The Nucleotide Sequence at the 3′-Linked End of Bacteriophage MS2 Ribonucleic Acid*

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ABSTRACT: Enzymatic labeling of the 3′-linked terminus of MS2 ribonucleic acid (RNA) with polynucleotide kinase is possible only after phosphomonoesterase treatment of the nucleic acid. Pancreatic ribonuclease degradation of the labeled product results in the formation only of labeled trinucleoside tetraphosphate, indicating an initial sequence of two purines followed by a pyrimidine.

Alkaline degradation of RNA bearing a uniform ^{32}P label shows that in the virus the terminal nucleoside is present as the 5′-triphosphate. The natural initial trinucleoside hexaphosphate has been isolated from pancreatic ribonuclease digests and the structure was determined to be pppGpGpUp.

The determination by Holley *et al.* (1965) of the complete nucleotide sequence in yeast alanine tRNA clearly marks the greatest advancement to date in the study of the exact chemical structure of a nucleic acid. The efforts involved in the correct alignment of a sequence of seventy to eighty nucleotides are enormous, and extension of the techniques used by Holley to the study of viral RNA sequences of a thousand or many more nucleotides does not yet appear possible. This is indeed unfortunate, since a small RNA-containing virus should be a source of a pure, homogeneous, biologically active, single species of mRNA, an ideal macromolecule for chemical study.

The bacterial virus MS2 (Strauss and Sinsheimer, 1963) is especially well suited to chemical examination of its RNA, since it is relatively easy to prepare with or without a radioactive label, and can be a practical source of homogeneous infective RNA with a molecular weight of 1.05 million, corresponding to a chain length of about 3300 nucleotides. MS2 RNA is an active messenger and appears to direct the synthesis of its own coat protein (Nathans *et al.*, 1966). No evidence reporting minor nucleotide components to be present has been found in the literature, while the usual four nucleotides are present in approximately equimolar amounts. Fiers *et al.* (1965a,b) have made a quantitative evaluation of short nucleotide sequences, and speculated on the characteristic patterns found.

While attempts at a complete sequence determination of MS2 RNA are not yet likely to be fruitful, studies of the nucleotides at each end of the molecule appear to be more hopeful. Such work could be of interest with regard to questions such as the extent of end-group phosphorylation, the relationship of observed nucleotide sequences to the initiation and termination of peptide chains, and possible correlation of these nucleotide sequences with amino acid sequences at the ends of the viral coat protein.

The 5′-linked end (also referred to as the right hand or 3′-hydroxyl end) of MS2 RNA has been examined by Sugiyama (1965); a second paper will deal with an extension of his studies (D. Glitz, A. Bradley, and H. Fraenkel-Conrat, in preparation). Here the nucleotide sequence at the 3′-linked end (or the left hand or 5′-hydroxyl end) of the chain will be examined.

Experimental Section

Preparation of MS2 and Its RNA. The virus was grown and isolated following in general the techniques of Strauss and Sinsheimer (1963), but with a few exceptions. The bacterial host was a T-phage-resistant mutant of *Escherichia coli* C-3000, isolated by Mrs. A. Bradley and designated 72-B2. The lysate was clarified by centrifugation for 15 min at 1000*g* before overnight ammonium sulfate precipitation. The ammonium sulfate precipitate was extracted with Genesolv D (Rueckert and Duesberg, 1966) and the aqueous portion was layered on top of 2-ml pads of concentrated sucrose solution (1.5 ml of 65% sucrose followed by 0.5 ml of 25% sucrose, both in the standard buffer containing 0.1 M NaCl, 0.05 M Tris-HCl, and 0.01 M EDTA, pH 7.6) in 12-ml centrifuge tubes. The resulting gradients were spun at 40,000 rpm for 2.5 hr in a Spinco Model L centrifuge; the virus was sedimented into the sucrose pad causing it to become translucent. As much as possible of the liquid above the sucrose was removed by pipet, and the lower layer with any pelletted materials dispersed in it was dialyzed for 3 hr against two or three 1-l. changes of buffer.

