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Isolation and Characterization of Terminal Polynucleotide Fragments from Bacteriophage Ribonucleic Acids*

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ABSTRACT: Procedures have been developed for the isolation and purification of polynucleotide fragments deriving from the terminals of large ribonucleic acids. The method depends on the selective periodate oxidation of the 2′,3′-diol groups of the terminal fragment after cleavage of the ribonucleic acid, and the specific binding of this fragment to aminoethylcellulose.

After the unoxidized polynucleotide fragments have been eluted from the cellulose the terminal fragment is released by a β-elimination reaction and is thus isolated in a form which lacks its terminal nucleoside. The method was developed initially with studies on dinucleoside phosphates and was then applied to the isolation of terminal polynucleotides from the ribonuclease T_1 digests of three bacteriophage ribonucleic acids. The fragments obtained from the bacteriophages, f2 and Qβ, have been characterized by alkaline and enzymatic hydrolyses and shown to have the compositions $(A_2U_2C_4)$-Cp and (C_9U_4)-Cp, respectively.

As an initial phase in a study of the relation between the primary structure and function of large RNA molecules, work in this laboratory has been directed toward the development of methods for the determination of nucleotide sequences near the terminals of these molecules (Lee and Gilham, 1965, 1966; Weith *et al.*, 1968; Weith and Gilham, 1967, 1969). It had been anticipated earlier that sequence studies near the terminals would be greatly facilitated by the fact that large ribonucleic acids such as those occurring in ribosomes and viruses possess terminals whose unique character can be maintained even after the molecules have been cleaved into small fragments. For example, if the RNA molecule is cleaved by one of the cyclizing ribonucleases or by alkali, the fragment which contains the left-hand terminal is distinguished by the fact that it possesses a phosphorylated 5′-hydroxyl group while the fragment corresponding to the right-hand end differs from all other fragments in that it contains an unphosphorylated 2′,3′-diol group. In the present work, the uniqueness of this terminal diol group together with the reactivity of its oxidation product are exploited in a method which involves the selective immobilization of terminal polynucleotides on aminoethylcellulose.

The specific oxidation of the terminal diol group of polyribonucleotides by periodate has been used in a number of laboratories as the basis for various methods of fractionation. For example, Zamecnik *et al.* (1960) have used this reaction in a method for tRNA fractionation where the change in solubility caused by the condensation of oxidized tRNA with a hydrazide allows the removal of those tRNA molecules containing free 2′,3′-diol groups from those containing a particular aminoacyl group at the 2′ or 3′ positions. A similar type of fractionation of a tRNA mixture was achieved when the tRNA molecules lacking an aminoacyl substituent were oxidized with periodate and then removed by absorption to aminoethylcellulose (Zubay, 1962). Yolles (1964) has also studied the binding of oxidized polyribonucleotides to a cellulose derivative (*p*-hydrazinobenzylcellulose) and, in addition, he investigated a procedure, involving displacement with benzaldehyde, for the recovery of the bound material. Unfortunately, this approach produced rather low yields in both the binding and recovery steps. Methods for the release of oxidized polynucleotides bound to aminoethylcellulose have also been studied (Habermann *et al.*, 1966; Temmerman, 1967). In these procedures the release was effected by elution of the cellulose with hydrochloric acid. However, in view of the expected instability of polynucleotides under such conditions, it seems that this approach may not be applicable to the isolation of polynucleotides of any considerable length.

The aim of the present work was to study methods for the

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efficient binding and release of oxidized polynucleotides on aminoethylcellulose under conditions that would be expected to maintain the primary structure of the molecules concerned, and to apply this approach to the sequence analysis of the structures near the terminals of large ribonucleic acids. A brief report of this work has been published (Lee and Gilham, 1966).

Experimental Procedures

Materials. Samples of the bacteriophages were obtained from Dr. N. D. Zinder (f2), Dr. S. Spiegelman (Qβ), and Dr. I. Watanabe (GA). Tricine buffer and ribonuclease T_1 were purchased from Calbiochem, Los Angeles, Calif. Alkaline phosphatase and pancreatic ribonuclease were the products of Worthington Biochemical Corp., Freehold, N. J. The DEAE-cellulose and the DEAE-Sephadex were pretreated according to the directions of the respective manufacturers and the triethylammonium and propylammonium bicarbonates were prepared by cooling an aqueous solution of the appropriate redistilled amine and bubbling in carbon dioxide until the pH of the solution was 7.5.

