Attempts over the past 10 years to identify the terminal nucleotide in tobacco mosaic virus ribonucleic acid (RNA) (1-3) had met with little success until the recent demonstration by Sugiyama and Fraenkel-Conrat (4) that 1 mole of adenosine was liberated from 1 mole of RNA by carefully controlled alkaline hydrolysis. The fact that the terminal residue does not have a monoesterified phosphate on C-2' or C-3' should make it possible, theoretically, to distinguish any nucleotide group in only certain internucleotide bonds being cleaved. If the specificity of the ribonuclease (RNase) used for the digestion were known, the sequence of a number of nucleotides in the RNA molecule might be established if the group released from the end of the chain could be identified. An attempt to apply this approach to the determination of the sequence of some of the nucleotides in tobacco mosaic virus RNA is described in this paper.

By virtue of the size of the tobacco mosaic virus RNA molecule, which contains approximately 6400 nucleotides, it is impractical to carry out this work with anything but radioactive RNA. C14-labeled tobacco mosaic virus RNA was digested with three different RNases, pancreatic RNase, Taka-Diastase RNase T1 (5, 6), and Bacillus subtilis RNase (7), and the hydrolysis products were then fractionated to allow identification of certain nucleotides whose base to phosphate ratio was $n/n-1$ (where $n$ is the number of purine plus pyrimidine bases in the fragment). Such compounds must have originated from the end of the RNA chain. The results confirm that adenosine is a terminal group and allow certain conclusions to be drawn with regard to the nature of the second and third nucleotides in the RNA chain.

**EXPERIMENTAL PROCEDURE**

Tobacco mosaic virus labeled with C14 was isolated by differential centrifugation from infected tobacco plants that had been grown in an atmosphere containing 50 to 100 mc of C14O2. Generally labeled C14-RNA was prepared from the virus by the phenol method as modified by Fraenkel-Conrat, Singer, and Tsugita (8). The preparations were the same as those described by Sugiyama and Fraenkel-Conrat (4).

Pancreatic ribonuclease (five times crystallized) was obtained from Worthington Biochemical Corporation.

Samples of Taka-Diastase ribonuclease T1 (RNase T1) were very generously supplied by Dr. P. Berg, Stanford Medical School, Palo Alto, California, and by Dr. G. W. Ruszinsky, National Cancer Institute, Bethesda, Maryland.

Bacillus subtilis ribonuclease was a gift to Dr. H. Fraenkel-Conrat from Dr. S. Nishimura, Japanese Foundation for Cancer Research, Tokyo.

Unlabeled nucleosides were commercial products. The di-nucleoside monophosphates, ApA, UpA, CpA, ApU, and ApC, were kindly provided by Dr. H. Witzel of this laboratory.

Chromatographic Solvents—Solvent 1, isopropanol-water (70:30 by volume) with ammonia in the vapor phase (9); Solvent 2, isopropanol-acetic acid-water (85:5:10); Solvent 3, isopropanol-acetic acid-water (70:25:5).

General Procedure for Enzymatic Hydrolysis and Separation of Products—Approximately 200 pg (800,000 c.p.m.) of C14-RNA were hydrolyzed with the appropriate enzyme in the presence of 3 to 5 moles of phosphate buffer, pH 7.7, and then known amounts of unlabeled markers were added. The mixture was freeze-dried, redissolved in a small volume of water, and spotted onto a large sheet of Whatman No. 3MM paper for chromatography in the first dimension in Solvent 1 for a period of 24 hours. If the four nucleosides only were being isolated, then the portion of the chromatogram that moved ahead of adenylic acid was cut away and chromatographed in the second dimension in Solvent 2 for approximately 20 hours, the solvent being allowed to drip off the bottom of the paper. This gives a reasonably good separation of the nucleosides and moves them well away from any nucleoside 2',3'-cyclic phosphates and dinucleoside monophosphates (Fig. 1a). If a separation of both nucleosides and dinucleoside monophosphates was desired, then the area moving ahead of adenylic acid was cut away and chromatographed in the second dimension in Solvent 3 for 20 hours, and the area containing cytidine, uridine, and adenosine was run in Solvent 2 in the second dimension (Fig. 1, a and b).

After two-dimensional chromatography, the spots were eluted with water, concentrated, and electrophoresed on Whatman No. 3MM paper for 2 hours at pH 3.5 and 18 volts per cm as a third and final step of purification.

The spots were then eluted in 0.01 N HCl, and the recovery of added markers was determined spectrophotometrically. Re-
coveries were fairly constant from experiment to experiment and always fell within the range of 65 to 85% of the amount initially added. The samples were plated and the radioactivity was determined with a Nuclear-Chicago D47 Micromil gas flow counter in conjunction with scaler, automatic sample changer, and printer-timer. After correction of the radioactivity for self-absorption and by the recovery factor for the added marker, the amount of the nucleoside or dinucleoside monophosphate derived from the tobacco mosaic virus RNA could be calculated from the known specific activities of the nucleosides.