Cesium chloride (Trona, American Potash and Chemical Corp., Los Angeles, Calif.) was added to the bag

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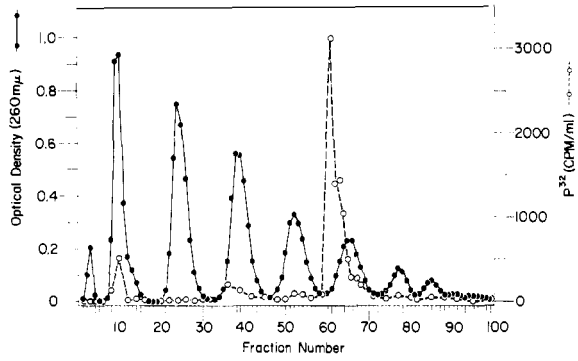

FIGURE 1: Chromatography of polynucleotide kinase labeled RNA after pancreatic ribonuclease degradation. MS2 RNA digest (5 mg) was taken up on a 15 × 1 cm diameter column of DEAE-cellulose and eluted with 1300 ml of 7 M urea–0.02 M Tris-HCl (pH 7.5), 0.0–0.4 M linear gradient of NaCl. Fractions (10 ml) were collected at a flow rate of 0.4 ml/min.

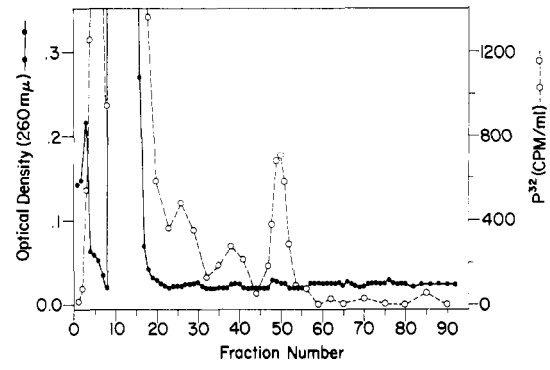

FIGURE 2: Chromatography of an alkaline digest of ^{32}P-labeled MS2 RNA. KOH-digested RNA (10 mg) (5×10^7 cpm) chromatographed on a 14 × 1.2 cm diameter column of DEAE-cellulose, using a gradient of 0.0–0.3 M NaCl in 850 ml of 7 M urea and 0.02 M Tris-HCl buffer. Fractions (8 ml) were collected at a flow rate of 0.3 ml/min.

contents at a level of 0.55 g/ml, and centrifuged at least 18 hr in the cold in a Spinco 40 rotor at 37,000 rpm. If necessary, the virus band was recentrifuged as above until free of materials banding at a different density. Salts were removed by dialysis against the standard buffer. Virus uniformly labeled with ^{32}P was prepared using the same procedure and medium, except that 1–2 mCi/l. of inorganic phosphate was added to the culture at the time of virus infection.

RNA was extracted at 0° using an equal volume of buffer-saturated phenol in the presence of a few drops of 4% bentonite (Singer and Fraenkel-Conrat, 1961). In general three phenol extractions were employed, and the RNA was finally precipitated from 0.1 M NaCl two or three times using ethanol.

Alkaline and Enzymatic Treatment of RNA. Alkaline hydrolyses were performed using 1 M KOH at room temperature for 20–24 hr. The hydrolysate was neutralized with Dowex 50 (H^+), made slightly basic with ammonia solution, filtered to remove resin, and the filtrate and washings were dried in a stream of air.

Digestions with pancreatic ribonuclease (Worthington Biochemical Co.) or ribonuclease T_1 (Sankyo Ltd., purchased from Cal Biochem) were carried out at 37° in 0.05 M Tris-HCl (pH 7.4). Approximately 0.1% (by weight) of enzyme was used, usually added in two portions over a course of 18–24 hr. RNA was treated with *E. coli* alkaline phosphomonoesterase (a gift of Dr. B. Vallee) at a level of 0.2% for 1 hr at 37°. The buffer contained 0.1 M Tris-HCl and 0.01 M $MgCl_2$ (pH 8.0). The reaction was stopped by extraction with an equal volume of buffer-saturated phenol.

Preparations of polynucleotide kinase (Richardson, 1965; Takanami, 1967) were obtained as gifts from Dr. C. C. Richardson and Dr. M. Takanami; the labeling procedure followed exactly the conditions employed by Takanami (1967). ATP labeled in the γ position with ^{32}P was prepared according to Glynn and Chappell (1964). After enzymatic labeling using polynucleotide kinase the reaction products were purified by zonal centrifugation. Samples (1 ml) containing 1–2 mg of MS2 RNA were layered on 28-ml sucrose density gradients (5–20% sucrose in 0.02 M Tris-HCl, pH 7.5) and centrifuged for 18 hr at 24,000 rpm at 4° in an SW 25 rotor and Spinco Model L centrifuge.

