclic dinucleotides (2!) and free dinucleotides, although the cyclic dinucleotides probably contained mostly dipyrimidine sequences (see Discussion). Similar calculations were performed from the ^{3}H-cytidine- and ^{3}H-guanosine-labeled RNA preparations. From the base ratio of the viral RNA, the minimum probable number of ^{3}H-uridine-, ^{3}H-cytidine-, and ^{3}H-guanosine-labeled oligonucleotides (and therefore labeled bases), obtained from 1 mol of RNA were calculated (see text). To obtain the number of adenylate residues present in the viral RNA, the adenylate residues associated with each oligonucleotide were computed (see text), again (*) assuming that the peak preceding the ^{3}H-uridine-labeled dinucleotides (2!), represented dipyrimidine nucleotides lacking adenosine. The total nucleosides recovered from 1 mol of RNA were then determined and by multiplying by the individual nucleotide molecular weights, the molecular weight of VSV-1 RNA was calculated. Total nucleotides = 11,278 (±382). Molecular weight = $3.82 \pm 0.14 \times 10^6$.

[b] Four analyses; 6.1×10^6 counts per min per analysis.

[c] Three analyses; 4.4×10^6 counts per min per analysis.

[d] Two analyses; 5.5×10^6 counts per min per analysis.

dinucleotide ApGp or trinucleotide ApApGp were greater (~10%) than that expected for just the ^{3}H-adenosine in pyrimidine-containing nucleotides, and by base analysis were found to contain both ^{3}H-adenylic acid as well as some ^{3}H-guanylic acid. This suggested that these peaks probably also contained 2′,3′ cyclic ApGp (ApGp!) and 2′3′ cyclic ApApGp (ApApGp!), respectively.

When digests of equal amounts of ^{3}H-uridine- and ^{3}H-cytidine-labeled RNA were cochromatographed on DEAE, the oligonucleotide isopleths were recovered as single peaks. However, when digests of ^{3}H-cytidine- and ^{3}H-guanosine-labeled VSV RNA were cochromatographed, double peaks for each oligonucleotide were obtained with the ^{3}H-cytidine-labeled nucleotides eluting before the corresponding sized ^{3}H-guanosine-labeled nucleotides, indicating that the smaller peaks in Fig. 5 were mostly due to ^{3}H-adenosine-labeled, pyrimidine-containing nucleotides.

Base ratio analysis of VSV-1 and VSV-III RNA. A preparation of VSV virus which gave both B (VSV-1) and defective T particles (VSV-111) was grown in monolayers of BHK/21 cells, the virus was harvested, and the B and T particles were resolved by centrifugation in gradients of sucrose. RNA was extracted from the isolated VSV-1 and VSV-111 virions and

finally purified by electrophoresis in swollen 2.1% polyacrylamide gels (4). Individual 1-mm gel slices were counted with a hand Geiger-Müller counter to locate the VSV-1 and VSV-111 RNA bands, and then suspended in absolute ethanol to precipitate the RNA within the gel slice and elute SDS. After 15 min, the contracted gel slice containing 98% of the radioactivity was removed, dried, and incubated in a sealed tube with 0.3 ml of 0.3 M KOH at 37 C for 18 h to digest the RNA. During the incubation the gel slice expanded to a volume of approximately 0.3 ml. After hydrolysis, 0.11 ml of 1 M HCl was added and the mixture was frozen at −20 C for 24 h. On thawing, the gel slice, which had now shrunk back to approximately one-third its original size, was removed, counted, and discarded. Samples of the hydrolysate, which accounted for 95% of the total initial radioactivity, were subjected to paper electrophoresis at pH 3.5, and the distribution of label was determined amongst the four ribonucleoside monophosphates (18). From 10 separate analyses of each RNA species, the base ratios were calculated to be 25.2 ± 0.5% AMP, 18.0 ± 0.5% CMP, 22.4 ± 0.5% GMP, and 34.4 ± 0.5% UMP for VSV-1 RNA, and 24.2 ± 0.5% AMP, 19.9 ± 0.5% CMP, 21.7 ± 0.5% GMP, and 34.2 ± 0.5% UMP for VSV-111 RNA. From these results it was determined that the average molecular weight of a nucleotide from VSV-1 RNA was 338.6 whereas that from VSV-111 RNA was 338.0.