Growth and Purification of Bacteriophages. The RNA phages were grown in 40-l. batches using the methods described by Overby *et al.* (1966) except that *Escherichia coli* C3000 was used as the host cell. The phages were isolated by modifications of the methods described by Rushizky *et al.* (1965) and Robinson *et al.* (1969). At the end of the 6–8-hr virus growth period each lysate (40 l.) was transferred to glass vessels and quickly chilled to 0°. EDTA (disodium salt, 70 g) and chloroform (300 ml) were added and the total mixture was stirred for 1 hr. All subsequent operations were carried out at 4°. Ammonium sulfate (12 kg) was added to the lysate and the stirring was continued for 1 hr. The mixture was allowed to stand for about 8 hr and then passed through a Sharples centrifuge (AS-14) at a flow rate of 20 l./hr with a rotor speed of 15,000 rpm. The resulting pellet was suspended in 0.001 M EDTA buffer (pH 7.0, 3 l.) and then stirred for 3 hr. The mixture was centrifuged (Lourdes 3RA rotor; 7500 rpm; 45 min); the supernatant was collected and ammonium sulfate (900 g) was added to the solution with stirring. When the salt had dissolved the stirring was continued for a further 2 hr and the suspension was then centrifuged (3RA head; 7500 rpm; 45 min). The pellet was suspended in 0.001 M EDTA buffer (pH 7.0, 1 l.) with stirring and then dialyzed against two 6-l. portions of the same buffer for 12 hr. Insoluble material was removed by centrifugation (Lourdes 3RA rotor; 7500 rpm; 45 min) and the phage was then obtained from the supernatant by centrifugation in the Spinco Model L ultracentrifuge (21 rotor; 21,000 rpm; 8 hr). The clear pellets of phage were combined and suspended in 0.1 M sodium acetate–0.001 M EDTA buffer (pH 6.0, 50 ml), and any insoluble material was removed by centrifugation (Lourdes 9RA rotor; 15,000 rpm; 30 min). The phage concentration was determined spectrophotometrically in 0.1 M sodium phosphate buffer (pH 7.0) using the value, 8.2 ODU_{260}/mg derived for the bacteriophage R17 (Enger *et al.*, 1963). The yields of purified phage determined in this way varied from 1.5 g to 3.5 g/40 l. of lysate.

Bacteriophage Ribonucleic Acids. A. PREPARATION. The RNA of each phage was extracted by a modification of a previously described method (Robinson *et al.*, 1969). All operations were carried out at 4° using glassware which had been cleaned with strong acid and alkali before use to avoid contamination of the preparations with nucleases. Bentonite was purified according to Fraenkel-Conrat *et al.* (1961) and was suspended in the RNA extraction buffer: 0.1 M sodium acetate–0.001 M EDTA (pH 6.0), such that 1 ml of the suspension contained 30–40 mg dry weight of bentonite. The phage solution (1 g in 50 ml of the RNA extraction buffer) was mixed in a polyethylene bottle with the bentonite suspension (15 ml), 10 M lithium chloride (7 ml), 20% sodium dodecyl sulfate solution (1.6 ml), and redistilled phenol (75 ml) which had been previously equilibrated with the RNA extraction buffer. The mixture was shaken by hand for 10 min and the resulting emulsion was broken by centrifugation in polyethylene tubes at 15,000 rpm for 20 min. The water-rich upper layers were combined in another polyethylene bottle and subjected to a second extraction with a mixture of buffer-saturated phenol (75 ml) and bentonite suspension (15 ml). The mixture was shaken and centrifuged and the water-rich layers were removed as before. The phenol-rich layers from the first extraction were then extracted with a mixture of the RNA extraction buffer (65 ml) and 10 M lithium chloride (7 ml). The mixture was shaken and centrifuged as before and the water-rich layers from this mixture were used to extract the phenol-rich layers from the second extraction. Finally the two water-rich extracts were combined and centrifuged in polyethylene tubes at 105,000*g* for 1 hr to remove bentonite. The supernatant (135 ml) was mixed with 4 M sodium acetate, pH 6.0 (10 ml), and cold ethanol (400 ml) was added. The mixture was kept overnight at −20° and the resulting precipitate was collected by centrifugation at 3000*g* for 10 min. The precipitate was washed three times with cold ethanol and dried *in vacuo*. The RNA (*ca.* 300 mg) was then dissolved in water (30 ml) and reprecipitated by the slow addition of sodium acetate trihydrate (22.5 g) with stirring. The mixture was allowed to stand overnight and the precipitate was collected by centrifugation as before and then washed with cold ethanol until all the excess sodium acetate had been removed. After drying *in vacuo* the product was obtained as a fine white powder in 70–90% yield. Yields were based on the assumption that these phages contain 34% by weight of RNA. Since the RNA product is usually obtained in a state which contains a variable amount of water the yields of RNA were estimated by determining the total ODU_{260} obtained and converting this into weight using the ODU_{260}/mg values derived below for the sodium salt of each ribonucleic acid.