Chromatography and Electrophoresis. DEAE-cellulose chromatography in the presence of 7 M urea followed the principles of Tomlinson and Tener (1962). Urea solutions were first deionized with mixed-bed resin and then buffered with 0.02 M Tris-HCl (pH 7.5). Column dimensions and gradients of sodium chloride were determined by the separations to be made. Eluents were desalted as described by Rushizky and Sober (1962).

Paper electrophoresis was carried out in a device as described by Markham and Smith (1952) using 0.05 M ammonium formate (pH 3.5) and Whatman No. 3MM paper. The usual solvent for descending paper chromatography was *n*-propyl alcohol–concentrated NH_4OH–H_2O (6:3:1).

Other Methods. Radioactivity levels were measured with a variety of devices. A gas-flow strip counter (Nuclear-Chicago Actigraph III) was used to locate and roughly approximate ^{32}P on paper chromatograms or electrophoresis strips. Quantitative data were obtained by liquid scintillation counting with a Beckman LS-200 or Packard Tri-Carb instrument. Aqueous samples (0.1–0.2 ml/5 ml of fluid) were counted in Bray's (1960) medium, while samples on paper were measured in a fluid containing 5 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-2′-(5′-phenyloxazole)benzene per l. of toluene.

Analytical ultracentrifugation was carried out at 2–4° in 0.1 M NaCl buffer using a Spinco Model E and its ultraviolet optical system. RNA homogeneity and sedimentation coefficients were evaluated from tracings made with a Joyce-Loebl recording microdensitometer.

Results

Virus and RNA Preparation. MS2 bacteriophage prepared using the techniques outlined appeared to be of a quality similar to that described in the literature. The preparations were essentially homogeneous in the analytical ultracentrifuge and infective to *E. coli* C 3000

(72-B2) at levels of 12–20% of the physical particles present. The yield of virus was generally about 50 mg/l. of lysate. RNA was extracted from the virus in 80–95% yields and was always at least 80% homogeneous in the analytical ultracentrifuge.

Labeling of MS2 RNA with Polynucleotide Kinase. Attempts to label untreated MS2 RNA using polynucleotide kinase and ^{32}P-labeled ATP were uniformly unsuccessful. After isolation from the sucrose gradient, less than 0.05 mole of ^{32}P/mole of viral RNA was found to have been incorporated. Treatment of MS2 RNA with *E. coli* alkaline phosphomonoesterase before the polynucleotide kinase reaction increase the degree of labeling considerably, the levels ranging from 0.2 to 0.8 mole of ^{32}P per mole of RNA in various experiments. The specific activity of ^{32}P across the peak of viral RNA obtained upon zonal centrifugation was nearly uniform, with a slight increase on the slower sedimenting side of the peak. Some degradation of the RNA, presumably due to nucleases in the phosphatase or kinase preparations, was usually apparent but not a major problem in most cases.

Pancreatic ribonuclease degradation of the labeled MS2 RNA was followed by DEAE-cellulose chromatography in the presence of urea (Tomlinson and Tener, 1962). The resulting pattern of optical density and ^{32}P distribution is presented in Figure 1. The greatest portion of the ^{32}P, equal to about 85% of the radioactivity incorporated or 0.7 mole of ^{32}P/mole of RNA initially treated with kinase, is most closely associated with the fifth optical density peak, consisting of materials bearing a net charge of −6 at this pH. A trinucleoside tetraphosphate of the form *p*Pu*p*Pu*p*Py*p* is most consistent with the chromatographic behavior and enzymatic formation of the labeled fragment.

Nature of the Base at the 3'-Linked End of MS2 RNA. About 10 mg of uniformly ^{32}P-labeled MS2 RNA containing 5×10^7 cpm was hydrolyzed with alkali and chromatographed on DEAE-cellulose. A quantity of ^{32}P was eluted after the main mononucleotide peak, as is seen in Figure 2. In various experiments the amount of ^{32}P eluted in a position equivalent to the peak at tube 50 of Figure 2 ranged from 0.07 to 0.13% of the total radioactivity. Such a quantity of ^{32}P is equal to 2.5–4.2 moles of phosphate/mole of nucleic acid of 3300 nucleotide chain length, or to a 60–105% recovery of the nucleoside tetraphosphate end group to be proposed.

