3. D. Weinblum and H. E. Johns, *ibid.* **114**, 450 (1966).
4. N. Camerman, S. C. Nyburg, D. Weinblum, *Tetrahedron Lett.* **42**, 4127 (1967).
5. J. R. Einstein, J. L. Hosszu, J. W. Longworth, R. O. Rahn, C. H. Wei, *Chem. Commun.* **20**, 1063 (1967).
6. D. Weinblum, *Biochem. Biophys. Res. Commun.* **27**, 384 (1967); A. J. Varghese and S. Y. Wang, *Nature* **213**, 909 (1967).
7. J. Karle and I. L. Karle, *Acta Cryst.* **21**, 849 (1966).
8. Most of the experimental work was performed at the University of Toronto, and we thank Professor S. C. Nyburg for the use of facilities in his laboratory and for encouragement. We also thank D. Weinblum for supplying the material.

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Pyrophosphate in the 5′ Terminal Position of a Viral Ribonucleic Acid

Abstract. *A pancreatic ribonuclease digest of carbon-14–labeled Satellite Tobacco Necrosis Virus RNA was fractionated, according to charge, by column chromatography. Individual fractions were dephosphorylated with alkaline phosphomonoesterase and rechromatogramed. The fraction originally containing oligonucleotides with seven negative charges separated into two components corresponding to five and two negative charges, respectively, and therefore must have contained a terminal trinucleotide 5′-pyrophosphate, in addition to the internal hexanucleotides. Other fractions when similarly treated were found to contain only internal oligonucleotides.*

The 5′ hydroxyl end (*1*) of naturally occurring RNA (*2*) molecules is most commonly found to be esterified to a monophosphate (*3*). Yet it has recently been shown that bacteriophage R17 RNA contains a triphosphate in the 5′ position (*4*). This latter finding is consistent with current ideas on the mechanism of RNA synthesis. The presence of a monophosphate in the 5′ position in many RNA's may therefore be a result of a subsequent cleavage of a triphosphate, suggesting that in vivo the 5′ end of RNA's may be subject to specific and limited dephosphorylations. Thus, limited and specific dephosphorylation of certain species of RNA could lead to the removal of a single phosphate group, resulting in an RNA containing a pyrophosphate at the 5′ end.

This report presents evidence that Satellite Tobacco Necrosis Virus RNA (STNV RNA), as prepared in our laboratory, contains such a 5′ terminal pyrophosphate group.

Virus preparations were made from Turkish Tobacco plants (variety Xanthi), which were grown in a chamber in an atmosphere containing 10 mc of $^{14}CO_2$. The virus was isolated as described (*5*), by using nonradioactive infected plant extracts as carrier in sufficient quantities to avoid the loss of labeled virus. The purified virus solution was adjusted to 0.1*M* $(NH_4)_2CO_3$ immediately prior to isolation and purification of the RNA (*6*). Ribonucleic acid from STNV (180 O.D. units, 790,000 count/min) was digested at 37°C for 24 hours in 0.1*M* tris HCl buffer, *p*H 7.5, with 0.6 mg of pancreatic ribonuclease (RAF, 7da, phosphate free, Worthington, Freehold, N.J.). For column chromatography, DEAE-cellulose (Cellex D, Bio-Rad, Richmond, Calif.) was purified by repeated washing with 3*M* NaCl and then 2*M* $(NH_4)_2CO_3$. Columns for the separation of enzyme digests were packed under slight pressure (⅓ atm) with this DEAE-cellulose, equilibrated in 7*M* urea and 0.01*M* tris HCl buffer, *p*H 7.5. The columns were then eluted with a linear gradient of NaCl in 7*M* urea, 0.01*M* tris HCl buffer, *p*H 7.5. Additional details are given in the figure legends. Oligonucleotides eluted from this column were freed of salts and urea by dilution with 4 volumes of H_2O, passage onto small (1- by 10-cm) DEAE-cellulose (carbonate form) columns equilibrated in 0.005*M* triethylammonium carbonate (TAC) (*7*) and subsequently eluted with 1*M* TAC. These fractions were then flash evaporated and lyophilized overnight to remove TAC. Each lyophilized fraction was dissolved in 1 ml of 0.1*M* tris HCl buffer, *p*H 8, and incubated for 2 hours at 37°C with 0.3 unit of *E. coli* alkaline phosphatase (BAPF, ribonuclease free, Worthington, Freehold, N.J.) per O.D. unit of oligonucleotide. Crystalline urea was then added to each mixture to a final concentration of 7*M* and, following the addition of appropriate markers, the sample was reapplied to DEAE-cellulose (chloride form) columns, and eluted with a NaCl gradient as above.

