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Nucleotide Distribution of *Escherichia coli* 16S Ribosomal Ribonucleic Acid*

Akira Muto

ABSTRACT: Chemical analyses have been performed on oligonucleotides arising from digestion of ^{32}P-labeled *Escherichia coli* 16S rRNA with pancreatic ribonuclease. The digestion products of the RNA were separated into individual oligonucleotides by two steps of column chromatography, first with DEAE-cellulose at neutral pH according to chain length, and second with Dowex 1-X2 at acidic pH according to nucleotide composition. The analyses indicated that several oligonucleotides existed in amounts of less than 1 mole, while the 5′-terminal sequence, pApApApUp, was present in *ca.* 1 mole in the RNA. These results suggest the internal heterogeneity in nucleotide sequence of *E. coli* 16S rRNA.

The molecular weight of the bacterial 16S rRNA has been estimated by physical and chemical methods, both giving 5.5×10^5. A 16S rRNA thus contains about 1500 nucleotides (Kurland, 1960; Green and Hall, 1961; Stanley and Bock, 1965a; Midgley, 1965; Nichols and Lane, 1967; Fellner *et al.*, 1970). The 16S rRNA preparations usually reveal physical homogeneity in the sedimentation or other physical analyses. On the other hand, DNA–rRNA hybridization experiments indicate that the 16S rRNA cistrons exist on bacterial chromosome in multiple (Yankofsky and Spiegelman, 1962, 1963; Attardi *et al.*, 1965; Oishi and Sueoka, 1965). It is then possible that the 16S rRNA, which appears to be homogeneous in terms of physical criteria, might be chemically heterogeneous. Several investigators have presented evidence for the heterogeneity of 16S rRNA in *Escherichia coli* (Aronson and Holowczyk, 1965; McIlreavy and Midgley 1967; Midgley and McIlreavy, 1967; Peacock and Dingman, 1968; Young, 1968; Maruyama and Mizuno, 1970). The recent sequence analysis by Fellner *et al.* (1970) also suggested the presence of occasional "point" heterogeneity in the 16S rRNA molecules.

It is the purpose of this paper to present further evidence for the chemical heterogeneity of *E. coli* 16S rRNA.

Experimental Section

A. Cells and Culture Conditions. Escherichia coli Q13, RNase I less mutant derived from K12 strain (Gesteland, 1966), was incubated at 37° in the phosphate-free Tris–salts–glucose medium (Mitsui *et al.*, 1963) containing 0.05% Casamino Acids (Difco) and 0.05% yeast extracts (Difco). In the exponentially growing phase, 0.1 mCi/ml of carrier-free [^{32}P]orthophosphate was added to the culture and the incubation was continued for about 3.5 generations (doubling time 70 min). Cells were harvested by centrifugation, washed with TM buffer,[1] and frozen at −20°.

B. Preparation of Ribosomes and rRNA. All operations described below were carried out at 4°. Crude extracts of the ^{32}P-labeled cells, which were prepared as described in the previous paper (Muto, 1968), were applied onto 5–20% linear sucrose gradient made in TM buffer (27 ml) and centrifuged at 20,000 rpm for 16 hr in an SW 25-1 rotor (Beckman-Spinco ultracentrifuge). About 1-ml fractions were collected from the bottom of the tube. A 0.02-ml portion was removed from each fraction, dipped on a filter paper of 1 cm^2, and dried. After measurements of radioactivity in a liquid scintillation spectrometer, the fractions containing 30S ribosomal subunits were combined, diluted threefold with TM buffer, and submitted to RNA preparation (Muto, 1968). The RNA prepared from 30S ribosomal subunits was further purified by centrifugation through 5–20% sucrose gradient at 25,000 rpm for 20 hr as described above. The ^{32}P-labeled 16S rRNA so prepared was collected together with about 5 mg of nonlabeled rRNA by ethanol precipitation and stored at −20° until use.

C. Pancreatic RNase Digestion. The ^{32}P-labeled 16S rRNA preparation was dissolved in 0.01 M Tris-HCl buffer (pH 7.5) containing 0.002 M EDTA. The RNA concentration was adjusted to 5 mg/ml with an addition of nonlabeled rRNA. About 10^7 cpm/mg of [^{32}P]RNA was digested with pancreatic RNase (RNase A; Worthington Biochemical Corp.; phosphate-free, RFA 8GD) using an enzyme to substrate ratio of 1:100 (w/w), at 37° for 13 to 18 hr in the presence of one drop of chloroform. The digests were diluted

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[1] Abbreviations used are: TM buffer, 0.01 M Tris-HCl–0.1 mM magnesium acetate (pH 7.5); BBOT, 2,5-bis[2-(5-*tert*-butybenzoxazolyl)]-thiophene (Packard).

TABLE I: Conditions of Dowex 1-X2 Column Chromatography for the Separation of Nucleotides Arising from Pancreatic RNase Digestion of 16S rRNA According to Nucleotide Composition.

Nucleotide Length	Dowex 1-X2 Resin (Mesh)	Column Size (cm)	Elution Condition
1	200–400	0.5 × 13	0.0025 N HCl (100 ml) to 0.01 N HCl–0.1 M NaCl (100 ml)
2	200–400	0.5 × 13	0.01 N HCl (200 ml) to 0.01 N HCl–0.4 M NaCl (100 ml)
3	−400	0.7 × 15	0.01 N HCl (400 ml) to 0.01 N HCl–0.6 M NaCl (200 ml)
4	−400	0.7 × 18	0.01 N HCl–0.05 M NaCl (500 ml) to 0.01 N HCl–0.75 M NaCl (250 ml)
5	−400	0.7 × 18	0.01 N HCl–0.05 M NaCl (500 ml) to 0.01 N HCl–0.75 M NaCl (250 ml)
6	−400	0.7 × 18	0.01 N HCl–0.08 M NaCl (500 ml) to 0.01 N HCl–0.75 M NaCl (250 ml)
7	−400	0.7 × 18	0.01 N HCl–0.05 M NaCl (550 ml) to 0.02 N HCl–0.35 M NaCl (550 ml)
8	−400	0.7 × 18	0.01 N HCl–0.05 M NaCl (500 ml) to 0.02 N HCl–0.75 M NaCl (250 ml)
9	−400	0.7 × 18	0.01 N HCl–0.06 M NaCl (500 ml) to 0.02 N HCl–0.6 M NaCl (250 ml)
10	−400	0.7 × 18	0.02 N HCl–0.05 M NaCl (500 ml) to 0.02 N HCl–1.0 M NaCl (250 ml)

fivefold with 7 M urea–0.02 M Tris-HCl (pH 7.5) and immediately applied onto a DEAE-cellulose column.

D. Fractionation of Oligonucleotides. SEPARATION ACCORDING TO CHAIN LENGTH. In general, the procedure of Tomlinson and Tener (1963) was used. DEAE-cellulose (Bio-Rad Laboratories; Cl^- form) was packed by gravity to a 0.7 × 20 cm column equilibrated with about five column volumes of 7 M urea–0.02 M Tris-HCl (pH 7.5). The products of pancreatic RNase digestion were applied onto the column. After washing it with a small volume of 7 M urea–0.02 M Tris-HCl (pH 7.5), elution was performed with a linear gradient of NaCl from 0.025 to 0.25 M made in 7 M urea–0.02 M Tris-HCl (pH 7.5) (total volume 1.1 l.) at room temperature. After the end of the gradient elution, residual materials were eluted with 1 M NaCl in the same buffer and finally with 1 N KOH. Fractions (5.5 ml) were collected every 30 min. A 0.1-ml portion removed from each fraction was dissolved in 5 ml of BBOT–toluene scintillator containing ethylene glycol monomethyl ether (Muto, 1968), and subjected to radioactivity measurements in a liquid scintillation spectrometer.

