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Chemical Studies on Mixed Soluble Ribonucleic Acids from Yeast

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The products of alkaline digestion of a mixed s-RNA preparation, from growing yeast, were analyzed by DEAE-cellulose and paper chromatography, and by electrophoresis. The isolated components were estimated spectrophotometrically and the composition of the s-RNA was determined. Eighty per cent of the s-RNA chains were shown to have adenosine in the 3′-terminal position and the remainder had cytidine. More than 90% of the 5′- termini were phosphorylated and consisted of 78% pGp..., 10% pUp..., 7% pAp..., and 5% pCp.... A pancreatic ribonuclease digest of the same s-RNA preparation was subjected to chromatography on DEAE-cellulose in the presence of 7 M urea, thereby fractionating the components on the basis of net negative charge. Phosphorylated 5′-terminal sequences were eluted along with nonterminal fragments containing two more purine residues. They were then isolated as previously described by R. V. Tomlinson and G. M. Tener (1962, *J. Am. Chem. Soc. 84*, 2644; 1963, *Biochemistry 2*, 697, 703). The distribution of the major and minor bases in the mono-, di-, tri-, ... nucleotides was determined and the composition of the s-RNA was compiled from the results. Some of the sequences arising from phosphorylated 5′- termini were identified: 11.5% of the molecules terminated in pUp..., 6.5% in pCp..., 26% in pGpCp..., 0.7% in pApUp..., 0.4% in pGpUp..., and less than 0.1% in pApCp. Mixed trinucleoside tetraphosphates and tetranucleoside pentaphosphates from 5′- ends were also isolated and some evidence for the existence of the terminal sequences $pGp(Gp)_4Up...$ and $pGp(Ap)(Gp)_3Up$ was also obtained.

Soluble ribonucleic acid (s-RNA) has been the subject of intensive investigation ever since the discovery by Hoagland *et al.*, in 1957, that this RNA fraction, in the presence of the appropriate enzyme systems, is capable of accepting acyl-activated amino acids and transferring them to protein.

Present evidence indicates that s-RNA is a mixture of short-chain polyribonucleotides of molecular weight 20,000–30,000 (Osawa, 1960; Luborsky and Cantoni, 1962), each probably able to accept only one amino acid (Hoagland *et al.*, 1957, 1958; Berg and Offengand, 1958). Active s-RNA molecules are believed to ter-

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minate at one end, the 3′- terminus, in a ...pCpCpA[1] sequence, to which the amino acid is coupled (Zachau *et al.*, 1958; Hecht *et al.*, 1958). The opposite or 5′-end has been shown by Zillig *et al.* (1960) and Singer and Cantoni (1960) to be predominantly pGp.... More recently Ralph *et al.* (1962, 1963), using a yeast s-RNA preparation, demonstrated that pGp... was by far the most common 5′- terminal moiety but obtained evidence for the existence of pAp... and pUp... ends also.

The column chromatographic procedure of Tomlinson and Tener (1962, 1963a), by which the products of nuclease action can be fractionated according to their net charge, provides the basis for a technique whereby the terminal sequences of nucleic acids can be isolated without previous chemical treatment (Tomlinson and Tener, 1963b). Thus in a ribonuclease digest of s-RNA those sequences arising from the 5′-termini and containing an additional phosphate group in the 5′- position are eluted together with fragments from inside the chains which contain two more purine nucleotides. That is to say, pPyp is eluted with the trinucleotides; pPupPyp with the tetranucleotides, and so on. Treatment of these peaks with phosphomonoesterase to remove primary phosphate groups reduces the charge of the end-sequences by four (at *p*H 7.8), whereas the nonterminal fragments lose only two charges. As the terminal sequences in each peak then differ from the nonterminal sequences of that peak by two charges, they can be readily isolated by chromatography on DEAE-cellulose in the presence of 7 M urea. This approach and the well-established methods of alkaline degradation, and column and paper chromatography have been used (a) to obtain a complete base analysis of this s-RNA mixture, (b) to study the distribution of major and minor base components in the mono-, di-, tri-,... nucleotides produced by ribonuclease digestion, and (c) to identify the 5′-terminal moieties and to investigate the adjacent nucleotide sequences. A brief report of part of this work has previously appeared (Bell *et al.*, 1963).

Experimental Procedures

Materials.—DEAE-cellulose was purchased from Brown Co., Berlin, N. H. Dowex 1-X8 and Dowex 50W-X4 were obtained from Dow Chemical Co. Midland, Mich., and Amberlite IRC-50 from Rohm and Haas, Ltd., Philadelphia, Pa.

Pancreatic ribonuclease and alkaline phosphomonoesterase from *E. coli* were obtained from Worthington Biochemical Corp., Freehold, N. J. Snake venom phosphodiesterase was prepared from commercial lyophilized venom (*Crotalus adamanteus*, Ross Allen's Reptile Institute, Silver Springs, Fla.) by the method of Koerner and Sinsheimer (1957). The product had no detectable phosphomonoesterase activity under the conditions described for its use.

Tris-hydrochloride buffer was prepared by titrating a solution of tris(hydroxymethyl)aminomethane to the desired *p*H with hydrochloric acid.

[1] The abbreviations recommended by the Journal of Biological Chemistry for the nucleosides and their derivatives are used throughout, i.e., A for adenosine, C for cytidine, G for guanosine, U for uridine, X for xanthosine, T for thymine riboside, and ψU for pseudouridine. N is used as a general, nonspecific nucleoside symbol. The nucleoside-(2′)3′-phosphates are represented as Np and the (2′)3′,5′-diphosphates as pNp. ApGp represents adenylyl-(3′ → 5′)-guanosine-3′-phosphate. Pu = purine nucleoside, Py = pyrimidine nucleoside. The R_{Ap} (or R_A) of a compound indicates its movement relative to Ap (or A) in a chromatographic or electrophoretic system.