Aliquots of aqueous solutions were mixed with 15 ml of Bray's scintillation liquid (*8*) and counted for radioactivity in a Beckman LS-250 scintillation counter.

Figure 1 shows an elution profile of a pancreatic ribonuclease digest of STNV RNA. The oligonucleotides are separated on DEAE-cellulose according to their charge in 7*M* urea (*9*). Fractions −5, −6, −7, and −8 (numbers representing the number of negative charges) were desalted, dephosphoryl-

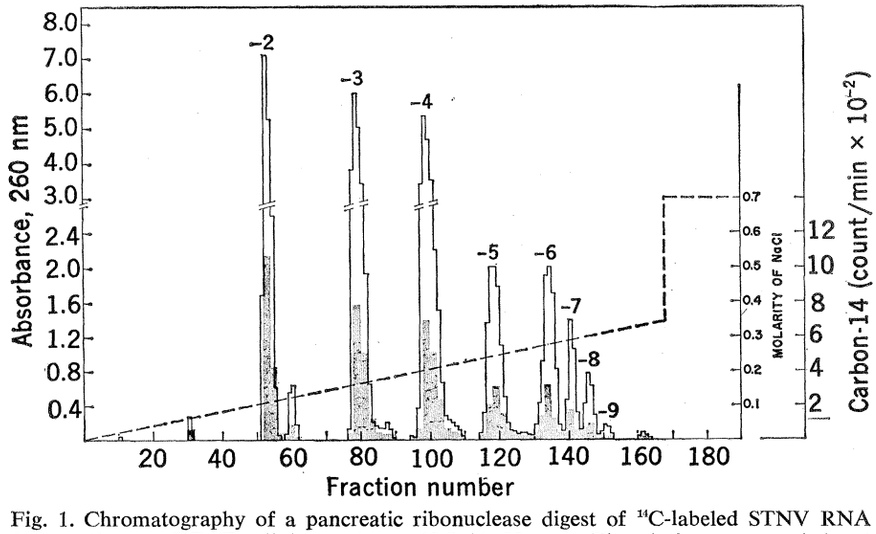

Fig. 1. Chromatography of a pancreatic ribonuclease digest of ^{14}C-labeled STNV RNA (see text) on a DEAE-cellulose column (0.8 × 55 cm). The elution was carried out with a gradient of NaCl concentration (broken line) in 7*M* urea, 0.01*M* tris HCl; total volume 500 ml. Fractions of 3 ml were collected. Aliquots of each fraction were measured for absorbance at 260 nm (solid line) and radioactivity (shaded area). The numbers above the peaks represent the number of charges of each oligonucleotide eluted in the peak.