RESULTS AND DISCUSSION

Hydrolysis with Pancreatic RNase—This RNase hydrolyzes specifically the esters of pyrimidine nucleoside 3'-phosphates (10). Thus, if the terminal group in tobacco mosaic virus RNA is adenosine (4) and the penultimate nucleotide is either cytidylic or uridylic acid, then hydrolysis with pancreatic RNase should liberate 1 mole of adenosine per mole of RNA. On the other hand, if the penultimate nucleotide is a purine nucleotide but the third residue is a pyrimidine nucleotide, then 1 mole of adenosine per mole of RNA is liberated. To determine whether this difference could be attributed to the fact that some terminal adenosine groups are linked to purine nucleotides in the RNA chain and thus are resistant to cleavage by RNase, the RNA was first digested with the enzyme and the digest was then adjusted to 0.1 M with respect to alkali, and hydrolysis was allowed to proceed for a further 20 to 44 hours. It can be seen in Table II that this treatment did not significantly increase the amount of adenosine liberated, although the amounts of both guanosine and uridine increased slightly, probably owing to some dephosphorylation by the alkali. The reason for the difference between the data obtained by alkaline hydrolysis and those obtained by RNase hydrolysis is still not clear.

In the course of the latter experiments, it was observed that the presence of unlabeled marker nucleosides during alkaline hydrolysis of RNA considerably increased the apparent number of C14-nucleosides produced (cf. Sugiyama and Fraenkel-Conrat (4)). Such an effect was not apparent in the case of enzymatic hydrolysis. This [OH-] catalyzed exchange reaction, which probably takes place at the level of the nucleoside 2',3'-cyclic monophosphates, is clearly demonstrated by the data in Table II. The amount of each of the four labeled nucleosides produced goes up considerably when the RNA is hydrolyzed by alkali in the presence of unlabeled nucleoside markers. However, if the RNA is first digested with RNase and then with alkali, only the purine nucleosides show an increase. This is explained by the fact that after RNase hydrolysis, only the purine nucleotides are still esterified through the C-3'-phosphate group and, that adenosine is indeed the terminal 5'-linked group of tobacco mosaic virus RNA and that the nucleotide adjacent to adenosine is either cytidylic or uridylic acid.

The amount of adenosine liberated was nearly the same whether the conditions of hydrolysis were very mild, as indicated by the fact that uridylic acid was present in the digestion products mainly in the form of uridine 2',3'-cyclic phosphate, or very prolonged. In the latter case, a slow hydrolysis of adenylic acid esters occurs (11), and adenosine 2',3'-cyclic phosphate and ApA-cyclic-p appear in small amounts among the hydrolysis products.

Whereas the data of Sugiyama and Fraenkel-Conrat (4) indicated a value of almost exactly 1 mole of adenosine per mole of RNA, our figures consistently showed a value of slightly less than 1 mole of adenosine. To determine whether this difference could be attributed to the fact that some terminal adenosine groups are linked to purine nucleotides in the RNA chain and thus are resistant to cleavage by RNase, the RNA was first digested with the enzyme and the digest was then adjusted to 0.1 M with respect to alkali, and hydrolysis was allowed to proceed for a further 20 to 44 hours. It can be seen in Table II that this treatment did not significantly increase the amount of adenosine liberated, although the amounts of both guanosine and uridine increased slightly, probably owing to some dephosphorylation by the alkali. The reason for the difference between the data obtained by alkaline hydrolysis and those obtained by RNase hydrolysis is still not clear.

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therefore, only the purine nucleotide esters will undergo hydrolysis and be able to participate in exchange reactions when the alkali is added.

**Hydrolysis with RNase T1**—This enzyme hydrolyzes specifically the esters of guanosine 3'-phosphate (6). From the results above, we know that guanosine does not occupy either the terminal or the second position in the tobacco mosaic virus RNA chain. There is one chance in four that it occupies the third position, and, if it does, then hydrolysis of the RNA with RNase T1 should give rise to 1 mole of either CpA or UpA. Table III shows the amounts of CpA, UpA, and the four nucleosides formed when tobacco mosaic virus RNA was digested with RNase T1. It is clear that guanylic acid cannot be the third residue in the chain. If a considerable excess of this RNase is used for the digestion (50 times the amount recommended by Rushizky, Sober, and Knight (12) for complete digestion), the value for guanosine shows a small increase (Table III, Experiment 2). The reason for this is not apparent unless it is that the enzyme preparation contains a minute amount of a phosphatase.

**Hydrolysis with B. subtilis RNase**—Evidence presented by Nishimura (7) indicates that this RNase preferentially hydrolyzes the esters of purine nucleoside 3'-phosphates. If adenylic acid should occupy the third position in the chain, then hydrolysis of tobacco mosaic virus RNA with B. subtilis RNase should liberate 1 mole of either CpA or UpA per mole of RNA. It can be seen (Table III) that, although small amounts of these compounds were detected among the hydrolysis products, the level was well below 1 mole per mole of RNA. It would therefore appear that the third residue is neither adenylic nor guanylic acid and must be a pyrimidine nucleotide.