Optical density measurements were made using a Cary Model 11 spectrophotometer. One optical density (OD) unit is defined as that amount of material per ml which in a 1-cm light path, at 271 mμ (unless otherwise specified), gives a spectrophotometric reading of one. All ultraviolet-absorption spectra were recorded under neutral, acidic, and alkaline conditions. The molar extinction values used (for readings at 271 mμ and neutrality) were: A, 9500; C, 9000; G, 9500; U, 8300; ψU, 7700; 2-*N*,*N*-dimethyl-G, 9500; 1-methyl-G, 9000; 5-Methyl-C, 8000; and T, 9200. These were derived from the values published by Littlefield and Dunn (1959) and Dunn (1960, 1961). For the probably complex "methyl-A," the unknown compounds α, β, Z_1, Z_2, and Z_3, and for the mixture labeled "pNp and/or alkali-stable NpNp" a value of 9500 was chosen.

Enzyme Incubation.—Phosphomonoesterase digestions were carried out in 0.01 M Tris hydrochloride, *p*H 8.0–0.01 M magnesium chloride, for 18 hours at 37°, unless otherwise stated. Snake venom phosphodiesterase incubations were performed in 0.1 M ammonium carbonate, *p*H 8.5–0.01 M magnesium chloride for 24 hours at 37°. Chloroform was added to all enzymic digestions to inhibit bacterial growth.

Chromatography and Electrophoresis.—Whatman No. 40 paper was used, in a descending technique at 20°–22°, for all chromatograms. The solvents used were: (A) water-saturated butanol with ammonia in the vapor phase; (B) isobutyric acid (66 ml), water (33 ml), and concd ammonia (1 ml); and (C) isopropanol (70 ml), concd ammonia (10 ml), and water (20 ml).

Paper electrophoresis was performed on Whatman 3 MM or No. 40 paper using water-cooled high voltage equipment (Shandon).

DEAE-cellulose columns were prepared as described by Tomlinson and Tener (1963a).

Ammonium carbonate was removed from column eluates and the like by repeated evaporation to dryness under vacuum at bath temperatures below 40°. (See under Discussion for precautions and modifications).

Results

Preparation of s-RNA.—Cantoni *et al.* (1962) noted that s-RNA prepared from freshly grown yeast contained increased amounts of adenosine in the 3′-terminal position. Therefore the isolation procedures of Holley *et al.* (1961) and Monier *et al.* (1960) were modified to allow the yeast to grow some time before the addition of phenol. Active dry baker's yeast (500 g) was stirred slowly into a solution of 75 g of sucrose in 3 liters of water, and the mixture was incubated for 1 hour at 37°. An equal volume of phenol (88%) was added and the mixture was stirred for 2 hours at room temperature. It was then centrifuged at 1500 × *g* at room temperature for 30 minutes, and the aqueous (upper) phase was taken. This material was recentrifuged and the supernatant was filtered through Celite. To the filtrate was added 0.1 volume of 20% potassium acetate, *p*H 5.0, and then 2.5 volumes of ethanol. The precipitate was allowed to stand overnight at −10° and the crude s-RNA thus obtained was collected by centrifugation at 0°. The pellet was dissolved in 30 ml of 0.1 M Tris-hydrochloride buffer, *p*H 7.5, and the solution was extracted twice with ether. It was then adjusted to *p*H 9.0 with ammonia and left for 1 hour at room temperature to ensure the removal of bound amino acids (cf. von Ehrenstein and Lipmann, 1961). The solution was diluted to 600 ml with water and applied to a DEAE-cellulose column (4 × 30 cm). The column was washed with water

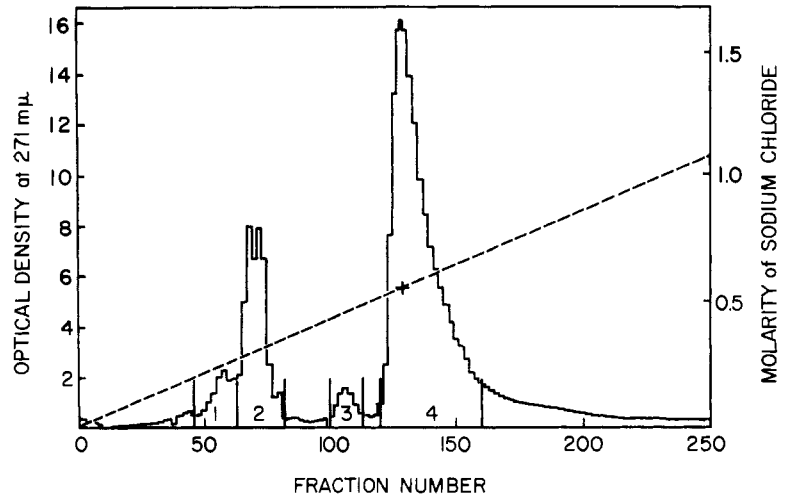

FIG. 1.—Chromatography of a crude yeast s-RNA preparation (about 8000 OD units at 271 mμ) on DEAE-cellulose (chloride) 0.0025 M Tris hydrochloride, *p*H 8. Column dimensions, 4 × 30 cm; fraction volume, 21 ml; flow rate, 3.5 ml/minute.

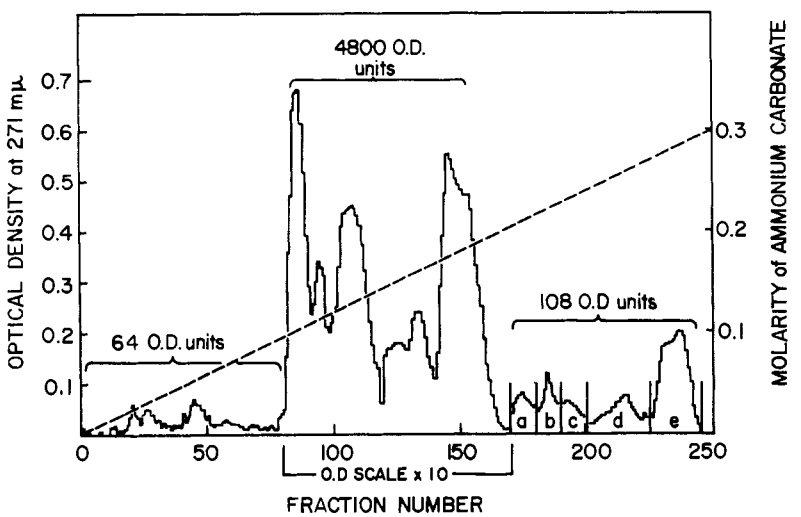

FIG. 2.—Chromatography of an alkaline digest of purified yeast s-RNA on DEAE-cellulose (carbonate). Column dimensions, 2 × 25 cm; fraction volume, 20 ml; flow rate, 2.5 ml/minute.