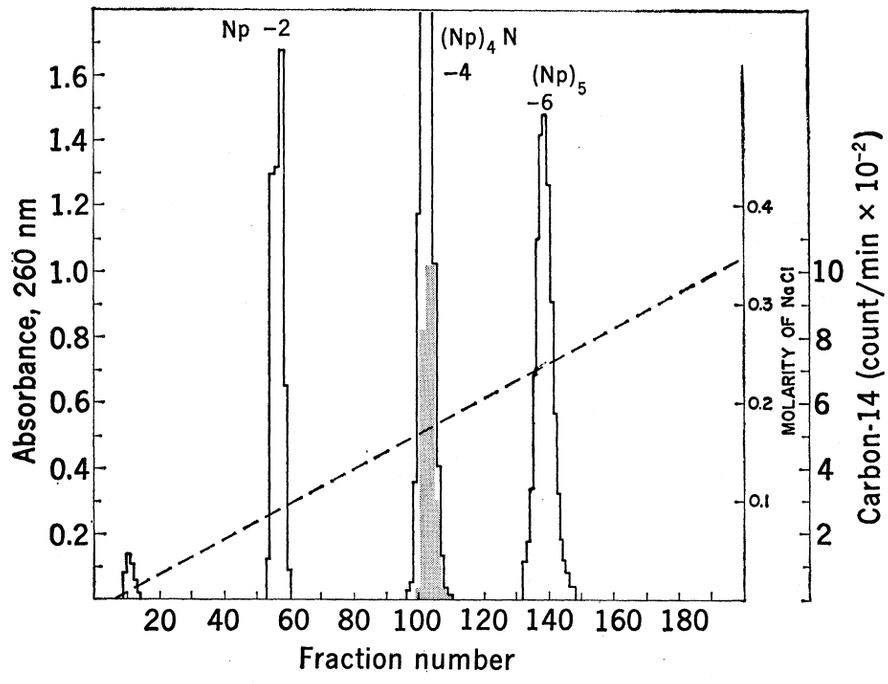

Fig. 2 (right). Rechromatography of alkaline phosphomonoesterase–treated (see text) peak −6 of Fig. 1. After addition of 14 O.D. units of nonradioactive $(NP)_6$ marker (obtained from a pancreatic ribonuclease digest of yeast RNA) and 7 O.D. units of NP (uridylic acid) the mixture was applied to a DEAE-cellulose column (0.65 × 50 cm) and eluted in 7*M* urea with a NaCl gradient (broken line, total volume 400 ml) as described in the text. Fractions of 2 ml were collected. Aliquots of each fraction were measured for absorbance at 260 nm (solid line) and radioactivity (shaded area).

ated with phosphomonoesterase, and then reanalyzed by DEAE column chromatography in 7*M* urea.

Preliminary investigations of the STNV RNA have suggested that the 5′ terminal fragment obtained from a pancreatic ribonuclease digest is a trinucleotide (*10*). If this 5′ terminal oligonucleotide carries some specific degree of 5′ phosphorylation, for example, pNpNpNp, it will chromatogram on the basis of its negative charges along with the internal oligonucleotides, in this case $(Np)_5$ carrying a −6 charge. Following the method of Tomlinson and Tener (*11*), treatment of this −6 fraction with alkaline phosphomonoesterase followed by separation on DEAE-cellulose in the presence of urea should yield two ^{14}C fractions, the $(Np)_4N$ internal oligonucleotides, and a $(Np)_2N$ 5′ terminal trinucleoside diphosphate. Similar alkaline phosphomonoesterase treatment of other fractions lacking a 5′ phosphorylated terminal oligonucleotide will yield only one ^{14}C fraction corresponding to the expected 3′ dephosphorylated oligonucleotides $(Np)_nN$.

Figure 2 is an elution profile of the dephosphorylated fraction −6 of Fig. 1 to which a −6 marker, $(Np)_5$, and a −2 marker, Np, were added. The absence of counts in the −6 marker demonstrates that the alkaline phosphomonoesterase digestion went to completion yielding a 3′ dephosphorylated product which has been shifted down two negative charges. No counts are found in any other part of the profile. Analogous results are obtained with fractions −5 and −8 of Fig. 1. All of these fractions must, therefore, contain only internal oligonucleotides.