Calculation of the molecular weight of VSV-1 RNA. The distribution of label amongst the various oligonucleotides recovered from digests of ^{3}H-uridine-, ^{3}H-cytidine-, or ^{3}H-guanosine-labeled VSV-1 RNA was determined (Fig. 3 to 5). The sum of counts in each peak from the ^{3}H-uridine-labeled RNA digest was corrected for the content of ^{3}H-cytidine-containing nucleotides, and the minimum total number of oligonucleotides was computed assuming that the septanucleotide isopleth corresponded to one. The average result for four analyses (and standard deviations) is given in Table 1. Since the dinucleotides through septanucleotides contained unlabeled adenine, the minimum number of adenylic nucleotides was also computed (assuming that the peak designated 2! was in fact principally dipyrimidine nucleotides [see Discussion]). Therefore, the calculated number of adenylate residues was $483 - 116 + 2 \times 108 + 3 \times 25 + 4 \times 6 + 5 \times 3 + 6 \times 1$, i.e., 703 (±43) nucleotides.

Similar calculations were performed for the ^{3}H-cytidine and ^{3}H-guanosine RNA digests, and the results are also given in Table 1.

In these calculations it was assumed that only one sequence of one of the large oligonucleotides was obtained from 1 mol of RNA. Clearly, where there were two or more identical sequences then the total number of oligonucleotides would be twice or more this calculated minimum number. Since the number of oligonucleotides labeled by cytidine, uridine, or guanosine also represented the number of corresponding bases in the RNA, an estimate of the base ratio of the viral RNA can be used to obtain a fit between these calculated numbers of each base. It is clear from the results presented in Table 1 that the best fit could be obtained if there were twice the number of uridine oligonucleotides, thrice the number of cytidine oligonucleotides, and the same number of guanosine oligonucleotides. The base ratio of such a fit gave 31% UMP, 19% CMP, 27% AMP, and 22% GMP (compare above). The total number of nucleotides was then calculated to be 11,278 (±382) and hence from the formula weights of the nucleotides, the weight of the whole RNA was calculated to be $3.82 \pm 0.14 \times 10^6$.

As an alternate method of computation, the minimum sum of oligonucleotides derived from one base-labeled RNA was divided by the fractional content of that base in the RNA to give an estimate of the total nucleotides present. By this approach the minimum sum of all nucleotides, calculated from the results of the ^{3}H-uridine RNA digest, was 1766/0.344 = 5,134, whereas that for the ^{3}H-cytidine- and ^{3}H-guanosine-labeled RNAs was 3,961 and 11,210, respectively. Since these results should be identical, a fit can be obtained from two complements of the uridine-labeled nucleotides, three of the cytidine-labeled nucleotides together with one of the guanosine-labeled nucleotides. The average of these three estimates was 11,120 (±811) nucleotides, and by multiplying by the average nucleotide weight (338.5 daltons) the weight of the viral RNA was calculated to be 3.76 (±0.27) $\times 10^6$—in essential agreement with the other calculation.

Molecular weight of VSV-111 RNA. Combined nuclease digests of ^{3}H-uridine-, ^{3}H-guanosine-, or ^{3}H-cytidine-labeled VSV-111 RNA were performed as described for VSV-1 RNA (Fig. 6). Since each VSV-111 RNA preparation contained between 10 and 13% VSV-1 RNA, as revealed by polyacrylamide gel electrophoresis, the computations of the number of oligonucleotides released from 1 mol of VSV-111 RNA were corrected for the contaminant VSV-1 RNA as determined in Table 1. The results for the oligonucleotides recovered from VSV-111 RNA are given in Table 2. Again in these calculations it was assumed that only one sequence of one (or more) of the large oligonu-

cleotides was obtained from 1 mol of RNA. It was concluded that the observed numbers of nucleotides labeled by each base was comparable to the base ratio of the RNA, giving a value of 18.7% CMP, 27.0% AMP, 20.9% GMP, and 33.3% UMP. Therefore, the total number of nucleotides derived from 1 mol of RNA was calculated to be 3638 (±109) and hence the weight of VSV-111 RNA was 1.23 (±0.04) × 10^6.

When the minimum sums of ^{3}H-uridine-, ^{3}H-cytidine-, or ^{3}H-guanosine-labeled nucleotides were divided by the respective fractional base composition of the whole RNA, values for the total nucleotides in 1 mol of VSV-111 RNA were calculated to be 3,541, 3,427, and 3,502, respectively (i.e., 3490 ± 58). Therefore, the weight of VSV-111 RNA was computed as 1.18 ± 0.02 × 10^6, again in essential agreement with the previous calculation.