and then subjected to a linear gradient of sodium chloride 0.0–1.5 M (total volume, 8 liters) containing 0.0025 M Tris hydrochloride, *p*H 8.0. The elution profile is shown in Figure 1. The components of peaks 1 and 2 were shown by paper chromatography with known compounds in solvent B, and by phosphorus-to-OD ratios, to be UTP, ATP, and GTP. The s-RNA was precipitated from peak 4 by addition of 3 volumes of cold ethanol and the precipitate was allowed to stand overnight in the cold. It was then collected by centrifugation, washed with 80% and 90% ethanol, and dried *in vacuo*. The yield was 400 mg.

Digestion of s-RNA with Alkali.—s-RNA (235 mg) was digested with 0.3 N sodium hydroxide for 18 hours at 37° (Markham and Smith, 1952). The digest was neutralized with Dowex 50 (hydrogen) resin and applied to a DEAE-cellulose (carbonate) column (2 × 25 cm). The column was subjected to a linear gradient of ammonium carbonate, 0.0–0.3 M (total volume, 5 liters), *p*H 8.7. The elution profile is shown in Figure 2. Tubes 1–75 were pooled as fraction 1. A 2-ml aliquot was taken from each of tubes 77–165 and the remaining liquid was pooled as fraction 2. Tubes 170–245 were pooled as shown to give fractions 3a, 3b, 3c, 3d, and 3e.

After removal of ammonium carbonate, fraction 1 was subjected to paper chromatography, along with authentic ribonucleoside markers, in solvent A, for 30 hours. The ultraviolet-absorbing areas were cut out and eluted quantitatively with water, and the spectra of the eluates were recorded. The fraction contained 80% adenosine and 20% cytidine.

The 2-ml aliquots from tubes 77–165 were freed of ammonium carbonate and applied to paper, and the chromatograms were developed for 30 hours in solvent B (see Fig. 3). The ultraviolet-absorbing areas were cut out and eluted with water, and the spectra of the eluates were recorded. For further confirmation of identity, those eluates which had the same spectral properties and were derived from similar positions on the chromatograms were pooled and lyophilized. The resultant residues were dissolved in 0.01 M Tris-hydrochloride buffer, *p*H 8.0–0.01 M magnesium chloride, an excess of phosphomonoesterase was added, and the mixtures were incubated for 18 hours at 37°. The contents of each tube was transferred quantitatively to paper and chromatographed with authentic nucleoside markers for 30 hours in solvent A. The ultraviolet-absorbing areas were cut out and eluted with water, and spectra were obtained at various *p*H values, as before. In addition to the four major ribonucleotides and pseudouridylic acid, 5-methyl-Cp, Tp, "methyl-Ap" (point of methylation not known), 1-methyl-Gp, 2-*N*,*N*-dimethyl-Gp, and xanthylic acid

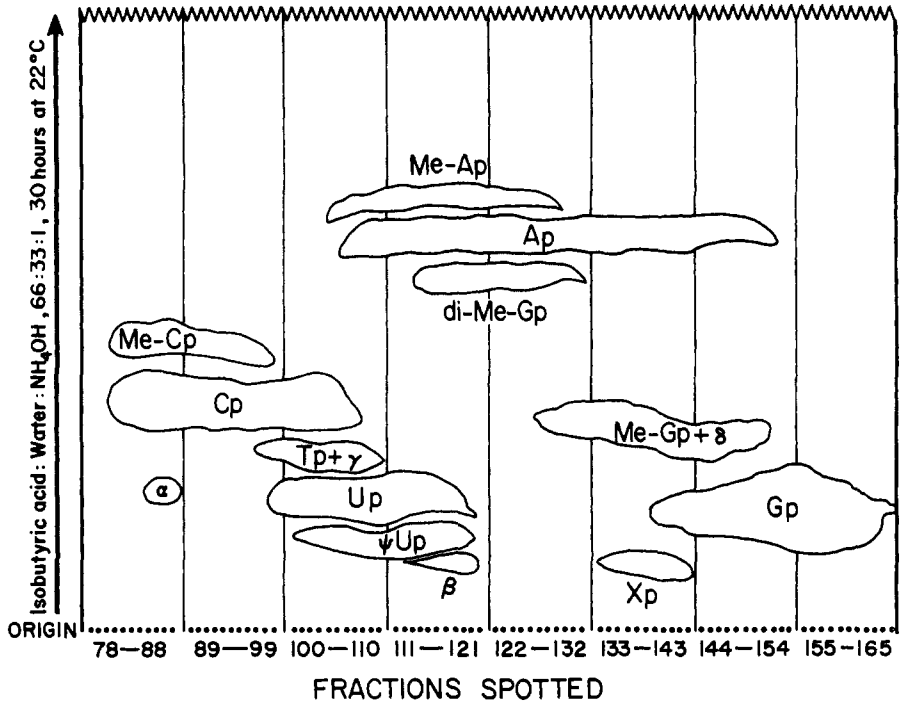

FIG. 3.—Composite diagram of the chromatograms obtained after paper chromatography of 2-ml samples from each of the tubes 78–165 of Fig. 2.

were also present. These assignments were made on the basis of R_F and spectral data (Littlefield and Dunn, 1959; Dunn, 1960, 1961). The spectrum of the eluate from the area α was of the cytidylic acid type but with a maximum at 270 mμ at neutrality and 263 mμ in base. In the case of area β the spectrum obtained was similar to that for pseudouridylic acid with a maximum at 264 mμ under neutral conditions, but it underwent a smaller bathochromic shift in base to give a maximum at 275 mμ. The symbols γ and δ indicate the presence of unidentified components in the Tp and 1-methyl-Gp regions. They were detected by differences in the spectra of eluates from different sections along the ultraviolet-absorbing band.