In contrast, alkaline phosphomonoesterase treatment of fraction −7 followed by rechromatography gives a significant number of counts in the −2 position as well as the expected −5 position (Fig. 3). Unfortunately, in this experiment the alkaline phosphomonoesterase digested the unlabeled Np (−2) marker (*12*). The minor radioactive peak, therefore, had to be rechromatographed with the appropriate markers. The coincidence of the radioactivity and the −2 marker, as shown in Fig. 4, is evidence that the dephosphorylated terminal oligonucleotide has two negative charges.

The interpretation which is consistent with these data is that the 5′ terminal oligonucleotide released from

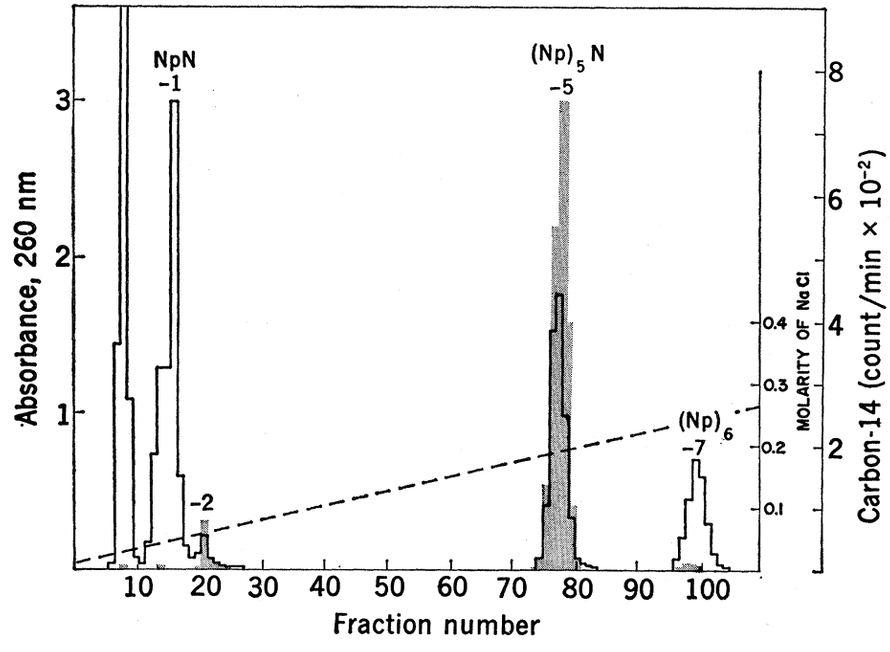

Fig. 3 (right). Elution profile of alkaline phosphomonoesterase–treated (see text) fraction −7 of Fig 1. After addition of 11 O.D. units of nonradioactive $(NP)_6$ marker (obtained from a pancreatic ribonuclease digest of yeast RNA), 6 O.D. units of each uridylic and cytidylic acid (NP) and 11 O.D. units of GpA, the mixture was applied to a DEAE-cellulose column (0.5 × 40 cm) and eluted in 7*M* urea with a gradient (broken line) of NaCl (0 to 0.35*M*, total volume 300 ml) as described in the text. Fractions of 2 ml were collected. Aliquots of each fraction were measured for absorbance at 260 nm (solid line) and radioactivity (shaded area). Total ^{14}C counts in peak −2 were 1455 (*14*).

STNV RNA by pancreatic ribonuclease contains a pyrophosphate at the 5′ hydroxyl end. Thus, the presence of seven negative charges on the original terminal oligonucleotide and the resultant two negative charges on the product of dephosphorylation can only be explained on the basis of a trinucleotide-5′ pyrophosphate (ppNpNpNp) in peak −7 of Fig. 1.

This interpretation is further supported by the radioactive counts. Assuming 1200 nucleotides in STNV RNA (*13*) the number of counts recovered in the minor peak of Fig. 3 is 73 percent of the number of counts theoretically expected for a trinucleotide (*14*). This high recovery and the absence of any measurable minor peaks of radioactivity in fractions −6 and −8 precludes the possibility of the 5′ pyrophosphate having originated from a random degradation of a 5′ triphosphate, or from a 5′ triphosphate that chromatogramed in an intermediate position between fractions −7 and −8.