**Table II**

*Effect of presence of unlabeled nucleoside markers during hydrolysis of C14-RNA on amount of C14-nucleosides produced*

<table>
<thead>
<tr>
<th>Hydrolysis conditions</th>
<th>Moles of nucleoside per mole of RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenosine</td>
</tr>
<tr>
<td>Unlabeled markers added after hydrolysis completed:*</td>
<td>1. RNA, 180 μg; RNase, 20 μg; 24 hours, 37°</td>
</tr>
<tr>
<td></td>
<td>2. RNA, 180 μg; RNase 20 μg; 24 hours, 37°; 50 μmoles of NaOH then added and digestion continued for 24 hours at 37°</td>
</tr>
<tr>
<td></td>
<td>3. RNA, 180 μg; NaOH 20 μmoles; 24 hours, 37°</td>
</tr>
<tr>
<td>Unlabeled markers added at the beginning of hydrolysis:*</td>
<td>4. As for Experiment 1</td>
</tr>
<tr>
<td></td>
<td>5. As for Experiment 2, except that NaOH hydrolysis was for 24 hours at 30°</td>
</tr>
<tr>
<td></td>
<td>6. RNA, 180 μg; NaOH, 25 μmoles; 24 hours, 30°</td>
</tr>
</tbody>
</table>

* The amounts of unlabeled marker nucleosides added per 180 μg of RNA were 0.16 μmole of adenosine, 0.13 μmole of guanosine, 0.23 μmole of cytidine, and 0.22 μmole of uridine.

**Table III**

**Nucleosides and dinucleotide monophosphates released from tobacco mosaic virus RNA by hydrolysis with RNase T1 and with B. subtilis RNase**

<table>
<thead>
<tr>
<th>Hydrolysis conditions</th>
<th>Molar amounts of nucleoside or dinucleotide monophosphate per mole of RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenosine</td>
</tr>
<tr>
<td>RNase T1</td>
<td></td>
</tr>
<tr>
<td>1. B3-RNA,* 180 μg; RNase, 0.25 μg; 6 hours, 24°</td>
<td>0.01</td>
</tr>
<tr>
<td>2. B3-RNA, 180 μg; RNase, 1.25 μg; 24 hours, 24°</td>
<td>0.03</td>
</tr>
<tr>
<td>B. subtilis RNase</td>
<td></td>
</tr>
<tr>
<td>3. B3-RNA, 180 μg; RNase, 1 μg; 1 hour, 24°</td>
<td>0.03</td>
</tr>
<tr>
<td>4. B3-RNA, 180 μg; RNase, 8 μg; 18 hours, 30°</td>
<td>0.07</td>
</tr>
</tbody>
</table>

*See (4) for description of RNA preparations.

This conclusion is valid despite the fact that control experiments have indicated that the specificity of the B. subtilis enzyme is not as simple as has been claimed by Nishimura (7). It has been found that the enzyme will hydrolyze GpC and GpA but not ApC or ApA. However, the enzyme readily cleaves ApApC and ApApA, yielding adenosine 2',3'-cyclic phosphate and either ApC or ApA, thus showing a requirement in the case of adenylic acid esters for a substrate larger than a dinucleotide. In the digestion of tobacco mosaic virus RNA there is no adenosine released; therefore, the terminal internucleotide bond must have remained intact. If the third residue in the chain were either adenylic or guanylic acid, then the enzyme would have hydrolyzed the second bond to give 1 mole of either CpA or UpA. Because these compounds were almost absent from the digest, our conclusion that neither purine nucleotide occupies the third position is justified. The small amounts of CpA and UpA detected in the digest may be due to partial, nonspecific hydrolysis of the interpyrimidine nucleotide bond or to the possibility that the dinucleotide monophosphates were contaminated by some dinucleotides.

**SUMMARY**

C14-labeled tobacco mosaic virus ribonucleic acid (RNA) has been hydrolyzed with a number of ribonucleases having different specificities. Examination of the various hydrolysis products provides evidence that adenosine is the 5'-linked terminal nucleotide of tobacco mosaic virus RNA, thus confirming an earlier observation made in this laboratory. The results also indicate that the penultimate nucleotide is either cytidylic or uridylic acid and that the third residue in the chain is also a pyrimidine nucleotide.

**Acknowledgments**—The author is indebted to Dr. H. Fraenkel-Conrat, Dr. C. A. Dacker, and Mrs. B. Singer for their many valuable suggestions.

* A more detailed examination of the specificity of this enzyme is at present being carried out in this laboratory.
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Identification of End Groups in Tobacco Mosaic Virus Ribonucleic Acid by Enzymatic Hydrolysis
Paul R. Whitfeld


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