Fractions 3a–3e were freed of ammonium carbonate and the residues from each were dissolved in 1 ml of water. One half of each solution was treated with phosphomonoesterase as described above, then both halves were subjected to paper electrophoresis in 0.05 M sodium citrate buffer, *p*H 5.0, for 1 hour at 50 v/cm. The spectra of the ultraviolet-absorbing components were determined as above.

These fractions were shown (Bell *et al.*, 1963) to contain nucleoside-(2′)3′,5′-diphosphates (59 OD units) and alkali-resistant dinucleotides (49 OD units). The latter do not necessarily contain 2′-*O*-methyl ribose (Smith and Dunn, 1959) as some normal dinucleotides have been shown to be resistant to the digestion conditions described (Lane and Butler, 1959a,b). The presence of these dinucleotides was tolerated in order to ensure that deamination of cytidine residues was kept to a minimum (Loring, 1955). The components of each subfraction were identified by their relative migrations on electrophoresis before and after phosphomonoesterase treatment and from spectral data (Table I). The results obtained from the analyses of fractions 1, 2, and 3 of the alkaline digest were combined to give the total s-RNA composition shown in column 1 of Table II.

Comparison of the total OD units recovered from the 3′- termini (as nucleoside) and from the 5′- termini (as nucleoside diphosphate) indicates that at least 90% of the 5′- termini are phosphorylated. These results also indicated an average chain length for this s-RNA of about 75–85 residues.

TABLE I
COMPOSITION OF FRACTIONS 3a–e (FIG. 2)

Compound	Peak a	b	c	d	e	Total
			(mole %)			
pUp	1.0	9.0	—	—	—	10.0
pCp	4.5	0.5	—	—	—	5.0
pAp	—	—	—	7.0	—	7.0
pGp	—	—	—	4.0	74.0	78.0
						100.0
ApCp	15.0	—	—	—	—	15.0
ApUp	5.0	13.0	—	—	—	18.0
ApAp	—	—	2.5	10.5	—	13.0
ApGp	—	—	19.0	17.5	—	36.5
GpCp	—	—	—	—	7.5	7.5
GpUp	—	—	—	—	10.0	10.0
						100.0

Electrophoretic Analysis.—Two samples (about 10 OD units at 260 mμ) of an alkaline digest of s-RNA, prepared as above, were applied to Whatman No. 40 paper and subjected to electrophoresis in 0.1 M ammonium formate buffer, *p*H 3.5, for 3 hours at 80 v/cm. The components were located relative to reference compounds by ultraviolet light and eluted with water. Identical paper blanks were treated in the same manner and the nucleotides were determined spectrophotometrically by reading against these blanks. From these results the relative proportions of the total adenine, cytosine, guanine, and uracil derivatives were obtained and are shown in column 3, Table II.

Digestion of s-RNA with Pancreatic Ribonuclease.—Purified s-RNA (380 mg) was dissolved in 30 ml of 0.1 M Tris-hydrochloride buffer, *p*H 7.8. Ribonuclease (10 mg) was added and the mixture was incubated for 22 hours at 37°. It was then diluted with 120 ml of 7 M urea–0.005 M Tris hydrochloride, *p*H 7.8, and

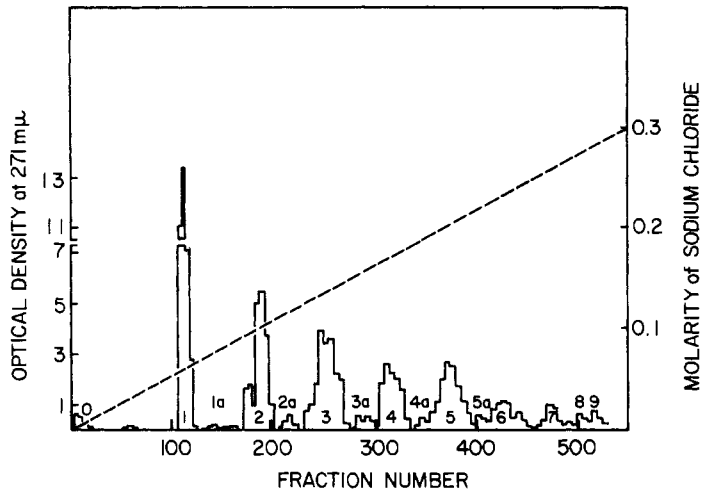

FIG. 4.—Chromatography of a ribonuclease digest of purified yeast s-RNA (about 7300 OD units at 271 mμ) on DEAE-cellulose (chloride) in the presence of 7 M urea and 0.005 M Tris hydrochloride, *p*H 7.8. Column dimensions, 2 × 100 cm; fraction volume, 14 ml; average flow rate, 1.5 ml/minute.

TABLE II
TOTAL ANALYSES OF S-RNA

Component	Analysis 1	Analysis 2	Analysis 3
A	16.3	17.1	19.2
"Methyl-A"	0.8	0.9	
C	29.0	28.0	28.8
5-Methyl-C	0.8	0.75	
α	0.15	—	
G	26.5	27.4	28.8
2-*N*,*N*-Dimethyl-G	0.6	0.6	
1-Methyl-G	0.95	1.15	
δ		—	
U	17.6	19.0	23.2
ψU	3.3	3.0	
T	2.15	0.9	
γ		—	—
β	1.6	—	—
X	0.3	—	—
Z_1	—	0.05	—
Z_2	—	0.05	—
Z_3	—	0.1	—
pNp or alkali-stable NpNp	—	1.05	—

applied to a DEAE-cellulose (chloride) column (2 × 100 cm), previously equilibrated against the same solution. The column was washed with 250 ml of the equilibration solution and then subjected to a linear gradient of sodium chloride, 0.0–0.3 M (total volume, 8 liters). The solutions in the reservior and mixing chamber were 7 M in urea and 0.005 M in Tris hydrochloride, *p*H 7.8. The elution profile is shown in Figure 4. The spectra of all peaks and shoulders were recorded and the tubes were pooled as indicated on the chart. Urea and sodium chloride were removed from all pools, except numbers 0 and 1, by readsorption of the diluted (1:5) pools on DEAE-cellulose (carbonate) followed by washing with 0.01 M ammonium carbonate (cf. Rushizky and Sober, 1962). The nucleotide material was then eluted with 2 M ammonium carbonate. Peak 1 was diluted 1:5 and applied to a column of Dowex 1 (carbonate) resin (Cohn and Bollum, 1961). The column was washed with 0.02 M ammonium carbonate to remove urea and chloride ion and then with 2 M ammonium carbonate to elute the nucleotides. Peak 0 was diluted 1:5, adjusted to *p*H 4 with acetic acid, and applied to a column of Dowex 50 (ammonium) resin, and the column was washed with water. Urea and chloride ion were removed by the water wash, but no ultraviolet-absorbing material was eluted. The adsorbed material was then eluted with 0.02 M ammonium carbonate.