SUBFRACTIONATION ACCORDING TO NUCLEOTIDE COMPOSITION. The pooled nucleotide mixture of an equal chain length was diluted three- to fivefold with water and charged to a column of 0.5 × 2 cm Dowex 1-X2 (Bio-Rad Laboratories; Cl^- form). The Dowex resins charged by the nucleotides were then layered on top of a freshly prepared Dowex 1-X2 column previously equilibrated with the starting elution fluid. Elution was carried out with a concave (exponential) or a linear gradient of NaCl in the presence of 0.01–0.02 N HCl. The gradient was produced by mixing a dilute solution of NaCl (in HCl) in a mixing cylinder vessel with a concentrated solution of NaCl (in HCl) from reservoir cylinder. Concave gradients were produced using two cylinders, a mixing vessel and a reservoir, the base area ratio of which was 2:1. The column size and elution conditions are listed in Table I. In each case, the residual materials were eluted with 1 N KOH after the end of the gradient elution. ^{32}P radioactivity in each fraction was measured in a liquid scintillation spectrometer without addition of scintillator (Clausen, 1968).

After radioactivity measurements, desired radioactive peaks were desalted and submitted to alkaline and/or T_1 RNase hydrolyses. For desaltating, a modified method of Rushizky and Sober (1962) was used. An appropriate nucleotide fraction was diluted fivefold with water, adjusted to pH 7.5 with 6 N KOH, and passed through a column of DEAE-Sephadex A25 (Pharmacia, Uppsala, Sweden; 1.2 × 3 cm), equilibrated with 0.05 M ammonium bicarbonate. Residual salts were washed off from the column with 100 ml of 0.05 M ammonium bicarbonate. The nucleotide materials were then eluted with about 15 ml of 2 M ammonium bicarbonate. The effluent was concentrated with a rotatory flash evaporator below 40°, dissolved in water, and taken to dryness *in vacuo* repeatedly until all ammonium bicarbonate was removed.

E. Base Composition Analysis. A part (usually 0.5 ml) of the desired ^{32}P-labeled oligonucleotide fraction was hydrolyzed with 0.33 N KOH at 37° for 18 hr in the presence of nonlabeled carrier RNA. It is then neutralized with 60% perchloric acid in an ice bath, followed by removal of precipitated $KClO_4$ by centrifugation. Mononucleotides were adsorbed on a column of Dowex 1-X2 (200–400 mesh; Cl^- form; 0.5 × 13 cm) and eluted with an exponential gradient from 0.0025 N HCl (200 ml) to 0.01 N HCl–0.1 M NaCl (100 ml). After collection of 5-ml fractions, the radioactivity was measured directly without scintillator.

F. T_1 RNase Digestion. [^{32}P]Oligonucleotides were mixed with 2 mg of nonlabeled carrier RNA and treated with 100 units of T_1 RNase (Dai-ichi Kagaku Co. Ltd., Tokyo) in 1 ml of 0.02 M Tris-HCl–0.002 M EDTA (pH 7.5) at 37° for 1.5 hr. The digestion products were immediately charged on a column of Dowex 1-X2 (−400 mesh; Cl^- form; 0.7 × 18 cm) and eluted with an exponential gradient from 0.0025 N HCl (400 ml) to 0.01 N HCl–0.4 M NaCl (200 ml). Fractions (5 ml) were collected every 15 min. The nucleotides were eluted in a order of Cp, ApCp, ApApCp, Gp, ApApApCp, ApGp, ApApGp, ApApApGp.... for nucleotides having cytidylic acid 3′ terminal, or Up, ApUp, Gp, ApApUp, ApGp, ApApGp, ApApApUp, ApApApGp.... for nucleotides having uridylic acid 3′ terminal.

G. Determination of 5′-Terminal Fragment. The position of the 5′-terminal fragment of 16S rRNA upon the chromatogram was determined using pancreatic RNase digests of [5′-^{32}P]16S rRNA. For the preparation of [5′-^{32}P]16S

rRNA, the procedure of Takanami (1967a) was followed: 1 mg of purified 16S rRNA was treated with bacterial alkaline phosphatase[2] to remove 5′-phosphodiesters. The position from which phosphate had been removed was then phosphorylated with radioactive phosphate using [γ-^{32}P]ATP and polynucleotide kinase. The RNA was freed from nonreacted [γ-^{32}P]ATP by Sephadex G-200 column chromatography (column size 1 × 40 cm), and centrifuged through sucrose gradient to remove degradation products resulting from the enzyme treatments; 0.4 mg of 16S rRNA (about 1 mμmole) containing 0.9 mμmole of ^{32}P (5×10^5 cpm) was digested with pancreatic RNase in the presence of 5 mg of nonlabeled carrier 16S rRNA, and the products were analyzed with DEAE-cellulose and Dowex 1-X2 column chromatography as described in section D. The absorbance at 260 mμ for each fraction was measured to know the elution position of the radioactive 5′-terminal fragment.

The above experiment indicated that the 5′-terminal fragment was eluted together with one of the hexanucleotide components (peak VI-1; see Figure 9) having nucleotide composition of (3Ap,2Gp)Cp on the Dowex 1-X2 chromatogram. The terminal fragment was then isolated using a preparation of 16S rRNA homogeneously labeled with ^{32}P by the following way. The ^{32}P-labeled peak VI-1 was digested with T_1 RNase. By this treatment the component (3Ap,2Gp)-Cp was decomposed to smaller nucleotide fragments, while the terminal fragment remained undigested because of the lack of Gp in it (Takanami, 1967b). The fractionation of the T_1 RNase digest with Dowex 1-X2 column chromatography under the conditions described in section F revealed that the 5′-terminal fragment was eluted after the peak ApApApGp (see Table VIII).

H. Determination of Methylated Oligonucleotides. The cells of *E. coli* Q13 were labeled with L-[*methyl*-^{14}C]methionine (0.1 mCi) in 100 ml of Tris–salts–glucose medium supplemented with 0.05% casamino acids. Under the conditions, [^{14}C]methionine incorporated exponentially for about three generations (doubling time 140 min). The ^{14}C-labeled 16S rRNA was then prepared and was digested with pancreatic RNase with 5 mg of nonlabeled 16S rRNA. In order to determine the elution positions of methylated oligonucleotides upon the chromatogram, the digestion products were analyzed by the same method as in the case of ^{32}P-labeled nucleotides. ^{14}C radioactivity was measured as follows. A 0.3-ml portion was removed from each fraction and dissolved in 10 ml of BBOT–toluene scintillator containing ethylene glycol monomethyl ether. The radioactivity in the sample was then counted in a liquid scintillation spectrometer. The absorbance at 260 mμ for each fraction was also measured to know the elution positions of the radioactive components.

I. Isotopes and Counting Conditions. [^{32}P]Orthophosphate (6.7–7.5 Ci/g) and L-[*methyl*-^{14}C]methionine (355 μCi/mg) were obtained from The Radiochemical Centre, Amersham, England. To measure the radioactivity, Packard Model 2002 Tri-Carb liquid scintillation spectrometer was used throughout with the gain settings as follows: 1.5% for ^{32}P (with scintillator), 15% (without scintillator), and 12% for ^{14}C (with scintillator). The window settings were in all cases between 40 and 1000.

[2] Bacterial alkaline phosphatase, polynucleotide kinase, and [γ-^{32}P]-ATP (10^9 cpm/μmole) are kindly supplied by Drs. Sugiura and Takanami (Kyoto University).