It should be noted that a specific enzyme in *E. coli,* capable of removing the γ phosphate of an ATP terminus of a synthetic RNA, has been purified and investigated (*15*). In addition to this enzyme the presence of other enzymes which hydrolyze γ phosphate nonspecifically with respect to the nature of the base and which yield pyrophosphate-terminated RNA's has also been reported (*15*). Any of these enzymes, if present also in tobacco plants, could have generated a 5′ terminal pyrophosphate in STNV RNA.

Finally, subsequent chemical studies of the 5′ terminal oligonucleotide in this laboratory established its base sequence as AGU and further verified its trinucleotide composition (*16*).

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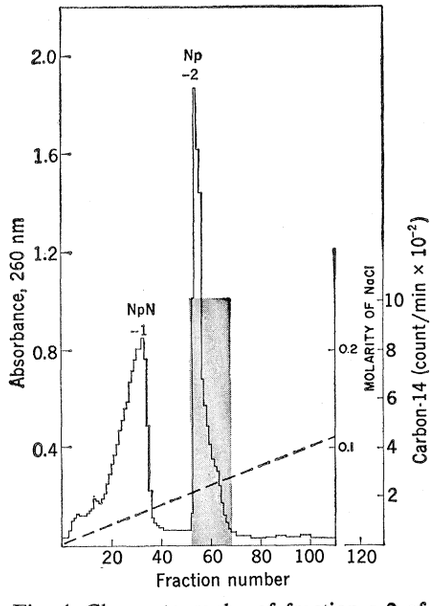

Fig. 4. Chromatography of fraction −2 of Fig. 3. Test tubes 20 to 23 of Fig. 3 were pooled, diluted with 15 ml 7*M* urea and, after the addition of 14 O.D. units of ApA (NpN) and 13 O.D. units of Cp (Np), applied to a DEAE-cellulose column (chloride form, 0.5 × 40 cm) equilibrated with 7*M* urea and 5 m*M* tris HCl, *p*H 7.5. The elution was carried out with a gradient (broken line) of NaCl (0 to 0.2*M*, total volume 300 ml), in 7*M* urea containing 5 m*M* tris HCl, *p*H 7.5. Fractions of 1.5 ml were collected and measured for absorbance at 260 nm (solid line). The eluents from tubes 10 to 38 and 52 to 68 were combined and desalted as described in the text. Aliquots of fraction −1 and −2 were counted for radioactivity (shaded area). After desalting the total counts in peak −2 were 1060 count/min (73 percent of peak −2 of Fig. 3).