Analysis of the Purified Peaks.—The residues after the removal of ammonium carbonate were dissolved in water, transferred to test tubes, and made up to known volumes.

Peak 0.—An aliquot containing 20 OD units was taken and subjected to paper chromatography for 40 hours in solvent A, along with authentic nucleoside markers. The ultraviolet-absorbing areas were cut out and eluted with water, and the spectra of the eluates were recorded.

Peaks 1–9.—Aliquots containing 20 OD units were taken from peaks 1–7, and aliquots containing 12.5 and 15 OD units, were taken from peaks 8 and 9, respectively. These solutions were taken to dryness *in vacuo*, redissolved in 0.25 ml of 0.3 N sodium hydroxide, and incubated for 24 hours at 37°. Each solution was then passed through a column of Amberlite IRC-50 (ammonium) resin (0.5 × 2 cm). The tubes were rinsed with 0.25 ml and then 0.5 ml of water and the washings were applied to the columns. The columns were blown dry, left for 10 minutes, then blown dry again. The column effluents were lyophilized, redissolved in 0.1 ml of water, and transferred quantitatively to paper. Less than 5% of the ultraviolet-absorbing material was lost during these manipulations. The chromatogram was developed for 30 hours in solvent B, and the spectra of the ultraviolet-absorbing components were determined as above. The eluates thus obtained were further treated in one of two ways. They were either (a) applied to paper and subjected to electrophoresis in 0.05 M ammonium formate buffer, *p*H 3.5, for 1.5 hours at 80 v/cm, or (b) incubated with an excess of phosphomonoesterase and then subjected to paper chromatography for 40 hours in solvent A. The ultraviolet-absorption spectra of all components were recorded. The movements of the various components relative to Ap and A are listed in Table III. The complete analyses of all the peaks are shown in Table IV.

Previous investigations by Tomlinson and Tener (1962, 1963a) have demonstrated that this chromatographic technique separates the constituents of a nuclease digest mainly on the basis of net negative charge, although base composition still plays a minor role. As shown by the Pu-Py ratios (as well as by

TABLE III
RELATIVE MOVEMENT OF NUCLEOTIDE COMPONENTS ON CHROMATOGRAPHY AND ELECTROPHORESIS

Nucleoside	R_{Ap} in Solvent B	R_{Ap} on pH 3.5 Electrophoresis	After Phosphomonoesterase R_A in Solvent A	
Ap	1.00	1.00	A	1.00
Cp	0.74	0.00	C	0.57
Gp	0.50	3.40	G	0.13
Up	0.48	5.10	U	0.28
ψUp	0.37	4.90	ψU	0.10
Tp	0.65	3.60		
1-Methyl-Gp	0.62			
2-*N*,*N*-Dimethyl-Gp	0.83			
5-Methyl-Cp	0.80		5-Methyl-C	0.58
"Methyl-A"	1.05	0.95		
Y_1	0.74			
Y_2	0.60			
Y_3	0.60			
Z_1	0.37			
Z_2	0.74			
Z_3	0.90		Z_3	0.13
Average Distance Moved by Ap =	32 cm	5 cm	Average Distance Moved by A = 18 cm	

chromatographic and electrophoretic data) peaks 0, 1, 2, 3, 4, and 5 did, indeed, contain largely nucleosides and mono-, di-, tri-, tetra-, and pentanucleotides, respectively. The fact that peaks 6, 7, and 9 (about 5% of the total digest) did not conform to this pattern may have been because of the difficulty of estimating accurately the very small amounts of pyrimidines present, or it may be that with long polymers of widely different compositions the charge differences are insufficient to permit a clear-cut separation (for peak 8, see Discussion).

Peaks 2a, 3a, 4a, and 5a (4.5% of the total digest) also appear to be anomalous and their presence remains unexplained. The first three peaks contain fairly large amounts of ψU although the percentage decreases from 2a to 4a. (A small amount of ψU in peak 5a may have been missed due to incomplete separation from U.) These peaks also contain two groups of unidentified compounds, Y_1, Y_2, and Y_3; Z_1, Z_2, and Z_3. The first group have ultraviolet spectra (see Figure 8) which bear a slight similarity to that of nicotinamide mononucleotide. It is not known whether these compounds are natural constituents of yeast s-RNA or arise during the isolation or analytical procedures. They do not appear to be simply contaminants as they were not detected when undigested samples of peaks 2a and 3a were subjected to chromatography (in solvent B) or to electrophoresis, at *p*H 3.5. They are released, however, on digestion by alkali or crude snake venom phosphodiesterase. A compound having the same spectrum was isolated from a snake venom phosphodiesterase–phosphomonoesterase digest of peak 3a by chromatography in solvent C (R_A 0.75). On electrophoresis at *p*H 7.8 it migrated half as far as the mononucleotides and this migration was unchanged by pretreatment with 1 N hydrochloric acid for 3 hours at 100°, although spectral changes compatible with glycosidic bond cleavage occurred. So far attempts to isolate and characterize this series of compounds have been unsuccessful.

The spectra of Z_1, Z_2, and Z_3 at neutrality (λ_{max} 257 mμ) were similar to that of adenylic acid but they differed slightly from it in their behavior in acid (λ_{max} 259 mμ) and alkali (λ_{max} 256 mμ). Their chromatographic and electrophoretic migrations did not correspond to those of any of the known methylated adenylic acids.

The total composition of the s-RNA was calculated from the data in Table IV and appears in column 2 of Table II. This analysis shows substantial agreement with those obtained by direct alkaline digestion of the s-RNA.