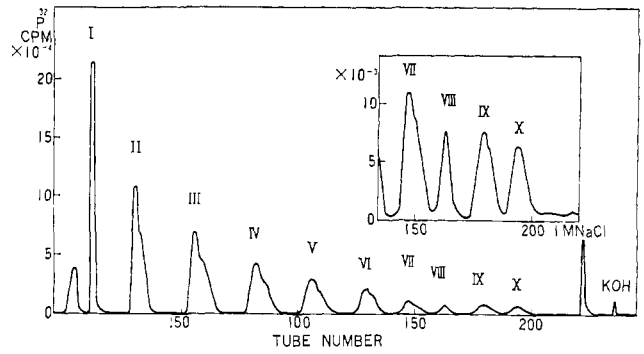

FIGURE 1: Chromatography of pancreatic RNase digests of ^{32}P-labeled *E. coli* 16S rRNA on a DEAE-cellulose column (0.7 × 20 cm). A linear gradient elution from 0.025 to 0.25 M NaCl in 7 M urea–0.02 M Tris-HCl, pH 7.5 (total volume 1.1 l.), followed by 1 M NaCl in the same buffer and then 1 M KOH. A 5.5-ml fraction was collected every 30 min. ^{32}P radioactivity of 0.1-ml portion from each fraction was measured.

Results

The experiments to be described below were performed at least twice with reasonable reproducibility in all cases as seen in Tables II–XIV.

A. Fractionation of Pancreatic RNase Digests of ^{32}P-Labeled 16S rRNA According to Chain Length. Figure 1 shows a typical chromatographic pattern of the pancreatic RNase digestion products of ^{32}P-labeled 16S rRNA upon a column of DEAE-cellulose. The first small peak consists of cyclic mononucleotides (mainly uridine cyclic monophosphate), followed by mono-, di-, tri-, tetra-, penta-, hexa-, hepta-, octa-, nona-, and decanucleotides (numbered with Roman numerals from I to X, respectively). The resolution of the chromatography was sufficient to determine the quantitative distribution of radioactivity in each peak, even in that of long nucleotides. Oligonucleotides longer than undecanucleotides were not detected; 2–3% of the radioactive materials charged to the column remained uneluted with the salt gradient used and was recovered by washing the column with 1 M NaCl–7 M urea–0.02 M Tris-HCl (pH 7.5) and 1 N KOH. These materials would be incompletely digested fragments, since (i) the amount of the materials varied depending upon the time of incubation with RNase, and (ii) rechromatography with DEAE-cellulose or Dowex 1-X2 column of the fraction showed that it consisted of a number of nonspecific components including short nucleotides.

Table II gives the distribution of radioactivity in each nucleotide fraction. The values are expressed as percentage of total radioactivity recovered in specific nucleotide components. The distribution of radioactivity was highly reproducible in repeated experiments, although a slight fluctuation in the amounts of octa-, nona-, and decanucleotide fractions was observed depending upon the time of RNase treatment.

B. Subfractionation According to Nucleotide Composition.

B-1. SUBFRACTIONATION OF SHORT NUCLEOTIDES. Nucleotide components corresponding to peaks I, II, III, and IV in

TABLE II: Distribution of Radioactivity on the DEAE-cellulose Column Chromatogram of the Pancreatic RNase Digests of ^{32}P-Labeled *E. coli* 16S rRNA.

Fraction	% of Radioactivity[a]	
	Expt 1[b]	Expt 2
I[c]	24.0	22.7
II	21.2	21.1
III	20.3	20.1
IV	13.5	13.9
V	8.9	9.0
VI	5.4	5.6
VII	3.0	3.1
VIII	1.0	1.3
IX	1.6	1.9
X	1.1	1.4

[a] Radioactivity eluted with 1 M NaCl and 1 N KOH was 2.1 and 2.9% of the total radioactivity recovered in expt 1 and 2, respectively. These values were subtracted in the above calculations. [b] The duration of the RNase treatment was 18 hr in expt 1 and 13 hr in expt 2. [c] Cyclic mononucleotides were included.

TABLE III: Subfractionation of Mononucleotides.

Peak	Composition	% of Radioactivity[a]	
		Expt 1	Expt 2
1-1	Cp[b]	52.4	51.6
1-2	Up	47.6	48.4

[a] Expressed as per cent of radioactivity recovered in the specific nucleotide peaks. [b] A methylated nucleotide (m-1) was included.

Figure 1 were subfractionated by chromatography on Dowex 1-X2 columns according to their nucleotide compositions. After the chromatography, 95–98% of radioactive materials of each fraction applied onto the column was recovered in the identified specific nucleotide peaks as shown in Table III, IV, V, and VI, respectively. The elution profiles were essentially the same as those of Solymosy *et al.* (1965), who analyzed the short fragments arising from pancreatic RNase digestion of turnip yellow mosaic virus RNA using Dowex 1-X2 column chromatography. The profiles of tri- and tetranucleotides also resembled that of the DEAE-cellulose column chromatogram reported by Rushizky and Sober (1964). The nucleotide composition of each component was confirmed by measuring the ultraviolet absorption spectrum in 0.01 N HCl (Stanley and Bock, 1965b) or by alkaline hydrolysis and T_1 RNase hydrolysis if necessary.

Mononucleotides. Two peaks, I-1 and I-2 corresponding to Cp and Up, were detected on the chromatogram (Table III). No other peaks were detected. The peak I-1 includes methylated nucleotide (m-1). (Determination of methylated nucleotides will be discussed in section D.)

TABLE IV: Subfractionation of Dinucleotides.

Peak	Composition	% of Radioactivity	
		Expt 1	Expt 2
II-1	ApCp	27.2	27.1
II-2	GpCp[a]	30.8	30.4
II-3	ApUp	16.2	16.3
II-4	GpUp	25.8	25.7
II-a	*b*	*c*	0.5

[a] Methylated nucleotides (m-3 and m-4) were included. [b] Methylated nucleotide (m-2). [c] Not determined.

TABLE V: Subfractionation of Trinucleotides.

Peak	Composition	% of Radioactivity	
		Expt 1	Expt 2
III-1	ApApCp	15.3	15.4
III-2	ApGpCp + GpApCp	28.0	28.1
III-3	ApApUp[a]	9.9	9.8
III-4	GpGpCp	14.5	14.1
III-5	GpApUp	9.4	8.7
III-6	ApGpUp	7.7	7.8
III-7	GpGpUp	14.2	15.3
III-a	*b*	1.0	0.9

[a] A methylated nucleotide (m-6) was included. [b] Methylated nucleotide (m-5).

TABLE VI: Subfractionation of Tetranucleotides.

Peak	Composition	% of Radioactivity	
		Expt 1	Expt 2
IV-1	ApApApCp	6.7	6.9
IV-2	(2Ap,Gp)Cp	16.7	16.4
IV-3	ApApApUp	3.1	3.7
IV-4	(Ap,2Gp)Cp	15.0	13.5
IV-5	(2Ap,Gp)Up	20.0	21.8
IV-6	GpGpGpCp	9.7	9.2
IV-7	(Ap,2Gp)Up	21.8	21.3
IV-8	GpGpGpUp	7.0	7.2

Dinucleotides. Four major peaks and one minor methylated nucleotide peak (m-2) were seen on the chromatogram in the following order: m-2, ApCp, GpCp, GpCp, ApUp, and GpUp (Table IV). The peak II-2 (GpCp) contains methylated nucleotides, m-3 and m-4.

TABLE VII: Subfractionation of Pentanucleotides.