DISCUSSION

The weight of the virion RNA of the B particle of vesicular stomatitis virus (VSV-1, Indiana strain), has been variously estimated by sedimentation or electrophoretic criteria as between 3.0 × 10^6 and 4.4 × 10^6 (4–6, 9, 13, 14). The results obtained by the procedure used in this investigation are in excellent agreement with these other estimates.

It should be stressed that, in applying these techniques to determining the molecular weight of any viral RNA, three criteria must be satisfied relating to the purity of the RNA, the labeling procedures, and the nuclease digestion conditions.

Purity of the viral RNA. In the experiments reported here, viral RNA was extracted from purified virions and purified by 4% agarose gel chromatography. The purity of the RNA was monitored by gel electrophoresis. Although a cloned stock of virus was used for preparing labeled virions, for some RNA viruses this procedure may not be sufficient for obtaining a homogeneous preparation of a paritcular viral RNA, as for instance for viruses which cannot be cloned or in mixtures such as the RSV-RAV or MSV-MLV mixtures, etc. However, even if such stocks contained two RNA species which were unrelated to each other by sequence, only where the smaller component represented 10% or more of the total particles would the method of analysis be inapplicable since on a molar basis less than 10% of a unique oligonucleotide would be difficult to detect and the error caused by the presence of that amount of nonhomologous RNA would only overestimate the actual molecular weight by 10%. Where 10% or more of a heterologous RNA species was present, identical in size to the major component, this method of analysis would in most cases give molecular weight estimates higher than those observed by other procedures (sedimentation, electrophoresis, etc.), and such estimates should lead the investigator to suspect RNA mixtures. Wherever possible, therefore, the method should be applied to cloned viral preparations.

Labeling procedures. We have found that in vivo labeling of RNA must be monitored to be certain that the precursor nucleoside is principally recovered in the corresponding nucleotide in the viral RNA. Whether the recovery of label in other nucleotides is in part due to contaminant in the precursors or to intracellular conversion is not known.

For accuracy, in the eventual computations, a minimal number of counts must be used per digest (4 × 10^6 for VSV-1, 10^6 for VSV-111); consequently, in this investigation a compromise was made between having an RNA of low specific activity with all of the label in the progenitor base and an RNA of higher specific activity and some of the label in other nucleotides.

Usually virus obtained from a confluent monolayer of BHK cells in a 32-ounce prescription or Blake bottle, containing 50 ml of media and a total of 1 mCi of label, was enough for one DEAE column analysis, after purification of the viral RNA, etc.

Nuclease digestion conditions. The nuclease digestion conditions were controlled to minimize the amount of overdigestion or underdigestion of RNA. In several cases a certain amount of underdigestion was allowed (as for instance with the ^{3}H-uridine-labeled VSV-1 RNA).

Underdigestion was indicated by the presence in some digests of peaks of label preceding the mononucleotides or dinucleotides. It has been found with the ^{3}H-uridine-labeled RNA, that if the pancreatic RNase was increased to give a 10 (RNA) to 1 (pancreatic RNase) ratio, the peaks preceding the mono- or dinucleotides could be abolished. However, it has been shown that such a ratio of enzyme to RNA could cause overdigestion (Fig. 2), and consequently this ratio of enzyme to RNA was considered undesirable. It is noteworthy that not all RNA preparations showed the same degree of underdigestion even though the nuclease digestion conditions were identical (compare the ^{3}H-cytidine-labeled VSV-1 and VSV-111 profiles). Presumably this represented differences in the labeled RNA preparation (trace amounts of cations or SDS, etc.). Moreover, different preparations of ^{3}H-cytidine-labeled VSV-1 have shown different amounts of underdigestion under identical