Isolation of 5'-Terminal Sequences (Table V).—The presence of terminal sequences of the form $p(Pup)_nPyp$ in a peak should have been detected by the liberation of pNp compounds on alkaline digestion. Unfortunately the chromatographic systems used did not clearly distinguish between these compounds and alkali-resistant dinucleotides. However, the expected end-sequences were isolated from peaks 3, 4, 5, and 6 (Fig. 4) as follows: First the remaining material from each of the peaks was incubated with an excess of phosphomonoesterase and then the 5'-terminal sequences were separated by chromatography on DEAE-cellulose. They then contained two phosphate groups less than the nonterminal polymers and were thus found in the first major peak off the columns.

Peak 3.—After phosphomonoesterase treatment, peak 3 was diluted 1:5 and applied to a column of DEAE-cellulose (carbonate), (2 × 20 cm), and the column was washed extensively with water to remove the terminal material (present in this case as nucleosides). These were identified by chromatography in solvents A and B and by spectral data. The nonterminal sequences were eluted with 2 M ammonium carbonate.

Peaks 4,, 5 and 6.—These peaks were applied to DEAE-cellulose (chloride) columns (2 × 30 cm for 4 and 5, 2 × 20 cm for 6) and each was subjected to a linear gradient of sodium chloride 0.0–0.3 M (total volume, 4 liters). All solutions were 7 M in urea and 0.005 M in Tris hydrochloride, *p*H 7.8. The elution profiles obtained are shown in Figures 5, 6, and 7. Urea, sodium chloride, and ammonium carbonate were removed as above. For total analysis samples of the 5'-terminal sequences from peak 4 (5 OD units), peak 5 (15 OD units), and peak 6 (12 OD units) were incubated with a mixture of phosphomonoesterase and snake venom phosphodiesterase in 0.1 M ammonium carbonate buffer, *p*H 8.5, for 24 hours at 37°. The ammonium carbonate was removed and the nucleosides identified by paper chromatography in solvent A and from spectral data (Table V).

Peak 4.—In Figure 5, peak 1 contained the terminal sequences PupPy, and peaks 3, 4, and 5 contained the nonterminal sequences PupPupPupPy. Peak 2 appeared to contain nonnucleotide material. Peak 6 consisted of a small amount of unchanged starting material. Two aliquots of peak 1 (5 OD units each) were taken. One was subjected to paper chromatography in solvent B, the other to electrophoresis in 0.05 M formate buffer, *p*H 3.5, for 1.5 hours at 60 v/cm. In both cases the components were located, relative to reference compounds, by ultraviolet light and eluted with water. They were then identified and determined spectrophotometrically (see footnote, Table V).

Peak 5.—In Figure 6, peak 3 contained the terminal fragments PupPupPy, and peaks 6 and 7 contained the nonterminal PupPupPupPupPy. The presence of peaks 1, 2, 4, and probably 5 is indicative of the fact that breakdown of the pentanucleotides had occurred probably before treatment with phosphomonoesterase. Cleavage at various points along the pentanucleotide chain to yield fragments bearing 2',3'-cylic phosphates (resistant to phosphomonoesterase, Khorana, 1961) would give the type of elution pattern obtained. For example, PupPupPu-cyclic-p and PupPy would be

TABLE IV

ANALYSES OF PEAKS ISOLATED AFTER RIBONUCLEASE DIGESTION

Peak	0	1	2	2a	3	3a	4	4a	5	5ε	6	7	8	9
Percentage of Total OD (271 mμ) Recovered	1.35	27.30	20.60	1.40	21.70	1.50	10.40	0.75	8.70	0.80	3.35	1.25	0.40	0.60
Pu/Py[a]		0	1.0	(2.8)	2.0	(2.4)	2.7	(4.4)	4.1	(5.2)	7.1	7.8	4.3	6.4
Compound														
A	79.5	—	8.0	43.5	21.0	52.5	27.0	29.0	37.5	22.0	36.0	45.0	7.0	27.0
C	20.5	60.9	22.0	3.5	16.0	11.5	12.0	6.5	11.5	2.0	8.5	8.0		9.0
G			34.0	30.0	40.5	19.0	41.0	37.0	40.0	45.0	49.0	41.0	61.5	59.5
U		30.2	25.0	8.0	15.0	2.0	10.5	4.0	8.0	11.0	3.5	3.0	16.0	4.5
ψU		6.5	3.5	15.0		12.5		5.0						
T					2.0		4.0							
1-Methyl-G					5.5									
2-*N*,*N*-Dimethyl-G			3.0											
5-Methyl-C		2.5												
"Methyl-A"			4.5											
Y1														
Y2						[b]								
Y3														
Z1								8.5						
Z2								8.0						
Z3										16.5				
pNp and/or alkali-stable NpNp					[c]	2.5	5.0		3.0	3.5	3.0	3.0	15.5	

[a] This ratio was calculated on the basis of those components which had been identified. Due to the presence of unknown compounds those values in parentheses are less meaningful. [b] Indicates the presence of ultraviolet-absorbing material which did not resemble any known nucleic acid constituent. [c] Pyrimidine nucleoside diphosphates, although present (see text), were not detected by this means as they constituted only 1% of peak 3.

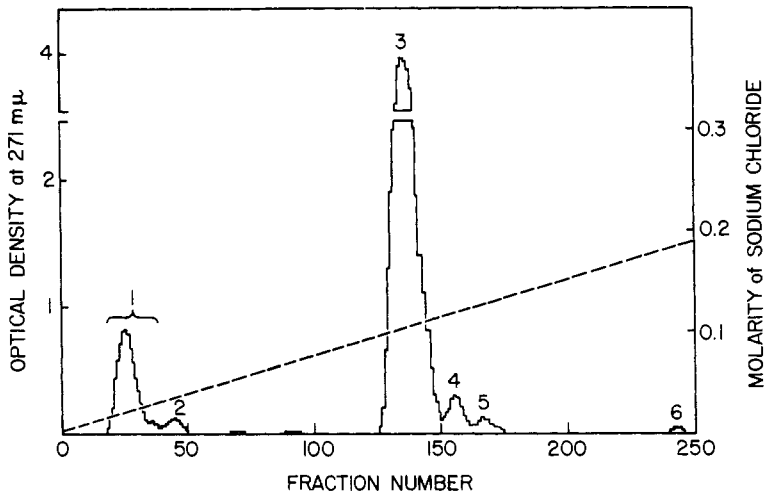

FIG. 5.—Chromatography of peak 4 of Figure 4 (650 OD units at 271 mμ), after treatment with phosphomonoesterase, on DEAE-cellulose (chloride) in the presence of 7 M urea and 0.005 M Tris hydrochloride, *p*H 7.8. Column dimensions, 2 × 30 cm; fraction volume, 10 ml; flow rate, 1.5 ml/minute.