B. BASE RATIOS. The nucleotide compositions of the ribonucleic acids were determined by a method developed in this laboratory for the analysis of oligonucleotides. The RNA (*ca.* 0.5 mg) was dissolved in 0.25 M sodium hydroxide (0.1 ml) and kept at 37° for 20 hr. The pH of the hydrolysate was adjusted to 9.5 by the addition of 0.25 M ammonium acetate (0.2 ml) and the mixture was applied to a DEAE-Sephadex (A-25-120) column (120 × 0.35 cm) which had been packed in 0.4 M ammonium acetate (pH adjusted to 9.0 with ammonia) and equilibrated with 0.05 M ammonium acetate (pH adjusted to 9.0 with ammonia). The 2′- and 3′-phosphates of cytidine and adenosine were then eluted in separate peaks with 0.1 or 0.2 M ammonium acetate (pH set at 10.0 with ammonia). Under these conditions the 2′- and 3′-phosphates of uridine and guanosine are retained and

these four products are subsequently eluted in separate peaks with 0.4 M ammonium acetate (adjusted to pH 9.0 with ammonia). The amounts of each nucleotide were then determined spectrophotometrically except that, in the case of the uridine and guanosine nucleotides, it was first necessary to lower the pH of the fractions by passing in water-saturated carbon dioxide in order to avoid reading the absorbance of these components within the range of their pK values. The results obtained for the ribonucleic acids of the three bacteriophages are given in Table I.

C. HYPERCHROMICITY AND ABSORPTIVITY. The ribonucleic acids were degraded to nucleotides by treatment with 0.25 M sodium hydroxide at 37° for 20 hr and the per cent hyperchromicity was taken as the per cent change in ODU_{260} (measured in 0.1 M sodium phosphate buffer, pH 7.0) resulting from this hydrolysis. The values obtained for the three ribonucleic acids are shown in Table I. All absorption measurements on the ribonucleic acids were carried out in 0.1 M sodium phosphate buffer (pH 7.0) to avoid the hyperchromic effect which occurs in solutions of low ionic strength. The per cent hyperchromicity values were used to calculate the absorptivities as shown below and these values are also listed in Table I.