References and Notes

1. The 5′ terminus is that end of a nucleic acid the terminal nucleoside of which is linked by its 3′ hydroxy group to the nucleotide chain.
2. Abbreviations used: RNA, ribonucleic acid; pp, pyrophosphate; p, phosphate; N, nucleoside; A, adenosine; G, guanosine; U, uridine; C, cytidine; p on the left of N indicates a 5′ phosphate; p on the right, a 3′ phosphate; STNV, Satellite Tobacco Necrosis Virus; O. D. unit, that amount of RNA per milliliter solution which produces an absorbance of 1 in a 1-cm light path cell at 260 nm; DEAE, diethylaminoethyl; TAC, triethylammonium carbonate; tris, tris (hydroxymethyl) aminomethane.
3. W. Zillig, D. Schachtschnabel, W. Krone, *Z. Physiol. Chem.* **318**, 100 (1960); M. Takanami, *J. Mol. Biol.* **23**, 135 (1967); G. G. Brownlee, F. Sanger, B. G. Barrel, *Nature* **215**, 735 (1967).
4. R. Roblin, *J. Mol. Biol.* **31**, 51 (1968).
5. B. Kassanis and H. L. Nixon, *J. Gen. Microbiol.* **25**, 459 (1961).
6. A. Gierer and G. Schramm, *Nature* **177**, 702 (1956).
7. M. Smith, D. H. Rammler, I. H. Goldberg, H. G. Khorana. *J. Amer. Chem. Soc.* **84**, 430 (1962).
8. G. A. Bray, *Anal. Biochem.* **1**, 279 (1960).
9. R. V. Tomlinson and G. M. Tener, *Biochemistry* **2**, 697 (1963).
10. M. E. Reichmann, A. Y. Chang, L. Faiman, J. M. Clark, Jr., *Cold Spring Harbor Symp. Quant. Biol.* **31**, 139 (1966).
11. R. V. Tomlinson and G. M. Tener, *Biochemistry* **2**, 703 (1963).
12. The activity of *E. coli* alkaline phosphatase in solutions of high urea concentrations was the subject of a separate study (E. Wimmer and M. E. Reichmann, in preparation).
13. M. E. Reichmann, *Proc. Natl. Acad. Sci. U.S.* **52**, 1009 (1964).
14. Incomplete dephosphorylation of the terminal nucleotide, as indicated by some radioactivity in peak —7 of Fig. 3, may have lowered the recovery of counts in peak —2. This may have been owing to a slow action of the alkaline phosphomonoesterase on the 5′ pyrophosphate group. A similar incomplete dephosphorylation of a 5′ triphosphate was observed with R17 RNA (*4*).
15. U. Maitra, S. N. Cohen, T. Hurwitz, *Cold Spring Harbor Symp. Quant. Biol.* **31**, 113 1966); U. Maitra, J. Dubnoff, *Federation Proc.* **26**, 349 (1967).
16. A. Y. Chang, E. Wimmer, M. E. Reichmann, in preparation.
17. Aided by NIH grant GM 12444.

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Circadian Rhythmicity in Amino Acid Incorporation in Euglena gracilis

Abstract. *A daily rhythm of amino acid incorporation exists in nondividing cultures of* Euglena *kept in a light-dark cycle. This rhythmicity persists for the two cycles measured in cultures transferred to constant conditions and is therefore probably circadian in nature.*

Circadian rhythms are a manifestation of an endogenous timing mechanism, a biological clock, which allows an organism to restrict a given activity to a particular part of the solar day. These clocks exist in nearly all eucaryotic organisms including unicellulars. In *Euglena gracilis,* the circadian clock controls phototactic response (*1*), motility in the dark (*2*), and, under certain conditions, cell division (*3*). In other organisms many biochemical rhythms have been found. Of particular interest are the reports of a circadian oscillation in RNA metabolism (*4*). Although such fluctuations do not in themselves indicate whether RNA metabolism is part of the clock or simply another parameter controlled by the clock, they may eventually acquire added significance in light of studies with actinomycin D which suggest that RNA synthesis is required for the normal operation of the clock (*5*).

I now report a circadian oscillation in amino acid incorporation into that fraction of *Euglena gracilis* which can be precipitated with hot trichloroacetic acid (TCA). Although this result, as with the fluctuations in RNA metabolism, does not in itself imply that protein synthesis is part of the basic clock mechanism, it is significant since the "slowing down" of the *Euglena* clock by an inhibitor of protein synthesis indicates that in some way the clock requires continued protein synthesis (*6*).

Several experiments demonstrated a daily fluctuation in the rate of amino acid incorporation in autotrophic nondividing cultures of *Euglena gracilis* Z maintained at constant temperature (25°C) in a cycle of 12 hours light (cool white fluorescent, 3850 lumen/m²) and 12 hours dark (LD 12:12). The cultures had been grown axenically in an inorganic salt medium under the same conditions of light and temperature (*6, 7*). Samples were removed from these cultures 10 minutes before a light-dark or dark-light transition. Amino acid incorporation was then measured either in the light (cool white fluores-