TABLE V

ANALYSIS OF END SEQUENCES ISOLATED AFTER RIBONUCLEASE DIGESTION

Type of end sequence obtained by ribonuclease digestion		pPyp	pPupPyp[a]	pPupPupPyp	pPupPupPupPyp
Percentage of total end recovered		20.5	30.5	42.0	7.0
Ratio Pu/Py		0	1.02	2.8	3.4
Nucleosides isolated after snake venom phosphodiesterase and phosphomonoesterase treatment (%)	A		0.5	19.5	12.5
	C	36.0	48.0	13.5	21.0
	G		50.0	54.0	65.0
	U	64.0	1.5	13.1	1.5

[a] This material was shown (see text) to consist of pGpCp, 95.5%; pApUp, 2.5%; pGpUp, 1.5%; and perhaps a trace of pApCp.

eluted in peaks 2 and 4 (or perhaps 5) and the breakdown products PupPu-cyclic-p and PupPupPy would, of course, be eluted along with the terminal sequences, thereby raising the Pu-Py ratio. The Pu-Py ratio actually obtained for this material indicated that this had in fact occurred and the multiplicity of components obtained on paper chromatography and electrophoresis made further analysis impossible (Bell *et al.*, 1963).

Peak 6.—The profile shown in Figure 7 illustrates that in this fraction the degree of contamination was much less (this is supported by the Pu-Py ratio) but lack of material prevented further investigation.

The total 5′-terminal nucleotide recovered amounted to about 90% of that expected on the basis of a length of 80 residues for this s-RNA (calculated from the ends isolated after alkaline digestion).

DISCUSSION

The pre-existing analytical technique of alkaline digestion followed by column and paper chromatography and electrophoresis is sufficient to allow a complete base analysis of s-RNA (including estimates of those components at the 3′- and 5′-termini) to be made. The analysis shown in Table I and Table II, column 1 was performed on sufficient material to permit the detection of small amounts of 5′-terminal moieties other than pGp. Thus 78% of the chains were shown to terminate in pGp. . ., 10% in pUp. . ., 7% in pAp. . ., and 5% in pCp. . .. However, if information regarding the primary structure of the s-RNA chains is to be obtained, methods must be available whereby the products of digestion of the s-RNA by specific nucleases can be isolated and studied. It was recently demonstrated (Tomlinson and Tener, 1962, 1963a,b) that this can be achieved by chromatography of the digest on DEAE-cellulose in the presence of 7 M urea, by means of which the digestion products are fractionated primarily on the basis of their net negative charge. In the case of s-RNA in which the 5′- termini are phosphorylated (to the extent of 90% or more in this preparation) each terminal sequence is eluted (at *p*H 7.8) in a peak containing nonterminal fragments having two additional purine nucleotides. The end-sequences can then be isolated simply by treatment with phosphomonoesterase and chromatography in the presence of urea, as before. The individual sequences present can then be determined by standard techniques. For studies on a single s-RNA species the nonterminal fragments could also be further fractionated by chromatography on Dowex 1, or DEAE-cellulose, in the normal way. In studying an unfractionated s-RNA preparation the multiplicity of nonterminal sequences obtained makes any attempt at further fractionation impractical.

By means of this new technique a pancreatic ribonuclease digest of s-RNA was fractionated and the distribution of the major and minor base components in the various charge groups studied (Table IV). The methylated components were found to be widely, but nonrandomly, distributed. No T was detected in the mononucleotide peak, demonstrating that this component must always occur adjacent to a purine residue.

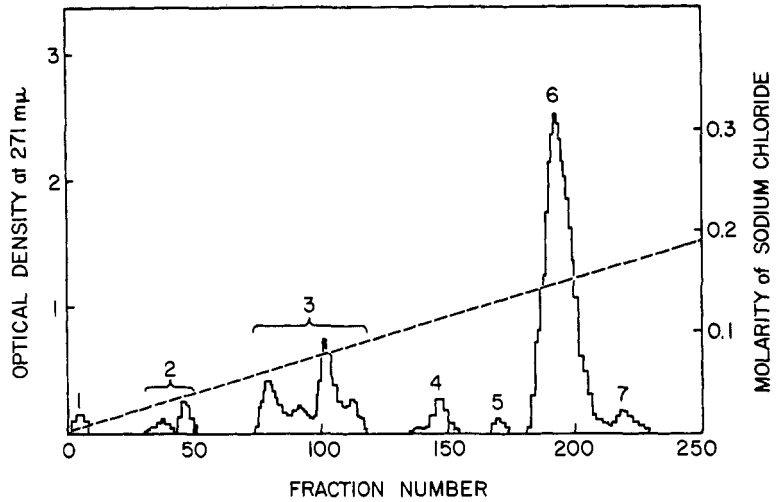

FIG. 6.—Chromatography of peak 5 of Fig. 4 (550 OD units at 271 mμ), after treatment with phosphomonoesterase, on DEAE-cellulose (chloride) in the presence of 7 M urea and 0.005 M Tris hydrochloride, *p*H 7.8. Column dimensions, 2 × 30 cm; fraction volume, 10 ml; flow rate, 1.5 ml/minute.