The postmononucleotide fractions (44–59) of Figure 2 were pooled, desalted, alkali digested as before, and again chromatographed, this time in the presence of 5 mg of a pancreatic ribonuclease digest of MS2 RNA used as an optical density marker and carrier. The resulting pattern is shown in Figure 3. Only the carrier pentanucleotide peak (charge = −6) shows an appreciable quantity of associated ^{32}P activity. A nucleoside tetraphosphate of the form *ppp*N*p* (a 3'-monophospho-5'-triphosphonucleoside) would be expected to chromatograph with pentanucleotides in this system. Labeled alkali-stable material bearing a net charge of −6 was also located in the expected position in elution patterns obtained after [^{32}P]MS2 RNA was degraded with ribonuclease T_1.

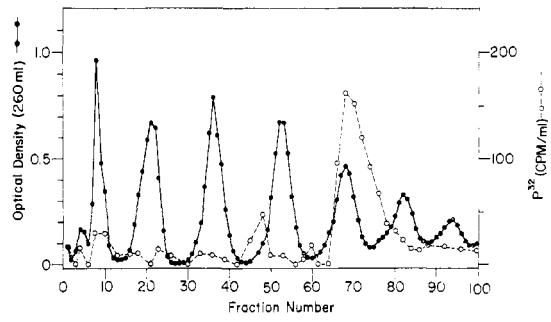

FIGURE 3: Chromatography of the KOH-stable MS2 RNA end group in the presence of a pancreatic ribonuclease digest. Unlabeled ribonuclease-digested MS2 RNA (5 mg) was mixed with 17,000 cpm of ^{32}P-labeled alkali-stable material and chromatographed on an 18 × 1.2 cm diameter column of DEAE-cellulose, using a 0.0–0.3 M NaCl gradient in 1000 ml of 7 M urea plus 0.02 M Tris-HCl (pH 7.5). Fractions (8 ml) were collected at a rate of 0.3 ml/min.

Pooled and desalted ^{32}P-labeled alkali-stable material from a number of experiments was digested with 20 μg of *E. coli* alkaline phosphomonoesterase for 30 min at 37° and chromatographed on DEAE-cellulose with urea containing buffers; carrier amounts of the mono-, di-, and triphosphates of adenosine and guanosine were added. The peaks of the mono-, di-, and triphosphate markers were separately desalted and dried. The monophosphates were separated by electrophoresis at pH 3.5, and the di- and triphosphates by paper chromatography. In each case the majority of the ^{32}P was found to be associated with the guanosine derivative, as is seen in the data of Table I.

The Nature of the Initial Trinucleotide of MS2 RNA. A 30-mg sample (5×10^7 cpm) of [^{32}P]MS2 RNA was digested with pancreatic ribonuclease and chromatographed on DEAE-cellulose in the presence of 7 M urea. Each optical density peak was individually isolated, desalted, and hydrolyzed in 1 M KOH. Each hydrolysate was then chromatographed as above in the presence of 2 mg of a pancreatic ribonuclease digest of unlabeled RNA, and the elution patterns were examined for the presence of ^{32}P in the area of the pentanucleotide marker peak. The quantity of such alkali-stable material found in each peak is plotted with the original pancreatic ribonuclease digestion pattern of optical density in Figure 4. The only peak containing appreciable quantities of alkali-stable material is that corresponding to a charge of −8. This result is consistent with the structure *ppp*Pu*p*Pu*p*Py*p*. Recovery of the trinucleo-

TABLE I: Identification of the Initial Nucleoside of MS2 RNA.