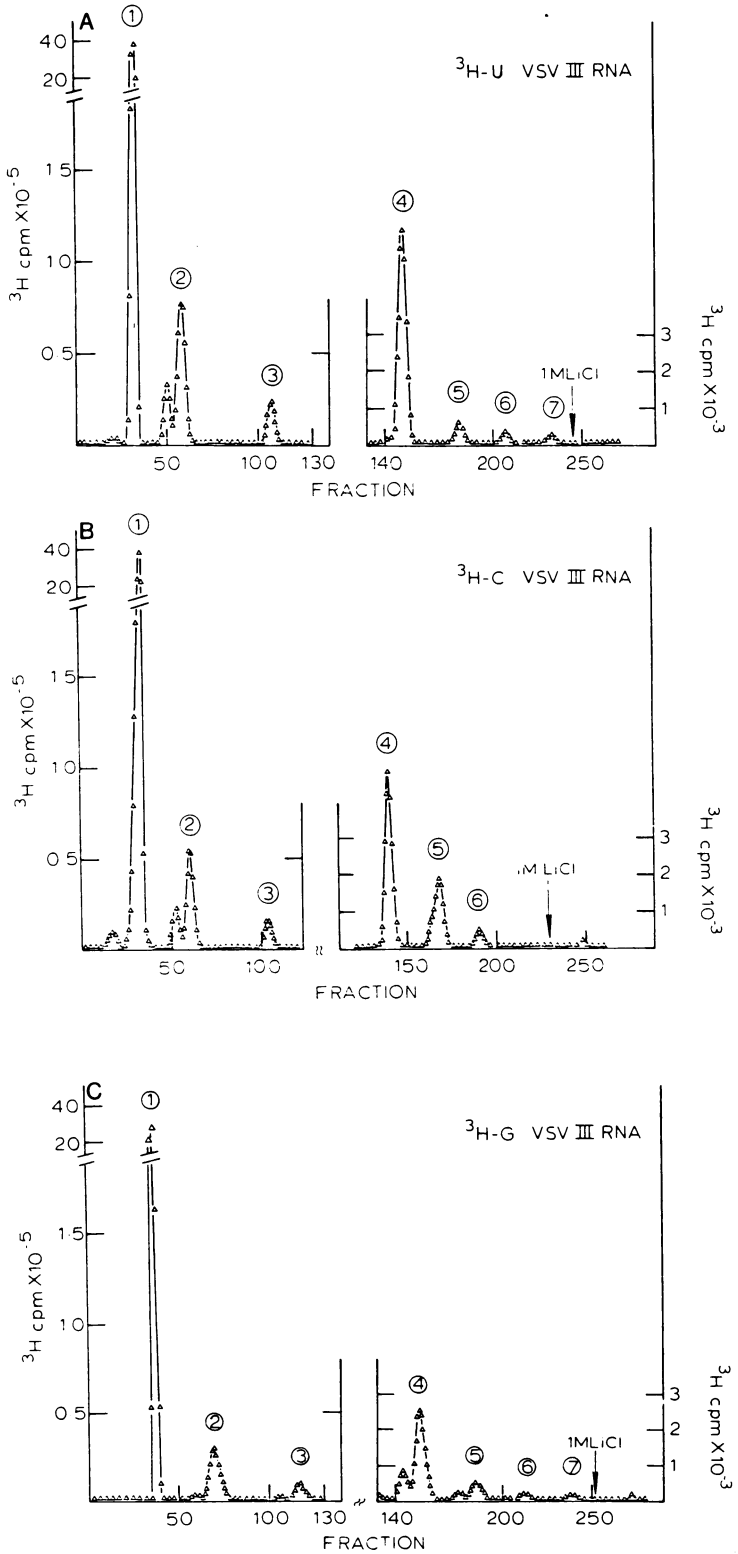

FIG. 6. *DEAE column chromatography of RNase A and T_1 digests of ^{3}H-uridine-, ^{3}H-cytidine-, and ^{3}H-guanosine-labeled VSV-111 RNA. Preparations of ^{3}H-nucleoside-labeled defective T virions of VSV (VSV-111) were prepared as described in Materials and Methods, and the RNA was extracted and purified by 4% agarose column chromatography. The base ratios and distribution of label between VSV-1 and VSV-111 RNA were determined,*

digestion conditions to those used in Fig. 4, although the results of the total moles of oligonucleotides obtained from 1 mol of RNA were calculated to be the same irrespective of the presence of these underdigested sequences (unpublished observations). It should be mentioned here that when the moles of mono- and dinucleotides were compared for these two preparations of ^{3}H-cytidine-labeled VSV-1 RNA, it was found that almost all the label found in the peak preceding the dinucleotides of the underdigested RNA were recovered in the mononucleotide peak of the more completely digested RNA, indicating that it was composed principally of dipyrimidine nucleotides with little cyclic ApCp! sequences. Consequently, in computing the adenosine residues associated with the various pyrimidine nucleotides, the peak preceding the dinucleotides was not counted (Tables 1 and 2).