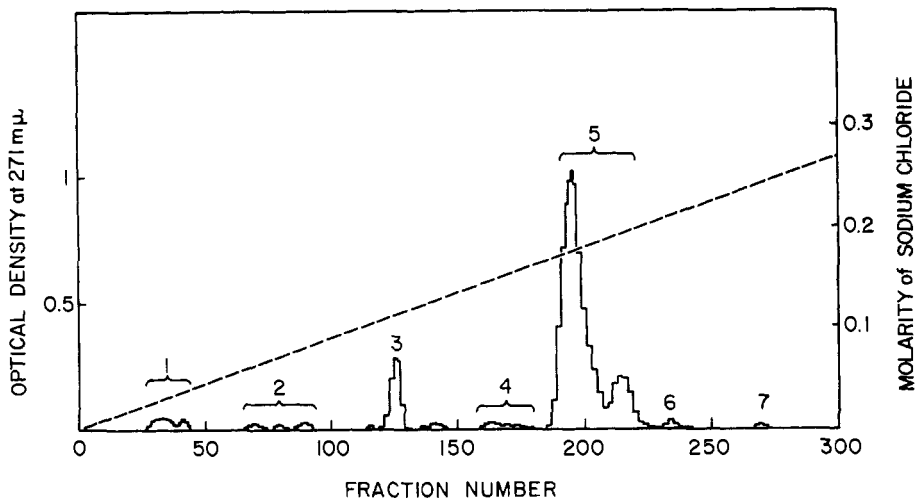

FIG. 7.—Chromatography of peak 6 of Fig. 4 (200 OD units at 271 mμ), after treatment with phosphomonoesterase, on DEAE-cellulose (chloride) in the presence of 7 M urea and 0.005 M Tris hydrochloride, *p*H 7.8. Column dimensions, 2 × 20 cm; fraction volume, 12 ml; flow rate, 1.7 ml/minute.

However, the difficulty of locating very rare constituents of a large peak should be stressed. An estimate of the composition of the whole s-RNA was compiled from these data and found to be in good agreement with those analyses obtained by other means (Table II). Sequences containing phosphorylated 5′- termini were isolated and analyzed (Table V). The figures obtained indicated that 11.5% of the chains terminated in pUp..., 6.5% in pCp..., 26% in pGpCp..., 0.7% in pApUp..., 0.4% in pGpUp..., and less than 0.1% in pApCp....

Identification of the individual 5′-terminal trinucleoside tetraphosphates (pPupPupPyp) was prevented by contamination with nonterminal material, arising by breakdown of the pentanucleotides with which these sequences were initially eluted (peak 5, Fig. 4). This almost certainly occurred during the removal of ammonium carbonate (prior to treatment of the peak with phosphomonoesterase) when the repeated evaporations to dryness under alkaline conditions could have been sufficient to cause cyclic phosphate formation and bond cleavage. Precautions that could be taken to avoid this are (a) use of a lower bath temperature, 20° or less, and (b) replacement of ammonium carbonate by the more volatile triethylammonium carbonate.

Due to lack of material rather than severe contamination, no individual pPupPupPupPyp sequences were identified. The fraction (peak 7, Fig. 4) which presumably contained the pentanucleoside tetraphosphate ends was accidently lost. However, the analysis of peak 8 (Figure 4 and Table IV) could be accommodated by an equal mixture of pGp(Gp)$_4$Up and pGp(Ap)(Gp)$_3$Up.

Any unphosphorylated 5′-terminal sequences would be indistinguishable from the nonterminal fragments in a pancreatic ribonuclease digest and so would not be detected. However, digestion with mung bean nuclease (Sung and Laskowski, 1962) would make these sequences unique and they could be isolated and studied in a manner analogous to the foregoing.

These findings, although similar to those recently obtained by Ralph *et al.* (1963) using a technique involving labeling of the 5′- termini with radioactive aniline, differ significantly in detail. They found that in a somewhat analogous yeast s-RNA preparation 60–70% of the chains terminated in pGp.... They also estimated that 40% of the chains had the sequence pGpCp... in common. It is not clear whether these differences are real or reflect the inadequacies of the analytical procedures.

We feel that this initial study, although by no means exhaustive, demonstrates the value of this approach to

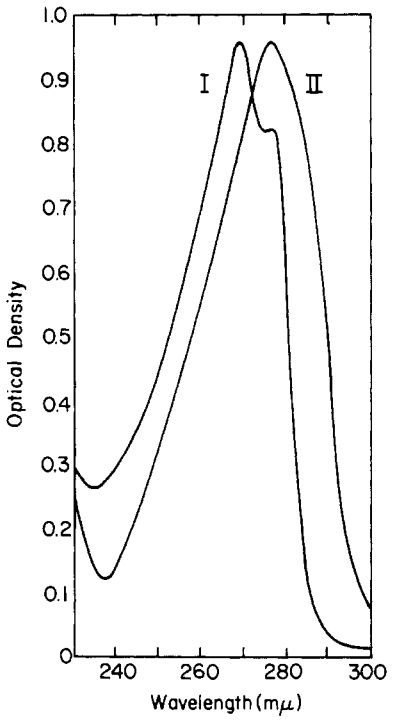

FIG. 8.—Ultraviolet-absorption spectra of the unknown compound Y_2 (Tables III and IV), I in 1 N alkali, and II in 1 N acid.

the determination of polynucleotide structure. However, further work on mixed s-RNA preparations would appear to be unprofitable and future efforts will be directed toward the isolation and study of a single s-RNA species.

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The Chemistry of Pseudouridine. III. The Structure of the A Isomers*

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Degradation of both pseudouridine A_F and A_S by periodate oxidation, borohydride reduction, and alkaline hydrolysis gave 5-(1′,2′-dihydroxyethyl)uracil. This establishes that the A isomers are anomeric forms of D-ribopyranosyluracil.

When pseudouridine-C is heated in either acid or alkali three new isomers, designated A_F, A_S, and B, are formed (Cohn, 1960; Chambers *et al.*, 1963). The structure of pseudouridine-C has been unequivocally established as 5-β-D-ribofuranosyluracil (Michelson and Cohn, 1962) and there is little doubt that pseudouridine-B is the α anomer of C. However there are at least four structures which are consistent with the data accumulated on the A isomers (Cohn, 1960; Shapiro and Chambers, 1961; Chambers *et al.*, 1963). Thus the A isomers could be pyranosyl (I) or furanosyl (IV) anomers or a combination of these forms. This paper describes a simple degradation scheme which clearly dis-

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