Absorption of Oxidized Polynucleotides on Aminoethylcellulose. Aminoethylcellulose (Whatman AE 11; 1 mequiv/g; 6 g) was suspended in 0.5 M *n*-propylammonium bicarbonate (250 ml) and kept at 45° for 90 min with occasional stirring. The solution was removed by decantation and the cellulose was suspended in 4 M sodium chloride (250 ml) and stirred for 10 min. The cellulose was collected, resuspended in 4 M sodium chloride (250 ml), and then allowed to settle. The cellulose was finally suspended in 4 M sodium chloride–0.01 M Tricine buffer (pH 8.0 at 20°) and the suspension was adjusted to pH 8.0 at 20°. Unless stated to the contrary all subsequent operations were carried out at 4°. This suspension was used to prepare the cellulose column (24 × 1.8 cm) which was then washed with the above sodium chloride–Tricine buffer (300 ml). The oxidized polynucleotide solution (*ca.* 12 ml) was run onto the column in 5 min and the column was then eluted with the sodium chloride–Tricine buffer at a flow rate of 2 ml/hr for 5 hr. The flow rate was then increased to 10 ml/hr and elution was continued until no further ultraviolet-absorbing material appeared in the eluate. The column was washed with 0.1 M sodium bicarbonate (pH 8.0, 125 ml) at a flow rate of 200 ml/hr to displace all chloride ions from the column. The sodium bicarbonate was then removed by washing the column with water (125 ml) at 200 ml/hr. The aminoethylcellulose was extruded from the column and mixed with 1 M *n*-propylammonium bicarbonate (60 ml) and the mixture was kept at 45° for 2 hr with occasional stirring. The mixture was poured into a column (2.5 cm diameter) and the propylammonium bicarbonate was allowed to drain through. The cellulose was then eluted with 1 M triethylammonium bicarbonate (150 ml) at room temperature. The combined propylammonium bicarbonate and triethylammonium bicarbonate eluates were evaporated to dryness *in vacuo*.

Oxidation and Absorption of Dinucleoside Phosphates. Adenylyl-(3′–5′)-uridine, uridylyl-(3′–5′)-adenosine, or guanylyl-(3′–5′)-adenosine (10–50 ODU_{260}) in 0.02 M sodium phosphate buffer (pH 7.0, 10 ml) was treated with sodium metaperiodate (107 mg) and, after the oxidant had dissolved, the solution was kept at 0° for 30 min. Sodium chloride (2.4 g) and potassium chloride (80 mg) were added and, when the salts had dissolved, the mixture was adjusted to pH 8 with concentrated sodium hydroxide solution. The mixture was kept at 0° for 15 min and the precipitate was removed by centrifugation. The precipitate was washed with two 1-ml portions of cold 4 M sodium chloride–0.01 M Tricine buffer (pH 8.0 at 20°). The supernatant and washings were then absorbed to the aminoethylcellulose column and the bound material was recovered as described above. The *n*-propylammonium and triethylammonium bicarbonate eluates were concentrated to dryness and the product was chromatographed on Whatman No. 3MM paper with *n*-propyl alcohol–concentrated ammonia–water (55:10:35) as solvent. The 3′-phosphates of adenosine, uridine, and guanosine deriving from the above three dinucleoside phosphates, respectively, were thus obtained in 65–75% yield.

TABLE I: Base Ratios, Hyperchromicities, and Absorptivities of Phage Ribonucleic Acids.

Ribonucleic Acid	Mole %				Hyperchromicity (%)	Absorptivity (ODU_{260}/mg)
	A	C	G	U		
Qβ	23.0	23.4	24.4	29.2	48	21.5
f2	22.2	25.4	26.8	25.4	49	21.2
GA[a]	22.5	24.7	24.6	28.2	45	21.8

[a] Experiments on GA RNA were carried out by G. T. Asteriadis.

Isolation and Purification of Terminal Polynucleotides from Phage Ribonucleic Acids. The phage RNA (300 mg) was dissolved in water (10 ml) and ribonuclease T_1 (1.5 mg) was added. The hydrolysis was allowed to proceed at 20° for 4 hr with the pH being maintained at 7.5 by the addition of 0.1 N sodium hydroxide with stirring. A few drops of toluene were added and the mixture was allowed to stand at 20° for a further 15 hr. The toluene was then removed by passing a stream of air over the mixture. Sodium metaperiodate (107 mg) was added and, after the oxidant had dissolved, the mixture was kept at 0° for 30 min. Sodium chloride (2.4 g) and potassium chloride (80 mg) were added and, when the salts were dissolved, the solution was brought to pH 8 with concentrated sodium hydroxide. The mixture was kept at 0° for 15 min and the precipitate was removed by centrifugation. The precipitate was washed with two 1-ml portions of cold 4 M sodium chloride–0.01 M Tricine buffer (pH 8.0 at 20°). The supernatant and washings were then absorbed to the aminoethylcellulose column and the terminal fragment recovered as described above. After the removal of the *n*-propylammonium and triethylammonium bicarbonates the product was dissolved in water and applied to a column of DEAE-cellulose (Whatman DE 23; HCO_3^- form; 55 × 0.5 cm). Chromatography was effected with 600 ml of water containing a linear gradient of triethylammonium bicarbonate (0.1–1.0 M) at a flow rate of 15 ml/hr and the elution was monitored by the measurement of the optical density of the eluate at 270 mμ. Two typical elution patterns are shown in Figure 1. The yields of terminal fragments purified in this way