Peak	% of Radioactivity		Alkaline Hydrolysis[a]				T_1 RNase Hydrolysis[a]	Deduced Composition
	Expt 1	Expt 2	Cp	Ap	Up	Gp		
V-1	3.7	4.2	*1.0*	4.2				ApApApApCp
V-2	9.2	9.9	*1.0*	3.1		1.1	Cp(*1.0*):ApCp(1.6):ApApGp(1.5): ApApApGp(1.0)	ApApGpApApCp + ApApApGpGpCp (1:2?)
V-3	12.6	12.0	*1.0*	2.3		2.2	Cp(*1.0*):ApCp(2.1):ApApCp(0.8):Gp(5.1): ApGp(2.4):ApApGp(0.6)	GpGpApApApCp + (ApGp,Gp)ApCp + (ApApGp,Gp)Cp(1:2:1)
V-4[b]	24.6	27.6		2.9	*1.0*	1.0	Up(3.1):Gp(14.5):ApGp(2.0):ApApUp(2.0): ApApApGp(2.0):ApApApApUp(2.0)	(3Ap,Gp)Up + ApGpApApApUp + GpApApApApUp(3:2:2?)
V-5a	6.3	6.6	*1.0*	1.3		3.0	Cp(*1.0*):Gp(2.2):ApGp(1.0)	(ApGp,Gp,Gp)Cp
V-5b	5.6	6.4	*1.0*	1.1		2.9	Cp(*1.0*):Gp(2.2):ApGp(1.0)	(ApGp,Gp,Gp)Cp
V-6	24.6	23.4		2.1	*1.0*	2.1	Up(3.7):Gp(9.1):ApUp(*1.0*):ApGp(1.6): ApApUp(2.3):ApApGp(2.3)	(2Ap,2Gp)Up + (Ap,2Gp)ApUp + GpGpApApUp(4:1:2?)
V-7	8.4	6.5		1.1	*1.0*	3.0	Up(1.2):Gp(5.3):ApUp(*1.0*)ApGp(1.1)	GpGpGpApUp + (ApGp,Gp,Gp)Up(1:1)
V-8	5.0	3.4			*1.0*	4.3		GpGpGpGpUp

[a] Expressed as the molar yield of each nucleotide relative to the italic nucleotide. [b] A methylated nucleotide (m-7) was included.

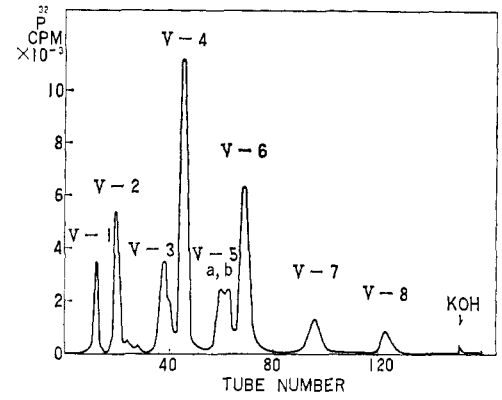

FIGURE 2: Rechromatography of the pentanucleotide fraction on a Dowex 1-X2 column (0.7 × 18 cm). A concave gradient elution from 0.1 N HCl–0.05 M NaCl (500 ml) to 0.01 N HCl–0.75 M NaCl (250 ml) followed by 1 N KOH (100 ml). A 5.0-ml fraction was collected every 15 min. The whole content of each fraction was used to measure the ^{32}P radioactivity.

Trinucleotides. Seven major components were eluted. Their nucleotide compositions were determined to be ApApCp, (Ap,Gp)Cp, ApApUp, GpGpCp, GpApUp, ApGpUp, and GpGpUp, respectively (Table V). In addition to the above, a small peak of methylated trinucleotide (m-5) was eluted between the peaks III-1 (ApApCp) and III-2 [(Ap,Gp)Cp]. The peak III-3 (ApApUp) contains methylated nucleotide (m-6). From the analysis of T_1 RNase hydrolysate, the peak III-2 was deduced to contain ApGpCp and GpApCp in a ratio of 1:1.

Tetranucleotides. Eight peaks were seen upon rechromatographing peak IV in Figure 1. The nucleotide compositions were determined to be ApApApCp, (2Ap,Gp)Cp, ApApApUp, (Ap,2Gp)Cp, (2Ap,Gp)Up, GpGpGpCp, (Ap,2Gp)Up, and GpGpGpUp, respectively (Table VI).

B-2. SUBFRACTIONATION OF LONG NUCLEOTIDES. The Dowex 1-X2 column chromatographic technique, which had been used for subfractionation of nucleotides of short chain length (Solymosy *et al.*, 1965; Ishikura *et al.*, 1966; Neelon *et al.*, 1967), was modified and applied to separate oligonucleotides of long chain length. As shown in Figures 2, 3, 5, 6, 7, and 8, oligonucleotides from pentamer to decamer could be separated into individual components according to their nucleotide compositions. The resolving power of the Dowex 1-X2 column in this system seems to be much greater than that of DEAE-cellulose.

In order to estimate the frequency of occurrence of the individual component in the 16S rRNA, the relative numbers of the subfractionated oligonucleotides were calculated from their molar ratio. Since some fractions consisted of isomers having the same base composition and having different nucleotide sequence, the number of the fragment in several isomers could be also estimated by analyzing the T_1 RNase hydrolysates of the fractions.

Pentanucleotides. Figure 2 shows the elution profile of the pentanucleotide fraction (peak V in Figure 1) with Dowex 1-X2 column chromatography. Eight peaks were eluted. The peaks V-3 and V-5 consist of incompletely separated two components. The peak V-4 contains methylated nucleotide (m-7). Table VII gives the distribution of ^{32}P radioactivity

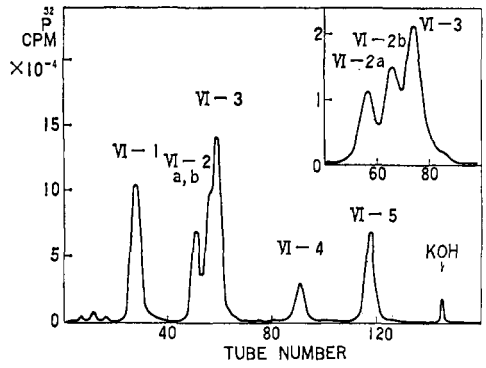

FIGURE 3: Rechromatography of the hexanucleotide fraction on a Dowex 1-X2 column (0.7 × 18 cm). A concave gradient elution from 0.01 N HCl–0.08 M NaCl (500 ml) to 0.01 N HCl–0.75 M NaCl (250 ml). The inserted figure is the rechromatographic pattern of the VI-2 and VI-3 region in Figure 3 on a Dowex 1-X2 column (0.7 × 18 cm) with a concave gradient elution from 0.01 N HCl–0.1 M NaCl (500 ml) to 0.01 N HCl–0.3 M NaCl (250 ml).

in the above eight components and the results of the analyses after alkaline and T_1 RNase digestion of these components. Pentanucleotides having base compositions ApApApApUp and GpGpGpGpCp were not detected. As shown in the last column of the table, some pentanucleotide fractions consist of isomers having different nucleotide sequences. For example, the T_1 RNase digestion of the peak V-7 [(Ap,3Gp)Up] yielded 1Up, 5Gp, 1ApUp, and 1ApGp. From this, the peak would contain at least two components, one having the sequence GpGpGpApUp and the other (ApGp,Gp,-Gp)Up, in a ratio of 1:1. The relative numbers of the pentanucleotides calculated from the ratio of radioactivity in each peak and the composition of these isomers are 1ApApApAp-Cp, 3(3Ap,Gp)Cp, 4(2Ap,2Gp)Cp, 7(3Ap,Gp)Up, 4(Ap,-3Gp)Cp, 7(2Ap,2Gp)Up, 2(Ap,3Gp)Up, and 1GpGpGpGp-Up. The sum of the relative fragment numbers of pentamers closely fits the expected value when 16S rRNA is assumed to be a homogeneous polynucleotide of 1500 nucleotide length (see Table XIV).

Ninety-three per cent of the radioactivity charged to the column was recovered in the above eight peaks, and the remaining radioactivity was distributed in nonspecific regions between the definite peaks and at the region of the 1 N KOH eluate.