Base ratio analysis of RNA. The base ratio of VSV-1 and VSV-111 RNA was determined by growing virus in cells in the presence of ^{32}P-phosphate. The analyses are in essential agreement with those obtained by Brown et al. (6). However, it should be mentioned that there is no assurance that this base ratio exactly reflects the nucleotide composition of the viral RNA, since it is assumed that the intracellular nucleotide pools of each triphosphate possess the same specific activity. Even though alkali hydrolysis would tend to mitigate the effect of unequal specific activities of the precursor nucleotides, depending on the nearest neighbor frequencies, the base ratio could still be an inaccurate representation of the nucleotide composition.

It is noteworthy that the viral RNA specific activities obtained from virus isolated after 24 or 48 h of infection are considerably different. We have attempted to grow cells through five

TABLE 2. *Oligonucleotides obtained by nuclease digestion of ^{3}H-uridine-, ^{3}H-cytidine-, or ^{3}H-guanosine-labeled VSV-111 RNA*[a]

Oligonucleotide	^{3}H-uridine RNA[b]	^{3}H-cytidine RNA[c]	^{3}H-guanosine RNA[d]
1 + 1!	775 (±20)	507 (±16)	544
2 (−2!)*	331 (−93)* (±24)	132 (−36)* (±5)	154
3	81 (±4.5)	28 (±1.0)	46
4	19.7 (±1.2)	9.0 (±0.1)	10.7
5	2.0 (±0.09)	5.0 (±0.07)	3.0
6	1.0 (±0.02)•	1.0 (±0.05)	1.0
7	1.0 (±0.01)	0	1.0
Minimum sum oligonucleotides	1,211 (±50)	682 (±22)	760
Probable number oligonucleotides	1,211 (±50)	682 (±22)	760
Minimum number adenylic acids	479 (±31)	204 (±6)	302
Probable number adenylic acids	479 (±31)	204 (±6)	302

[a] Preparation of ^{3}H-uridine-, ^{3}H-cytidine-, or ^{3}H-guanosine-labeled VSV-111 RNA were subjected to combined nuclease digestions (Fig. 6), and the molar ratio of oligonucleotides was determined as described in Table 1 and the text after correcting for the contaminant label attributable to VSV-1 RNA and label present in nonprecursor nucleosides. The minimum sum of oligonucleotides was concluded to be identical to the probable number of oligonucleotides since they were essentially in agreement with the base ratio determination. Again the peak preceding the dinucleotides (*, 2!) was considered as dipyrimidine nucleotides and was not counted in calculating the adenylate residues (see Discussion, Table 1). Total nucleotides = 3,638 ± 109. Molecular weight = 1.23 (±0.04) × 10^6

[b] Three analyses; 1.6 × 10^6 counts per min per analysis.

[c] Two analyses; 1.9 × 10^6 counts per min per analysis.

[d] One analysis; 1.1 × 10^6 counts per min per analysis.

and samples of each RNA were digested by nucleases and resolved by DEAE column chromatography as described in the text and Fig. 3. A, The ^{3}H-uridine-labeled VSV-111 RNA preparation (96% ^{3}H-UMP, 4% ^{3}H-CMP: 87% VSV-111 RNA, 12% VSV-1 RNA) had a specific activity of 6 × 10^6 counts per min per mg of RNA. B, The ^{3}H-cytidine-labeled VSV-111 RNA preparation (93% ^{3}H-CMP, 7% ^{3}H-UMP: 85% VSV-111 RNA, 13% VSV-1 RNA) had a specific activity of 6 × 10^6 counts per min per mg of RNA. C, The ^{3}H-guanosine-labeled VSV-111 RNA preparation (99% ^{3}H-GMP, 1% ^{3}H-AMP: 88% VSV-111 RNA, 11% VSV-1 RNA) possessed a specific activity of 3 × 10^6 counts per min per mg of RNA. The RNA to pancreatic RNase A ratio in the three digests were 400 to 20 for ^{3}H-uridine RNA, 400 to 10 for ^{3}H-cytidine RNA, and 400 to 20 for ^{3}H-guanosine RNA.

cell divisions in the presence of 5 μCi of ^{32}P-phosphate per ml of Eagle medium, and then infect them with VSV to be certain that the intracellular nucleoside triphosphate specific activities are identical. Although the cells divide and grow at approximately half the rate of unlabeled cells, we have so far not been able to obtain productive infections with VSV in such cells. The reason for this inability is not known.