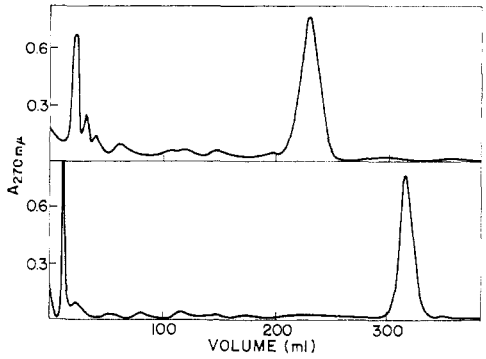

FIGURE 1: Elution patterns from the column chromatography of the terminal polynucleotides from f2 RNA (upper curve) and Qβ RNA. The column consisted of DEAE-cellulose (Whatman DE 23; HCO_3^- form; 55 × 0.5 cm) and the elution was effected with 600 ml of water containing a linear gradient of triethylammonium bicarbonate (0.1–1.0 M) at a flow rate of 15 ml/hr.

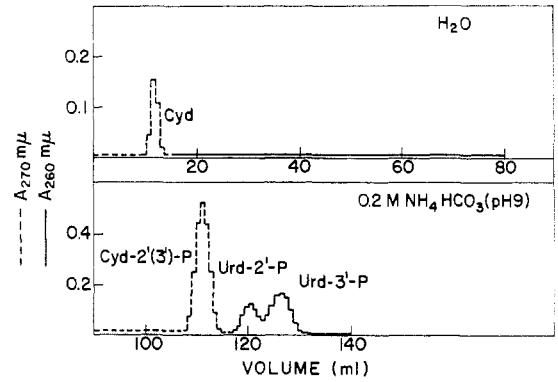

FIGURE 3: Fractionation of the products from the treatment of the Qβ terminal polynucleotide with phosphatase and alkali as described in the Experimental Section. The conditions for the column chromatography were identical with those used for the fractionation shown in Figure 2.

were 60–70%. The yields were calculated by reference to the theoretical values derived as described below.

Characterization of Terminal Polynucleotides. A. FRACTIONATION OF ALKALINE HYDROLYSIS PRODUCTS ON DEAE-SEPHADEX BICARBONATE. The polynucleotide (2.5–3.5 ODU_{260} was dissolved in 0.3 ml of Tris chloride buffer (0.03 M, pH 8.2) containing 0.1 mg of bacterial alkaline phosphatase (2 units). After incubation at 37° for 3 hr 1 M sodium hydroxide (0.1 ml) was added such that all the phosphatase solution in the tube came in contact with the alkali. The hydrolysis was allowed to proceed for 20 hr at 37° and then water (0.6 ml) was added. The pH of the mixture was adjusted to 8.8–9.0 by first passing in excess carbon dioxide and then heating the solution at 45° for about 10 min. The hydrolysate was fractionated on a DEAE-Sephadex column (A-25-120; HCO_3^- form; 100 × 0.4 cm) using solvents which contained 1% butanol to discourage microbial growth. Before use, the column was washed extensively with 0.2 M ammonium bicarbonate, pH 9.0, and finally with water (15 ml). The nucleosides were eluted with water at a flow rate of 2–3 ml/hr and the nucleotides were eluted with 0.2 M ammonium bicarbonate, pH 9.0, at the same flow rate. The nucleosides and nucleotides were recognized by their position of elution and were quantitated spectrophotometrically. Fractions containing uridine and guanosine and their respective nucleotides were first treated with excess water-saturated carbon dioxide in order to avoid reading the absorbance of these components within the range of their pK values. Typical elution patterns are shown in Figures 2 and 3.