Hexanucleotides. Six peaks were eluted through Dowex 1-X2 column chromatography (Figure 3). The first peak (VI-1) contains 5′-terminal nucleotides pApApApUp and hexanucleotides (3Ap,2Gp)Cp. As shown in Table VIII, the 5′-terminal fragment could be detected on Dowex 1-X2 column chromatography of the T_1 RNase hydrolysate of the VI-1 fraction. About 20% of the VI-1 radioactivity corresponds to the terminal fragment. Detailed discussion about this fragment will be made in the next section. Besides the 5′-terminal nucleotide fragment, the peak VI-1 contains at least three hexanucleotide isomers, (ApApApGp,Gp)Cp, (ApApGp,Gp)ApCp, and GpGpApApApCp, in a ratio of approximately 2:2:1. The hexanucleotides with the composition of (2Ap,3Gp)Cp were separated into two peaks on the chromatogram, the former (VI-2a) being (Ap-ApGp,Gp,Gp)Cp and the latter (VI-2b) GpGpGpApApCp. Figure 4 shows the elution profile on a Dowex 1-X2 column

TABLE VIII: Subfractionation of Hexanucleotides.

Peak	% of Radioactivity		Alkaline Hydrolysis				T_1 RNase Hydrolysis	Deduced Composition
	Expt 1	Expt 2	Cp	Ap	Up	Gp		
VI-1	25.3	24.4	*1.0*	3.4		2.0[a]	Cp(2.1):ApCp(2.1):Gp(7.6):ApApApCp(*1.0*): ApApGp(1.7):ApApApGp(1.7): pApApApUp(1.7)[b]	(ApApApGp,Gp)Cp + (ApApGp,Gp)ApCp + GpGpApApApCp + pApApApUp (2:2:1:2)
VI-2a	13.7	13.6	*1.0*	1.7		3.0	Cp(2.0):Gp(4.0):ApGp(2.2):ApApGp(*1.0*)	(ApApGp,Gp,Gp)Cp + (ApGp,ApGp,-Gp)Cp(1:1)
VI-2b	17.4	18.2	*1.0*	2.0		3.0	ApApCp(*1.0*):Gp(4.2)	GpGpGpApApCp
VI-3	23.0	22.4		2.8	*1.0*	2.1	Gp(2.8):ApUp(*4.0*):ApGp(8.6):ApApUp(2.9)	ApGpApGpApApUp + (ApGp,Gp)ApApUp (4:3?)
VI-4	6.2	6.2		1.8	*1.0*	2.9	Up(*1.0*):Gp(2.2):ApApGp(0.7)	(ApApGp,Gp,Gp)Up
VI-5	14.4	15.2		1.0	*1.0*	3.8	Up(4.0):Gp(15.4):ApUp(*1.0*):ApGp(3.9)	(ApGp,Gp,Gp,Gp)Up + GpGpGpGp-ApUp(4:1)

[a] pAp from the 5′-terminal fragment was included. [b] The 5′-terminal sequence.

TABLE IX: Subfractionation of Heptanucleotides.

Peak	% of Radioactivity		Alkaline Hydrolysis				T_1 RNase Hydrolysis	Deduced Composition
	Expt 1	Expt 2	Cp	Ap	Up	Gp		
VII-1	21.9	21.1	*1.0*	4.0		2.0	ApApCp(3.0):Gp(5.6):ApApApCp(*1.0*):ApGp(1.4):ApApGp(3.1)	(ApApGp,Gp)ApApCp + (ApGp,Gp)ApApApCp(3.1)
VII-2a	4.6	4.1	*1.0*	3.0		3.2	Cp(*1.0*):ApCp(0.5):Gp(2.0):ApGp(1.2):ApApGp(0.8)	(ApApGp,ApGp,Gp)Cp ?
VII-2b	16.0	18.5	*1.0*	3.7	(0.3)	3.4	Gp(3.4):ApApApCp(*1.0*)[a]	GpGpGpApApApCp
VII-3	17.1	16.8		2.8	*1.0*	2.8	Up(*1.0*):Gp(1.7):ApGp(1.0):ApApGp(0.8)	(ApApGp,ApGp,Gp)Up
VII-4	12.3	13.0	*1.0*	1.1		4.8	Cp(*1.0*):Gp(3.9):ApGp(0.9)	(ApGp,Gp,Gp,Gp,Gp)Cp
VII-5a	7.0	6.7	(0.2)	1.9	*1.0*	4.1	Up(*1.0*):Gp(3.6):ApGp(2.4)	(ApGp,ApGp,Gp,Gp)Up
VII-5b	7.6	7.0		2.3	*1.0*	4.4	Gp(5.5):ApApUp(*1.0*)	GpGpGpGpApApUp
VII-6	13.5	12.8		1.1	*1.0*	5.2	Up(*1.0*):Gp(4.0):ApGp(0.9)	(ApGp,Gp,Gp,Gp,Gp)Up

[a] About 25% of radioactivity in T_1 RNase digests of the peak VI-2b was detected also in the peaks Cp, ApCp, Up, and ApGp.

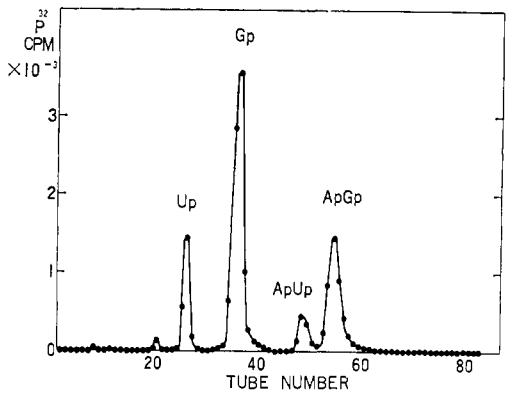

FIGURE 4: Chromatography of T_1 RNase digests of the VI-5 fraction in Figure 3 on a Dowex 1-X2 column (0.7 × 18 cm). The desalted VI-5 fraction was digested with T_1 RNase at 37° for 1.5 hr, which was then directly charged to the column and eluted with a concave gradient elution from 0.0025 N HCl (400 ml) to 0.01 N HCl–0.4 M NaCl (200 ml). A 5-ml fraction was collected every 15 min and the ^{32}P radioactivity was measured directly in a counting vial.

of T_1 RNase hydrolysate of the peak VI-5. The molar ratio of the digestion products is 4Up, 16Gp, 1ApUp, and 4ApGp. This indicates that the peak VI-5 consists of 4(ApGp,Gp,Gp,-Gp)Up and 1GpGpGpGpApUp. From the results mentioned above, the relative numbers of the hexamers may be 5(3Ap,-2Gp)Cp, 10(2Ap,3Gp)Cp, 7(3Ap,2Gp)Up, 2(2Ap,3Gp)Up, and 5(Ap,4Gp)Up. The molar ratio of pApApApUp (5′-terminal sequence of 16S rRNA) to the hexamers GpGpApAp-ApCp or GpGpGpGpApUp is approximately 2:1. This would mean that about 1 mole of the 5′-terminal nucleotide sequence (see next section) and only 0.5 mole of the latter two nucleotides are present in 1 mole of 16S rRNA.

Ninety-four to ninety-five per cent of the radioactive materials charged to the column was recovered in the above six peaks.

Heptanucleotides. Figure 5 is the chromatographic pattern of the heptanucleotide fraction (peak VII in Figure 1). Relatively high nonspecific (background) radioactivity was seen on the chromatogram (18% in expt 1 and 16% in expt

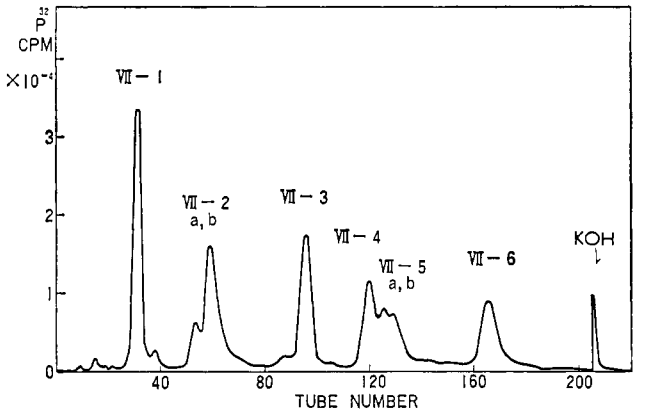

FIGURE 5: Rechromatography of the heptanucleotide fraction on a Dowex 1-X2 column (0.7 × 18 cm). A linear gradient elution from 0.01 N HCl–0.08 M NaCl (550 ml) to 0.02 N HCl–0.35 M NaCl (550 ml). A 5.4-ml fraction was collected every 18 min.