Nucleoside	^{32}P Recovered (cpm) as		
	Mono-phosphate	Diphos-phate	Triphos-phate
Adenosine	14.5	34.5	34.0
Guanosine	63.5	301.5	181.0

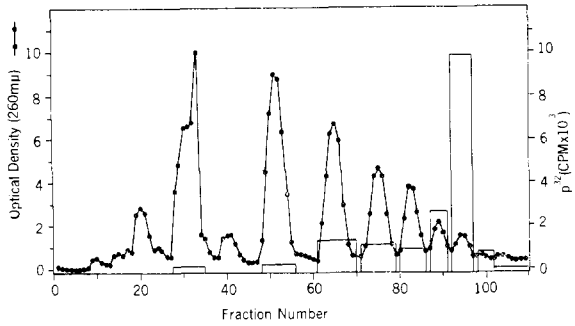

FIGURE 4. Location of the ribonuclease oligonucleotide containing the 3′-linked end of MS2 RNA. Pancreatic ribonuclease digest (30 mg) (5×10^7 cpm) of ^{32}P-labeled MS2 RNA was chromatographed on a 24 × 1.8 cm diameter column of DEAE-cellulose using 1500 ml of a 0.0–0.4 M NaCl gradient in 7 M urea–0.02 M Tris-HCl (pH 7.5). Fractions (10 ml) were collected at a rate of 0.5 ml/min. Each individual peak was then hydrolyzed in KOH and the digests were chromatographed on 10 × 0.8 cm diameter columns of DEAE-cellulose using 600 ml of linear 0.0–0.4 M NaCl gradient in buffered urea. KOH-stable materials of net charge −6 were counted and the data were plotted in relation to the original position of elution of the parent oligonucleotide peaks.

tide was 70–112% as estimated from the amount of ^{32}P which could be recovered as nucleoside tetraphosphate.

Using a second preparation of pancreatic ribonuclease degraded MS2 RNA labeled uniformly with ^{32}P, the peak containing the intact trinucleoside hexaphosphate was desalted and applied to paper for electrophoresis at pH 3.5. Under these conditions the end group *ppp*G*p* migrated with a mobility relative to picric acid of 1.3, and it was assumed that a triphosphorylated trinucleotide would also have a high mobility. Material moving with or in front of picric acid was eluted, concentrated, and electrophoresis repeated until a single symmetrical peak of radioactivity with a mobility relative to picric acid of 1.2 was isolated.

Alkaline hydrolysis of this material, followed by paper electrophoresis, resulted in the separation of three radioactive peaks: a major component of relative mobility 1.3 presumed to be *ppp*G*p*, and two smaller peaks corresponding exactly to uridylic and guanylic acid markers. A second sample of trinucleoside hexaphosphate was digested with ribonuclease T_1 and chromatographed on paper. Again three radioactive products were detected by strip counting; the major peak moved with and somewhat behind nucleoside triphosphate markers, while two smaller peaks were associated with guanylic and uridylic acid markers. Quantitative data are presented in Table II.

TABLE II: Degradation of the 3′-Linked End Trinucleotide of MS2 RNA.

Component	^{32}P Recovered (cpm)	
	Alkaline Hydrolysis	RNase T_1 Hydrolysis
Guanosine tetraphosphate	507	302
Adenylic acid	10	
Uridylic acid	110	81[a]
Cytidylic acid	11	
Guanylic acid	122	67

[a] Adenylic, cytidylic, and uridylic acids were not cleanly separated in the system used for chromatography, and the value represents the sum of all three components.

Discussion

Labeling of the 3′-Linked End Using Polynucleotide Kinase. These experiments resulted in the labelling of MS2 RNA only after treatment of the RNA with phosphomonoesterase, indicating that the end of the nucleic acid is naturally phosphorylated. A similar conclusion was reached by Takanami (1967) in the case of rRNA and of the RNA of the bacterial virus f2, a close relative of MS2. Once the natural phosphate end has been altered the MS2 RNA becomes a polynucleotide kinase substrate and a ^{32}P label can be attached to the end of the molecule. Again this result is in agreement with Takanami, and the same explanation is offered.

Pancreatic ribonuclease degradation of the kinase-labeled material results in the formation of a ^{32}P-containing product which chromatographs in the Tomlinson and Tener system along with pentanucleotides, indicating that the labeled fragment bears a net charge of −6 (see Figure 1). Since the label is present as a 5′-monophosphate group, and pancreatic ribonuclease results in the formation only of pyrimidine 3′-phosphate ended products, the sole possible structure for the fragment is that of a trinucleoside tetraphosphate, *p*Pu*p*Pu*p*Py*p*. No other chain length would allow the observed chromatographic properties, and no other base sequence could arise from pancreatic ribonuclease degradation.