B. FRACTIONATION OF ALKALINE HYDROLYSIS PRODUCTS ON DEAE-SEPHADEX ACETATE. The phosphatase digest was prepared as above and, after the incubation period, it was evaporated to dryness and the product was dissolved in 0.25 N sodium hydroxide (0.2 ml). The mixture was incubated at 37° for 20 hr and its pH was then adjusted to 9.5 by the addition of 0.25 M ammonium acetate (0.4 ml). The mixture was diluted with water (0.6 ml) and chromatographed on a DEAE-Sephadex (A-25-120) column with solvents containing 1% butanol. The column (120 × 0.35 cm) was packed in 0.4 M ammonium acetate (pH adjusted to 9.0 with ammonia) and then washed with 0.05 M ammonium acetate (pH adjusted to 9.0 with ammonia). After application of the hydrolysate, elution was carried out with the latter solvent. Under such conditions the nucleosides are eluted separately in the order cytidine, adenosine, uridine, and guanosine. The nucleotides are then eluted and quantitated as described above for the determination of the base compositions of the phage ribonucleic acids.

C. FRACTIONATION OF RIBONUCLEASE HYDROLYSIS PRODUCTS. The polynucleotide (2–3 ODU_{260}) was dissolved in 0.2 M ammonium bicarbonate, pH 9.0 (0.3 ml), and an aqueous solution of pancreatic ribonuclease (0.1 ml containing 0.05 mg) was added. The mixture was kept at 20° for 15 hr in the presence of a drop of chloroform. The products were fractionated on DEAE-Sephadex bicarbonate as described above for the products of the alkaline hydrolysis. The mononucleotides were eluted with 0.2 M ammonium bicarbonate, pH 9.0, and the dinucleotides with 0.4 M ammonium bicarbonate, pH 9.0, at 2–3 ml/hr.

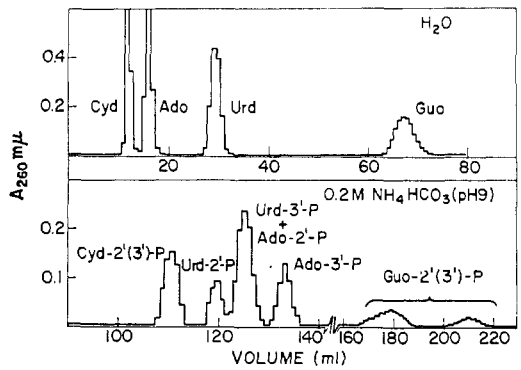

FIGURE 2: Fractionation of a mixture of the four nucleosides and the eight 2'(3')-nucleotides. The column consisted of DEAE-Sephadex (A-25-120; HCO_3^- form (pH 9.0); 100 × 0.4 cm) and the elution was effected with the solvents as shown at a flow rate of 2–3 ml/hr.

Results and Discussion

The bacteriophages whose ribonucleic acids were used in the present study were chosen from three serologically and

physicochemically distinct groups (Watanabe *et al.*, 1967a,b). The choice was made with the hope that, ultimately, information obtained with regard to similarities or differences in their nucleotide sequences might be related to similarities or differences in the functions of the ribonucleic acids and to the serological and physical differences in the phages themselves. Another factor in the choice of RNA resulted from the observation that these bacteriophages can be grown to very high yields using *E. coli* C3000 as the host, thus providing a ready supply of material for structural studies. In order to calculate both the percentage yields of RNA obtained from these phages and the percentage yields of terminal polynucleotides produced after cleavage of the molecules it was found useful to derive theoretical values for the absorptivity, Z (in optical density units/mg at 260 mμ), for the dry sodium salt of each RNA (measured in 0.1 M sodium phosphate buffer, pH 7.0) thus:

$$Z = \frac{100}{100 + x} \cdot \frac{a\epsilon^a_{260} + c\epsilon^c_{260} + g\epsilon^g_{260} + u\epsilon^u_{260}}{352a + 328c + 368g + 329u}$$

where x is the per cent hyperchromicity as defined above and a, c, g, u are the mole per cent values of adenosine, cytidine, guanosine, and uridine, respectively, for the composition of each RNA. ϵ^a_{260}, ϵ^c_{260}, ϵ^g_{260}, and ϵ^u_{260} are the molar absorptivities at 260 mμ for the 2′(3′)-phosphates of the same four nucleotides measured in 0.1 M sodium phosphate buffer (pH 7.0). The values of the absorptivities at 260 mμ used in these calculations were 15,000; 7,400; 11,700; and 9,900, respectively, and the absorptivity value obtained in this way for each RNA is shown in Table I.