TABLE X: Subfractionation of Octanucleotides.

Peak	Alkaline Hydrolysis				T_1 RNase Hydrolysis	Deduced Composition
	Cp	Ap	Up	Gp		
VIII-1		2.9	*1.0*	3.8	Gp(5.1):ApUp(*1.0*):ApGp(2.7):ApApUp(0.9)	(ApGp,ApGp,Gp,Gp)ApUp + (ApGp,Gp,Gp,Gp)ApApUp (1:1)

2). Peak VII-1 contains at least two isomers with different sequences, (ApApGp,Gp)ApApCp and (ApGp,Gp)ApApApCp, in a ratio of 3:1 (Table IX). The small peak referred to as VII-2a was assumed to be a mixture of nonspecific fragments by analysis of T_1 RNase digestion products of the peak. Peak VII-2b also contains about 25% of nonspecific radioactivity. The two small peaks detected behind VII-1 and before VII-3 were purine clusters which might be produced by some nonspecific action of RNase. The other heptamers were decided to be homogeneous by analyses of their nucleotide compositions after T_1 RNase hydrolyses. Isomers (ApGp,ApGp,Gp,Gp)Up and GpGpGpGpApApUp were partially separated (VII-5a and VII-5b). The fragments (ApGp,Gp)ApApApCp, (ApGp,ApGp,Gp,Gp)Up, and GpGpGpGpApApUp are calculated to exist only less than 1 mole/1 mole of 16S rRNA. The relative fragment numbers of isomeric heptamers are 4(4Ap,2Gp)Cp, 2(3Ap,3Gp)Cp, 3(3Ap,3Gp)Up, 2(Ap,5Gp)Cp, 2(2Ap,4Gp)Up, and 2(Ap,5Gp)Up.

Octanucleotides. Figure 6 shows the rechromatographic profile of the octanucleotide fraction (peak VIII in Figure 1). Only one main peak (VIII) having a base composition (3Ap,4Gp)Up was detected. The nucleotide composition analysis of T_1 RNase digestion products of this peak revealed that at least two different components having sequences (ApGp,ApGp,Gp,Gp)ApUp and (ApGp,Gp,Gp,Gp)ApApUp existed in nearly an equal amount (Table X). The radioactivity recovered in the peak VIII-1 was 77 and 70% of the charged materials in expt 1 and 2, respectively.

Nonanucleotides. The rechromatography of the peak IX in Figure 1 resulted in a separation into three distinct components (Figure 7), in a ratio of approximately 2:1:1 (Table XI). The analyses of T_1 RNase hydrolysates of these components showed that the first peak IX-1 was heterogeneous and consisted of at least two different components, (5Ap,3Gp)Cp and (4Ap,3Gp)ApCp, in a ratio of 1:1, while the other two appear to be homogeneous. The radioactivity recovered in the above three peaks was 86 and 85% of the charged materials in expt 1 and 2, respectively.

Decanucleotides. Three major peaks were eluted (Figure 8). All of them appear to be homogeneous when judged from their mononucleotide compositions and compositions of their T_1 RNase digests (Table XII). The minor peak (X-a) detected before the peak X-3 occurred in an amount too small (less than 0.05%) to be definitely a specific decamer in the 16S rRNA, although its sequence could be (ApGp,Gp,-Gp,Gp,Gp,Gp,Gp,Gp)Cp. The three main peaks X-1, X-2, and X-3 were detected in a ratio of about 4:2:3. Since the repeated experiments showed that the amount of the X-2 fraction varied depending upon the time of RNase digestion, the actual ratio could be 1:1:1. The radioactivity recovered in the peaks was 86% and 81% of the total radioactivity charged to the column in expt 1 and 2, respectively.

C. Determination of 5'-Terminal Fragment. In order to determine the chromatographic position of the 5'-terminal sequence of 16S rRNA, the pancreatic RNase digestion products of 5'-^{32}P-labeled 16S rRNA was chromatographed with a DEAE-cellulose column; 45% of ^{32}P radioactivity was detected in the hexanucleotide fraction (peak VI in Figure 1). This value is in agreement with the data of Takanami (1967b). The remaining radioactivity was irregularly distributed in the regions of shorter nucleotides. This is probably due to the partial breakdown of the terminal

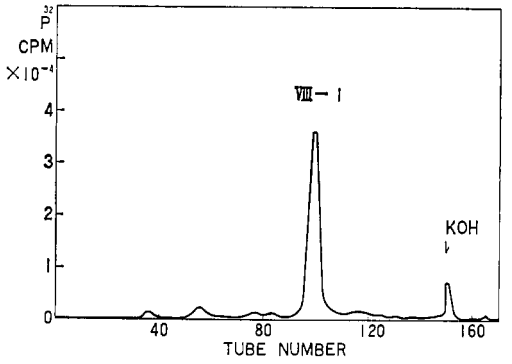

FIGURE 6: Rechromatography of the octanucleotide fraction on a Dowex 1-X2 column (0.7 × 18 cm). A concave gradient elution from 0.01 N HCl–0.05 M NaCl (500 ml) to 0.02 N HCl–0.75 M NaCl (250 ml). A 5-ml fractionwas collected every 15 min.

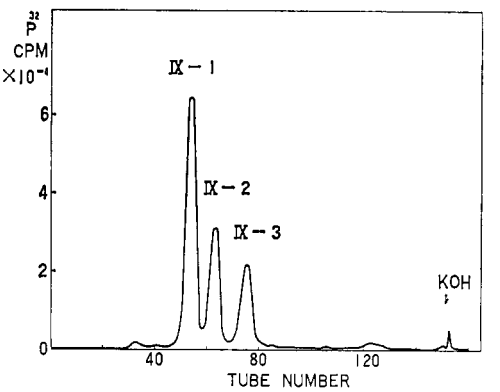

FIGURE 7: Rechromatography of the nonanucleotide fraction on a Dowex 1-X2 column (0.7 × 18 cm). A concave gradient elution from 0.01 N HCl–0.06 M NaCl (500 ml) to 0.02 N HCl–0.6 M NaCl (250 ml). A 5-ml fraction was collected every 15 min.

TABLE XI: Subfractionation of Nonanucleotides.

Peak	% of Radioactivity		Alkaline Hydrolysis				T_1 RNase Hydrolysis	Deduced Composition
	Expt 1	Expt 2	Cp	Ap	Up	Gp		
IX-1	53.0	51.2	*1.0*	4.8		3.0	Cp(*1.0*):ApCp(1.0):Gp(1.9):ApGp(1.8): ApApGp(1.6):ApApApGp(0.8)	(5Ap,3Gp)Cp + (4Ap,3Gp)ApCp (1:1)
IX-2	26.0	26.1		6.0	*1.0*	2.2	Up(1.1):ApApGp(*1.0*):ApApApApGp(0.7)	(ApApApApGp,ApApGp)Up
IX-3	21.0	22.7	*1.0*	4.3		4.1	Cp(*1.0*):Gp(2.4):ApApGp(1.7)	(ApApGp,ApApGp,Gp,Gp)Cp

TABLE XII: Subfractionation of Decanucleotides.