Nature of the Initial Purine Base. Alkaline hydrolysis results in the breakage of internucleotide linkages to form 2′(3′)-mononucleotides. One or more phosphates on the free 5′ position of a polynucleotide are stable to such hydrolysis (see Bremer *et al.*, 1965), and the resulting additional charges at neutral pH would be expected to make the nucleoside derivative chromatographically and electrophoretically distinctive. This predicted result is found. An alkali-stable fraction of ^{32}P is eluted with the carrier peak of pentanucleotides of Figure 3. In order to bear the necessary −6 charges the nucleoside derivative must bear a 5′-triphosphate as well as a 3′-monophosphate; it is designated *ppp*N*p* (where N represents the nucleoside).

The alkaline phosphomonoesterase of *E. coli* will attack any monoesterified phosphate grouping (Heppel *et al.*, 1962), and so enzymatic treatment of the derivative above would be expected to result in the formation of a variety of products (*pp*N*p*, *p*N*p*, N*p*, *pp*N, *p*N, and N). Separation by DEAE-cellulose chromatography to give charge groups of −2 (*p*N + N*p*), −3 (*pp*N), and −4 (*p*N*p* + *ppp*N) simplifies the product pattern some-

what and allows further fractionation and identification of N as guanosine. This conclusion is reinforced by the finding that nucleoside tetraphosphate appears in ribonuclease T_1 digests of MS2 RNA, since the enzyme is highly specific in its hydrolysis only at guanosine residues to give guanosine 3′-phosphate (Sato and Egami, 1957). The identification of the nucleoside tetraphosphate in T_1 digests is not completely clear cut, however, since the fractionation pattern with DEAE-cellulose is rather complex (see Bartos *et al.*, 1963).

The Initial Trinucleotide Sequence of MS2 RNA. The polynucleotide kinase experiments indicate a sequence in which two purines are followed by a pyrimidine. Since in the unaltered RNA the initial 5′ position is occupied by a triphosphate grouping, pancreatic ribonuclease digestion of intact RNA should result in the formation of a trinucleoside hexaphosphate. Such a fragment would bear a charge of −8 and would chromatograph in the vicinity of the heptanucleotide fraction of the digest. This result is seen in Figure 4, and an initial sequence of *ppp*G*p*Pu*p*Py*p* is thus indicated.

Both alkaline hydrolysis and digestion with ribonuclease T_1 result in the liberation of approximately equimolar quantities of guanylic and uridylic acids from the trinucleoside hexaphosphate. Four moles of phosphate per mole guanylic or uridylic acid are found associated with the nucleoside tetraphosphate *ppp*G*p*. The only sequence compatible with these data is *ppp*G*p*G*p*U*p* (see Table II).

Significance of the Sequence. It is of interest that the RNA in the intact virus retains the 5′-triphosphate terminus from the newly biosynthesized RNA (see Banerjee *et al.*, 1967; Bremer *et al.*, 1965; Maitra *et al.*, 1965). It is not degraded to the level of monophosphate as in tRNA (see RajBhandary *et al.*, 1964) or to a 5′-hydroxyl group as is the case with TMV–RNA (Fraenkel-Conrat and Singer, 1962). It is still conceivable that the triphosphate is altered during or after the process of infection, or even that it interferes with infection since only a minority of the physical virus particles are found to result in plaque formation. The only sure conclusion possible from the results at hand is that the majority of 3′-linked ends is present as the 5′-triphosphate. The identification of similar triphosphate-ended products of RNA polymerase reactions has been previously reported by at least three groups, cited above.

The initial triplet of MS2 RNA does not appear to be one of those implicated in chain initiation in protein synthesis. Stanley *et al.* (1966) have reported that MS2 RNA and other messengers require the presence of certain initiation factors for appreciable cell-free amino acid incorporation. The process of polypeptide initiation involves formylmethionine and its tRNA, and may be coded by the triplets UUG, AUG, and GUG (Salas *et al.*, 1967); no report has been located which suggests GGU as a possible starting signal. One may speculate that the initial triphosphorylated guanosine is not involved, and that a hypothetical guanosine in the fourth position provides a possible GUG initiation point. Or it is possible that the first initiation sequence is simply further along the chain.

The GGU sequence does not correspond to the code for alanine, the N-terminal amino acid of the MS2 coat protein (Lin *et al.*, 1967). Glycine is coded by GGU, while alanine would require a GCN triplet (see Singer and Leder, 1966). In short, no direct evidence for the meaning of the initial triplet is at hand.