In developing procedures for the isolation of terminal polynucleotides a number of experiments were carried out using the model compounds, adenylyl-(3′–5′)-uridine, uridylyl-(3′–5′)-adenosine, and guanylyl-(3′–5′)-adenosine. The various aspects of the isolation method were tested separately and optimum conditions were found for each step. For example, the most important factor involves the conditions necessary for the maximum binding to the aminoethylcellulose of the polynucleotide after it has been oxidized to the corresponding dialdehyde by periodate. It was important also, for the subsequent application of the method, that these conditions were such that those molecules which were unoxidized by periodate should not be bound to the cellulose matrix. These requirements were satisfied by carrying out the absorption step over a period of 5–8 hr in 4 M sodium chloride at pH 8 and at 4°. It was found that, although the reaction was not particularly pH dependent, the introduction of buffers such as bicarbonate or pyrophosphate inhibited the binding step. However, once the complex between the dialdehyde and the amino groups on the cellulose had formed it was shown to be quite stable to prolonged treatment with bicarbonate ions at low temperature. Another aspect of this part of the procedure concerns the problem of the excess periodate in the reaction. It was considered preferable to remove most of the excess oxidant by precipitation rather than to destroy it chemically by the addition of a glycol, since, in the latter case, the aldehyde groups so produced would also condense with the aminoethylcellulose, thus reducing the number of amino groups available for complex formation with the oxidized polynucleotide. The stability of the complex toward bicarbonate ions provides some experimental advantage in that it allows the displacement of all ionically bound chloride ions from the aminoethylcellulose prior to the release step and, since the bound polynucleotide is ultimately eluted with volatile bicarbonate salts, the product can be isolated in a salt-free condition.

Investigations into possible methods for the subsequent release of the bound molecules have shown that polynucleotides were more efficiently recovered by a cleavage reaction rather than by the application of conditions that might favor the dissociation of the polynucleotide–cellulose complex. The cleavage was effected by a β-elimination reaction under conditions similar to those defined by Neu and Heppel (1964) except that propylammonium bicarbonate was used as the amine for the elimination reaction. Control experiments carried out in solution have shown that the cleavage reaction proceeded essentially to completion with this amine salt. For example, the treatment of periodate-oxidized adenylyl-(3′–5′)-uridine with propylammonium bicarbonate yielded adenosine 3′-phosphate and this reaction proceeded almost equally well when the oxidized dinucleoside phosphate was bound to aminoethylcellulose prior to the cleavage reaction. Thus, this method of release results in the liberation of the polynucleotide in a form which lacks its terminal nucleoside, and the evaporation of the propylammonium and triethylammonium bicarbonate solutions that are used to elute the released polynucleotide from the cellulose yields the product in a salt-free condition ready for analysis.

The terminal polynucleotides obtained from ribonuclease T_1 digestion of the ribonucleic acids from the three bacteriophages have been isolated in this way. The fragments were then chromatographed on DEAE-cellulose in order to purify them and to obtain some estimate of their chain length. The elution patterns obtained for the f2 RNA and Qβ RNA are shown in Figure 1. The pattern obtained for the GA RNA was similar to that shown for f2 RNA and the fragment from this RNA is still under investigation. The ultraviolet-absorbing material appearing at the front of these chromatograms was found to be mainly nonnucleotide substances deriving from the aminoethylcellulose. In order to determine the efficiency of the whole process the theoretical yield of each terminal polynucleotide was calculated using the expression for the molar absorptivity of each RNA: MZ where M is the molecular weight of the sodium salt of the RNA and is here taken as 1.15×10^6 (*cf.* value of 1.1×10^6 calculated by Sinha *et al.* (1965) for the hydrogen form of the RNA from the closely related phage, R17). The theoretical yield of an isolated polynucleotide in optical density units at 260 mμ is then $\epsilon^n_{260}y/MZ$ where ϵ^n_{260} is the molar absorptivity at 260 mμ of the polynucleotide, and y is the number of ODU_{260} of RNA (measured in 0.1 M sodium phosphate buffer, pH 7) originally degraded by the ribonuclease. The molar absorptivities of the terminal polynucleotides have been calculated from the molar absorptivities of the known components and by the measurement of the hyperchromicity displayed by each polynucleotide on being degraded to its components by alkali. Thus the polynucleotide from f2 RNA had $\epsilon^n_{260} = 77{,}500$ and that from Qβ RNA had $\epsilon^n_{260} = 105{,}000$.