Peak	% of Radioactivity		Alkaline Hydrolysis				T_1 RNase Hydrolysis	Deduced Composition
	Expt 1	Expt 2	Cp	Ap	Up	Gp		
X-1	40.6	38.6	*1.0*	5.8		2.7	Cp(*1.0*):ApGp(0.8):ApApGp(0.9):ApApApGp(0.9)	(ApApApGp,ApApGp,ApGp)Cp
X-2	22.1	25.6	*1.0*	4.9		4.1	Cp(1.2):Gp(1.3):ApGp(*1.0*):ApApGp(1.8)	(ApApGp,ApApGp,ApGp,Gp)Cp
X-3	32.0	30.9		2.6	*1.0*	5.8	Up(*1.0*):Gp(4.2):ApGp(0.9):ApApGp(0.7)	(ApApGp,ApGp,Gp,Gp,Gp)Up
X-a	5.3	4.9	*1.0*	1.4	0.3	8.4	Cp(0.7):Gp(5.6):ApGp(*1.0*)	(ApGp,Gp,Gp,Gp,Gp,Gp,Gp)Cp ?

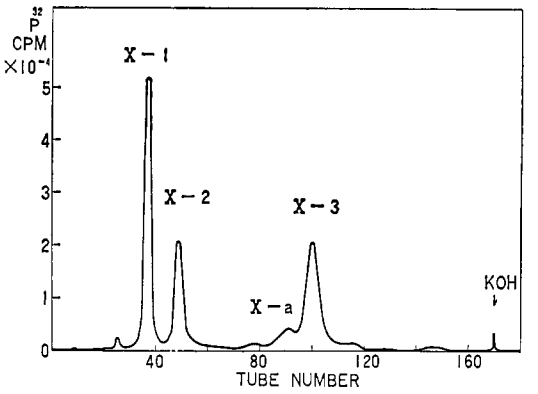

FIGURE 8: Rechromatography of the decanucleotide fraction on a Dowex 1-X2 column (0.7 × 18 cm). A concave gradient elution from 0.02 N HCl–0.05 M NaCl (500 ml) to 0.02 N HCl–1.0 M NaCl (250 ml). A 4.3-ml fraction was collected every 12 min.

fragments during the removal of 5′-terminal phosphate and/or the addition of ^{32}P to the 5′-terminal nucleotide. The hexanucleotide fraction was further rechromatographed on a Dowex 1-X2 column and the result was shown in Figure 9. More than 95% of radioactivity was found in the right half of the peak VI-1 [(3Ap,2Gp)Cp], indicating the existence of the 5′-terminal fragment in there. The 5′-terminal fragment could thus be isolated by rechromatography of the T_1 RNase hydrolysates of the peak VI-1 through a column of Dowex 1-X2, removing the digestion products of the hexamers (see section B).

The nucleotide composition of the 5′-terminal fragment was determined by alkaline hydrolysis of the fragment homogeneously labeled with ^{32}P, which had been obtained by T_1 RNase hydrolysis of the peak VI-1 in Figure 3. The composition was 52.3% Ap, 23.6% Up, and 24.1% pAp in mole per cent. Thus the 5′-terminal sequence pApApApUp determined by Takanami (1967b) was confirmed.

The radioactivity due to the 5′-terminal sequence in the peak VI-1 was 19% in expt 1 and 23% in expt 2, when deter-

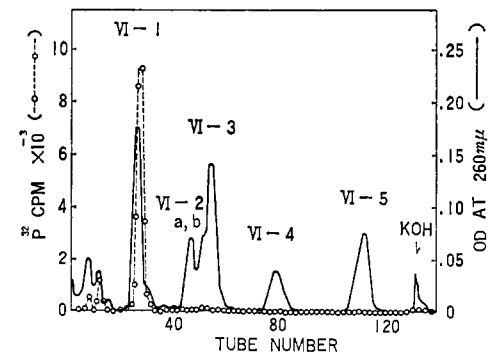

FIGURE 9: Chromatography of the hexanucleotide fraction prepared from pancreatic RNase digests of 5′-^{32}P-labeled 16S rRNA. The hydrolysates were first separated according to chain length and the hexanucleotide region was then subfractionated by a column of Dowex 1-X2 (0.7 × 18 cm). The conditions were the same as described in the legend of Figure 3. A 5-ml fraction was collected every 15 min. The whole content of each fraction was used to measure the ^{32}P radioactivity (O----O) and then the absorbance at 260 mμ (———).

TABLE XIII: Distribution of Methylated Nucleotides Arising from Pancreatic RNase Digestion of [*methyl*-^{14}C]16S rRNA.

Fraction	Elution position on the Column Chromatogram		% of Radioactivity	
	DEAE-cellulose	Dowex 1-X2	Expt 1	Expt 2
m-1	I[a]	Cp	15.9	14.8
m-2	II	Before ApCp	6.0	6.6
m-3	II	GpCp	1.6	3.0
m-4	II	GpCp	23.1	26.2
m-5	III	Between ApApCp and (Ap,Gp)Cp	20.2	20.3
m-6	III	ApApUp	11.3	12.0
m-7	V	(Ap,3Gp)Up	8.2	5.5
(m-8)	VII–VIII		3.3	3.8
(m-9)	1 M NaCl		10.4	7.9

[a] The number of peak shown in Figure 1.

TABLE XIV: Distribution of Radioactivity and Number of Nucleotide Fragments in Each Fraction Separated According to Chain Length.

Fraction	% of Radioactivity[a]	Rel No. of Bases[b]	Expected No. of Fragments[c]	Obsd Rel No. of Fragments[d]
I	23.2 ± 0.9	348.0	348.0	
II	20.9 ± 0.3	313.5	156.8	
III	20.3 ± 0.2	304.5	101.5	
IV	13.7 ± 0.3	205.5	51.4	
V	8.97 ± 0.10	134.6	26.9	29
VI	5.24 ± 0.23[e]	78.6	13.1	29
VII	2.97 ± 0.14	44.6	6.4	15
VIII	1.18 ± 0.14	17.7	2.2	2
IX	1.84 ± 0.14	27.6	3.1	4
X	1.33 ± 0.20	20.0	2.0	3
5′-Terminal fragment (pApApApUp)	0.29 ± 0.03[f]	4.4	0.9	1

[a] Average of five experiments. [b] The number of bases corresponding to each fraction is calculated on the assumption that the RNA contains 1500 bases/molecule. [c] The number of bases were divided by the appropriate chain length. [d] The sum of relative numbers of individual components fractionated by Dowex 1-X2 column chromatography (see text). [e] The content of the 5′-terminal fragment (*f*) was subtracted from the peak VI. [f] Average of two experiments.

mined from the chromatographic pattern of the T_1 RNase hydrolysates of the fraction. These values correspond to 0.26–0.33% of the total phosphates in the 16S rRNA. Thus 0.8–1.0 mole of pApApApUp was detected in one molecule of RNA having 1500 nucleotides. The result indicates the homogeneity of the 5′-terminal sequence of the 16S rRNA.