Acknowledgments

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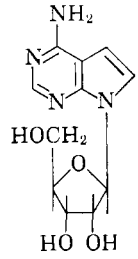
Nucleic Acids. V. Nucleotide Derivatives of Tubercidin (7-Deazaadenosine)*

Arthur R. Hanze

ABSTRACT: The syntheses of tubercidin 3′,5′-cyclic phosphate (cyclic 3,5-TuMP), the methyl ester of tubercidin 5′-phosphate (MepTu), tubercidinyl-(2′→5′)-thymidine (Tu^pT), tubercidinyl-(3′→5′)-thymidine (TupT), and thymidylyl-(3′→5′)-tubercidin (TpTu) are reported. An improved synthesis of tubercidin 5′-phosphate is given. The biological properties of these compounds are compared with those of tubercidin.

Tubercidin[1] I (7-deazaadenosine), a nucleoside described by Susuki and Marumo (1960), possesses both antitumor and antiviral activity and is highly cytotoxic (Acs *et al.*, 1964; Duvall, 1963; Owen and Smith, 1964).



Acs *et al.* (1964) studied the metabolism of tubercidin (Tu) in mouse fibroblasts and showed that Tu is incorporated into both RNA and DNA. They also reported that tubercidin 5′-triphosphate (TuTP) could replace ATP in the *Escherichia coli* RNA polymerase reaction and that it was used efficiently in heteropolymer as well as homopolymer formation. Nishimura *et al.* (1966) found that TuTP could replace ATP in the *E. coli* polymerase reaction with some thymidine-containing synthetic deoxypolynucleotides as templates but not with others. Ikehara and Ohtsuka (1965) prepared the trinucleoside diphosphate tubercidinyl-(3′→5′)-adenylyl-(3′→5′)-adenosine (TupApA) and showed that it stimulated the binding of Lys-$tRNA^{Lys}$ and Thr-$tRNA^{Thr}$ to ribosomes. In light of these interesting activities of Tu, its similarity to adenosine on the one hand and its high activity as an antitumor and antiviral agent on the other, we instituted a program to prepare some derivatives which might possess greater selectivity of action in biological systems.

One derivative in which we were particularly interested was the tubercidin analog of adenosine 3′,5-cyclic phosphate (cyclic AMP). Cyclic AMP has been shown (Sutherland and Rall, 1960) to occur widely in animal tissues. It promotes the accumulation of active phosphorylase in the tissues, causing increased glycogenolysis. For the synthesis of the analogous tubercidin 3′,5′-cyclic phosphate (cyclic 3,5-TuMP), tubercidin 5′-phosphate (pTu) was required. Although the synthesis of the latter compound has already been reported (Pike *et al.*, 1964), the yield was low and the isolation difficult. By protecting the amine function, we were able to double the over-all yield of pTu and isolate it by direct crystallization. The synthesis of this compound and its conversion into cyclic 3,5-TuMP is outlined in Figure 1. 2′,3′-Isopropylidenetubercidin (II) was benzoylated to give N^6,N^6,5′-tribenzoylisopropylidenetubercidin (III). Selective O debenzoylation gave N^6-benzoylisopropylidenetubercidin (IV). Phosphorylation of this compound by the Tener procedure (Tener, 1961) yielded the crystalline 5′-cyanoethyl phosphate (V), which was converted into isopropylidenetubercidin 5′-phosphate (VI) by treatment with aqueous methanolic ammonium hydroxide. Removal of the isopropylidene group with 80% aqueous acetic acid gave crystalline pTu VII directly. Reaction of VII with DCC in pyridine in the presence of 4-mor-

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[1] Abbreviations used: Tu, tubercidin; cyclic 3,5-TuMP, tubercidin 3′,5′-cyclic phosphate; MepTu, methyl ester of tubercidin-5′-phosphate; Tu^pT, 7-deazaadenylyl-(2′→5′)-thymidine; TupT, 7-deazaadenylyl-(3′→5′)-thymidine; TpTu, thymidylyl-(3′→5′)-tubercidin; pTu, tubercidin 5′-phosphate; pCpCpA, 5′-phosphorylcytidylyl-(3′→5′)-cytidylyl-(3′→5′)-adenosine; MT, methoxytrityl; pTAc, 5′-thymidylic acid 3′-acetate; ATP, adenosine triphosphate; DCC, dicyclohexylcarbodiimide.