Prior to the determination of their nucleotide sequences these fragments have been characterized with respect to their chain lengths and base compositions and also the identity of their terminal nucleosides. A technique which was developed

TABLE II: Products Obtained from Hydrolytic Experiments on Terminal Polynucleotides.

Terminal Polynucleotide	Phosphatase–Alkali Hydrolysis			Pancreatic Ribonuclease Hydrolysis		
	Products	mμmoles	Ratio	Products	mμmoles	Ratio
f2	Cp	170	4.0	Cp	83	3.0
	Up	98	2.3	Up	55	2.0
	Ap	91	2.1	ApCp	55	2.0
	C	39	0.9			
Qβ	Cp	252	9.0	Cp	230	10.0
	Up	116	4.1	Up	93	4.0
	C	27	1.0			

earlier allows the determination of all three of these properties in one experiment (Lee and Gilham, 1966). The polynucleotide is first dephosphorylated with alkaline phosphatase and then degraded with alkali. Control experiments have shown that the enzyme does not survive the alkaline treatment and that it does not have any activity during the alkaline hydrolysis. Thus, the terminal base is obtained in the form of a nucleoside and the others are liberated as 2′(3′)-nucleotides. Fractionation and quantitation of the products then yield the desired information. A useful chromatographic method employing the bicarbonate form of DEAE-Sephadex for the fractionation of all the possible products from such a reaction has been briefly reported (Weith *et al.*, 1968). The elution pattern obtained for the separation of a mixture of the four nucleosides and the eight nucleoside 2′(3′)-phosphates is shown in Figure 2 and, for comparison, the pattern obtained for the hydrolysis products from the terminal polynucleotide of Qβ RNA is also shown (Figure 3). More recently, an improved chromatographic method has been developed. In this system the acetate form of DEAE-Sephadex is used and by the appropriate changes in the ionic strength and pH of the eluting solvents the overlap between uridine and adenosine phosphates can be avoided. Thus, in the new system, the nucleosides are eluted separately with 0.05 M ammonium acetate (pH 9.0), and subsequently, the cytidine and adenosine nucleotides are eluted with 0.2 M ammonium acetate (pH 10.0). Finally, the uridine and guanosine nucleotides are displaced with 0.4 M ammonium acetate (pH 9.0).

The results of the alkaline hydrolyses were confirmed by pancreatic ribonuclease digestion and fractionation of the products on DEAE-Sephadex bicarbonate as described above for the alkaline hydrolysis products. The results of the various hydrolytic experiments are listed in Table II and these indicate that the terminal polynucleotides obtained from the ribonucleic acids of f2 and Qβ have the compositions: $(A_2U_2C_4)$-Cp and (C_9U_4)-Cp, respectively. These chain lengths are consistent with the positions of elution from the DEAE-cellulose columns shown in Figure 1 and they also agree with estimates of the molecular weights obtained for these fragments by sedimentation equilibrium (Weith *et al.*, 1968). In the meantime, both of these polynucleotides have undergone sequence analysis and the results of this analysis together with conclusions drawn from other experiments have shown that the ribonucleic acids of f2 and Qβ have the terminal structures: -G-U-U-A-C-C-A-C-C-C-A and -G-C-C-C-U-C-U-C-U-C-C-U-C-C-C-A, respectively (Weith and Gilham, 1967, 1969; Weith *et al.*, 1968). A more detailed discussion of this work will be the subject of a forthcoming publication.

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