D. Determination of Methylated Oligonucleotides. A preliminary study has been performed to determine the positions on the chromatogram of the methylated nucleotides produced by pancreatic RNase digestion of 16S rRNA. The digestion products of 16S rRNA labeled with [*methyl*-^{14}C]methionine were analyzed by the same procedure as in the case of ^{32}P-labeled nucleotides. On the first chromatogram where the pancreatic RNase digests were fractionated according to chain length, ^{14}C radioactivity was detected in the peaks of mono-, di-, tri-, and pentanucleotides. In addition to them, a small peak and a relatively large peak were detected between hepta- and octanucleotides and in the 1 M NaCl eluate, respectively. The latter two components seem to be nonspecific. On the second chromatogram of the nucleotides separated according to nucleotide composition, the most radioactivity in the mononucleotide fraction was eluted in the Cp region (m-1). Components of the dinucleotide fraction were separated into three peaks, a peak m-2 before the ApCp peak, a small peak m-3 slightly before the peak GpCp, and a large peak m-4 which is superimposed on the peak GpCp. Two distinct peaks were eluted upon rechromatography of the trinucleotide fraction, a peak m-5 between ApApCp and (Ap,Gp)Cp and another peak m-6 at the same position as ApApUp. A radioactive peak m-7 in the pentanucleotide fraction was found in the (3Ap,-Gp)Up region. These results indicate that the methylation

occurs at a small number of loci in 16S rRNA as shown by Fellner and Sanger (1968). The distribution of the ^{14}C radioactivity recovered in each peak mentioned above is listed in Table XIII. Further analyses of methylated nucleotides have not been done yet, since the most methylated nucleotides are eluted on the chromatogram together with a large amount of nonmethylated nucleotides, and thus their chemical characterization is hard to be done at present.

Discussion

Digestion of RNA with pancreatic RNase produces stacks of purine nucleotides followed by a terminal pyrimidine nucleotide. As shown in this paper, almost all the isomeric digestion products of ^{32}P-labeled 16S rRNA could be separated by two steps of ion-exchange column chromatography, first with DEAE-cellulose at neutral pH according to chain length and second with Dowex 1-X2 at acidic pH according to nucleotide composition. The individual fragments so separated, especially those from penta- to decanucleotides, were characterized by alkaline and T_1 RNase hydrolyses, followed by the determination of their nucleotide compositions. The content of each oligonucleotide component was also determined by quantitative measurement of ^{32}P radioactivity.

Since the 16S rRNA was known to be a single polynucleotide chain having about 1500 nucleotides, the content of each characterized oligonucleotide, including the 5′-terminal fragment, should represent its frequency of occurrence in the chain. If the 16S rRNA is chemically homogeneous, the 5′-terminal should occur once, and any other specific oligonucleotides arising from RNase digestion of the RNA should occur more than once in one molecule of the 16S rRNA. The results described in section C show that about 0.9 mole of the 5′-terminal sequence pApApApUp is detected in 1500 nucleotides, indicating that the 5′-terminal sequence of the 16S rRNA is largely homogeneous. The results agree with those reported by Takanami (1967b), Nichols and Lane (1967), and Fellner *et al.* (1970). Assuming that the 16S rRNA is homogeneous having 1500 nucleotides, the relative number of nucleotide fragments belonging to a given fraction on the DEAE-cellulose chromatogram, which consists of nucleotides having the same chain length, may be calculated by the ^{32}P radioactivity found in the fraction. Such number of the pentanucleotide fraction is 27 (Table XIV). As shown in section B, rechromatography on a Dowex 1-X2 column of the pentanucleotide fraction resulted in a separation into eight components according to their nucleotide compositions. The relative number of the individual components and their compositions are 1ApApApApCp, 3(3Ap,Gp)Cp, 4(2Ap,2Gp)Cp, 7(3Ap,Gp)Up, 4(Ap,3Gp)Cp, 7(2Ap,2Gp)Up, 2(Ap,3Gp)Up, and 1GpGpGpGpUp. The sum of these relative fragment numbers is 29 and this value is close to the calculated number 27. Similar situations may be found in the octa-, nona-, and decanucleotide fractions (Table XIV). This means that at least 1 mole of each nucleotide component belonging to these fractions occurs in 1 mole of the 16S rRNA, thus suggesting that a considerable part of the 16S rRNA chain including the 5′-terminal region may be homogeneous sequences.

On the other hand, several oligonucleotides found in hexa- and heptanucleotide fractions are detected only in a amount of less than 1 mole. The molar ratio of the hexamers, GpGpApApApCp or GpGpGpGpApUp, to the 5′-terminal fragment is approximately 0.5. The same is true in the molar ratio of the heptamers (ApApGp,Gp)ApApCp, (ApGp,ApGp,Gp,Gp)Up, or GpGpGpGpApApUp to the 5′-terminal. This would indicate the presence of partial heterogeneity in the nucleotide sequence of the 16S rRNA. The simplest explanation for the above results would be that there exist in *E. coli* 16S rRNA two kinds of molecules both of which have nucleotide sequences similar to but partially different from each other. Fellner *et al.* (1970) also demonstrated that some oligonucleotide fragments of 16S rRNA produced by T_1 RNase digestion existed in submolar amounts.

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Messenger and Template Activities of Chemically Modified Polynucleotides*

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ABSTRACT: Nitrous acid treatment of poly C, leading to 3–15% deamination, increased the messenger activity of the polymer in terms of proline incorporation and led to mutations in terms of serine, leucine, and phenylalanine incorporation, all effects correlated with the U content of the polymer. As templates these same samples were reduced in activity in terms of GTP incorporation, but showed some, though far from compensatory, ATP incorporation activity, both effects in approximate correlation to their U content. Hydroxylamine or methoxyamine treatment of poly C, to the point of yielding 0.5–2% of the N-4-substituted derivative of cytosine, caused marked losses in proline incorporation and no significant incorporation of other amino acids. When tested with RNA polymerase, such preparations caused less GTP incorporation, but increased ATP incorporation, resembling minimally deaminated poly C in both respects. Poly C methylated to about 5% with nitrosoguanidine or classical methylating agents was largely inactivated in the messenger test. Neither methylated poly C nor methylated poly U,G showed new amino acid incorporating specificities. As template, methylated poly C retained significant GTP incorporating activity and showed some activity in incorporating ATP, UTP, and CTP. These effects may account for the high mutagenicity of nitrosoguanidine observed under certain conditions. Poly C treated with ultraviolet light to 5% loss in absorbancy showed some loss in proline incorporation but no new amino acid incorporations. Its GTP incorporation was somewhat lowered, without definite effects on ATP, UTP, or CTP binding. Poly U irradiated with ultraviolet light up to 20% loss of absorbancy also showed no evidence for mutagenesis by either test method and comparatively little loss in its incorporating activities. It appears from these facts that cell-free amino acid incorporation supplies a reliable tool for the detection of mutagenic events if, and only if, these represent typical base replacements.

Triphosphate incorporation, in contrast, can reveal base changes which only simulate true base replacements, but gives reliable results only with poly C derivatives. This system has nevertheless shown good correspondence with the biological methods of detecting TMV mutants and may be useful in screening for potential mutagens.

The molecular basis of mutagenic events can at times be deduced from resultant protein or tRNA sequence changes but more often the chemical basis of the phenotypic alteration remains obscure. Since polyribonucleotides have been found to act as both messengers and templates in cell-free amino acid or ribonucleoside triphosphate incorporation systems, attempts have been made to use such systems for the identification of mutagenic reactions. The first reaction to be used for the modification of polynucleotides, the deaminating action of nitrous acid, has produced the expected mutagenic effects when the modified polynucleotide was used as messenger (Basilio *et al.*, 1962). The effect of deamination on the template activity of poly d(A-T) was also studied (Kotaka and Baldwin, 1964). Various other modifying reactions have since been reported to produce new coding or template activities in polynucleotides, some in line with expectation and others not. However, no comparative studies of the effects of different reactions as tested in both systems are known to us.

It is the purpose of the present paper to summarize our studies of the effects of treatment with nitrous acid, nitrosoguanidine, or classical alkylating agents, hydroxylamine or methoxyamine, and ultraviolet irradiation on both the coding and the template properties of poly C. We have attempted to obtain quantitative indications of the sensitivity of detecting mutagenic events in both systems by means of preparations of poly C that had been subjected to limited deamination. Using amino acid incorporation, we have not detected mutational events in the messenger activity of any of the modified poly C preparations excepting HNO_2-treated samples containing as little as 3% of U. When testing for new template activities in the presence of GTP, significant

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