Computations of RNA molecular weight from combined nuclease digestions. From the sum of the observed minimum number of oligonucleotides labeled by ^{3}H-uridine, ^{3}H-cytidine, or ^{3}H-guanosine, the weights of the viral RNAs have been calculated. Two methods of calculation have been employed. In one the number of total nucleotides was estimated by dividing the minimal sum of oligonucleotides labeled by a base by its fractional composition in the whole RNA. Since the total nucleotides must be the same whether the calculation is done for nucleotides labeled by cytidine, uridine, or guanosine, the three estimates for VSV-1 RNA put in a diaphantine equation in which the coefficients are integers indicated that there was probably thrice the minimal number of ^{3}H-cytidine-labeled oligonucleotides, and twice the minimal number of ^{3}H-uridine-labeled oligonucleotides per unit of ^{3}H-guanosine-labeled nucleotides in the VSV-1 viral RNA. The value obtained for the weight of the viral RNA was then calculated to be $3.76 \pm 0.27 \times 10^{6}$. This value is still a minimal estimate, and theoretically the actual value could be an integer multiple of this figure. However, as mentioned above, this estimate is in excellent agreement with the value obtained by other methods (4–6, 9, 13, 14), and particularly with the value of $3.6 \pm 0.2 \times 10^{6}$ obtained under denaturing or nondenaturing conditions by Mudd and Summers (13). The estimate of RNA molecular weight, derived as described above, relied on the base ratio determination of the viral RNA. Even though three bases were used for the calculations, no direct estimate of the number of adenylate residues were possible, so that errors introduced by an inaccuracy (e.g., an underestimate) of the base composition of one nucleotide could not be completely cancelled out by overestimates of the other two.

In the other method of computation, the base ratio of the viral RNA was used to obtain an essential agreement between the total number of the three individually labeled nucleotides, and subsequently the total numbers of all nucleotides including adenosine were calculated. The resulting weight of $3.82 \pm 0.12 \times 10^{6}$ is very similar to that obtained by the other method.

Application of combined nuclease digestions to determining the molecular weight, homology, and homogeneity of RNA viruses. In the preceding discussion it has been emphasized that the estimate of molecular weight obtained by combined nuclease digestions is a minimal one with the possibility that the actual weight is an integer multiple of that value. Even where the calculated minimum molecular weight agrees well with that obtained by other means (e.g., electrophoresis or sedimentation), as for the two RNA species analyzed here, there is no certainty that the RNA size could not still be a multiple of that value. One possible approach to solving this question is to look at the oligonucleotides liberated by one nuclease (e.g., RNase T_1), for sequences containing particular polyadenylic, pyrimidine nucleotides. For instance, a RNase T_1 digest of ^{3}H-cytidine-labeled VSV-1 RNA could be screened for the number of sequences containing $(Ap)_5C$. On the basis of the results presented in Table 1, we would predict that there were three such sequences. However, if six were found it would then be clear that the total molecular weight of VSV-1 was half the calculated value. Clearly, for the individual ^{3}H-labeled VSV-1 RNA preparations, the number of analyses of this sort are quite numerous so that this method would be reasonably reliable. As another approach, the terminal 3′ nucleoside or 5′ nucleotide could be sought by alkali digestion and their molar quantity could be determined. However, this approach may be complicated in situations in which there were more than one 3′ terminal nucleoside in the population of RNA molecules (as for the RNA bacteriophages), or if the 5′ terminal nucleotide was completely or partially dephosphorylated.

Despite these complications, the value of combined nuclease digestions for determining the molecular weights of RNA species lies in the accuracy of the method even for high-molecular-weight RNA. Potentially the analysis should be applicable to a variety of questions which are otherwise intangible, due to secondary structure or conflicting data (as for instance with the oncornaviruses or Sendai RNA; 7, 10), as well as to confirm the molecular weights of the RNA isolated from other VSV-defective virions (4, 15) or the recently reported long form of VSV (19).

We have recently been attempting to determine sequence homology among various rhabdovirus isolates by use of the transcription product RNA of VSV (Indiana) as a probe for relatedness. In unpublished observations (D. H. L. Bishop, P. Roy, J. F. Obijeski, H. F. Clark,

and H. G. Aaslestad), we can detect very little sequence homology between VSV (Indiana) and the virion RNA of VSV (New Jersey), Chandipura, Piry, rabies, and Kern Canyon viruses. The level of confidence of these determinations is about ±5% so that the possibility still remains that particular RNA sequences might be conserved among some or all rhabdovirus RNA genomes (e.g., 5′ or 3′ sequences, polymerase initiation sites, etc.). Such sequence conservation, if involving small numbers of nucleotides, could only be detected by nuclease digestion and sequence analysis procedures in which individual or combined nuclease digestion have a part to play.

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