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Chromatography of Mixed Oligonucleotides on DEAE-Sephadex

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Oligonucleotides as large as the octamer of different purine-pyrimidine ratios were separated according to their chain length by column chromatography on DEAE-Sephadex A-25 in 7 M urea–0.02 M Tris-Cl, *p*H 7.6, and increasing gradients of NaCl. Mixed tetranucleotides and pentanucleotides obtained in this manner were further subfractionated at *p*H 2.7 and found to be eluted according to their net charge. Potential applications of these procedures are discussed.

The fractionation of oligonucleotides from enzymatic digests of ribonucleic acid (RNA) is of importance for the elucidation of nucleic acid sequences. Tomlinson and Tener (1962, 1963) found that 7 M urea at neutral *p*H greatly facilitated DEAE-cellulose chromatography of mixed polynucleotides by resolving them according to their net charge or chain length. Subsequent investigations by Bartos *et al.* (1963) showed that such compounds were eluted in the same chromatographic position not only because of equal chain length but also because of identical purine-pyrimidine ratios. Thus, the trinucleotides ApApUp and GpGpCp (from pancreatic ribonuclease digests of RNA) emerged in one peak, while ApApGp and CpCpGp (from ribonuclease T_1 digest of RNA) did not.

It was also reported that tri- and tetranucleotides from pancreatic ribonuclease digests of RNA could be subfractionated according to their net charge at *p*H 3 by chromatography on DEAE-cellulose in 7 M urea and 0.1 M formic acid (Rushizky and Sober, 1964). However, this subfractionation procedure also failed with oligonucleotides of different purine-pyrimidine ratios.

As described here, the use of DEAE-Sephadex A-25 in 7 M urea at two different *p*H values permits the resolution of mixed oligonucleotides first according to chain length and subsequently by base composition as follows. At *p*H 7, where the dissociation of the amino groups of purines and pyrimidines is completely repressed and each of the four nucleotide residues has the same negative charge due to the phosphate groups, separation occurs as a function of chain length. However, at *p*H 2.7 where there are differences in the degree of dissociation of the amino groups of the nucleotides and the secondary phosphate groups are uncharged, separation of mixed oligonucleotides of equal chain length depends on the variation in net charge brought about by base composition. Applications of these procedures are discussed.

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Experimental Procedure

Reagents and Materials.—All spectrophotometric measurements were made in silica cells with a 1-cm light path in a Beckman DU spectrophotometer and are expressed as absorbancy (A). A Leeds and Northrup meter equipped with microelectrodes was used for *p*H determinations. Reagent-grade urea (Baker) was used without purification since the A_{260} of a 7 M solution was usually less than 0.05. Whatman No. 3MM paper was employed for descending paper chromatography with a solvent containing 40 g of ammonium sulfate per 100 ml of 0.1 M sodium phosphate, *p*H 7.0 (solvent A).

Enzymes.—Ribonuclease T_1, T_2, and *B. subtilis* ribonuclease were prepared as described (Rushizky and Sober, 1962a, 1963; Hartley *et al.*, 1963).

RNA.—High-molecular-weight RNA from yeast was isolated by the method of Crestfield *et al.* (1955). Bacteriophage MS 2 was grown on *E. coli* C-3000 and isolated as described by Strauss and Sinsheimer (1963). The sedimentation coefficient $s_{20,w}$ of a 0.01-ml sample of $A_{260} = 2.0$ was found to be 79.5 in 0.04 M potassium phosphate, *p*H 7, by the method of Vinograd *et al.* (1963). RNA of MS 2 was obtained as described by Strauss and Sinsheimer (1963); the A_{260} for 1 mg/ml at neutral *p*H in water was taken as 25.0.

Enzymatic Digests of RNA.—The preparation of ribonuclease T_1-digests of yeast RNA has been published (Bartos *et al.*, 1963). RNA from MS 2 was similarly digested except that 1 M ammonium carbonate (*p*H 7.6) was employed as the buffer. After hydrolysis (at 37°) the enzyme was removed with phenol and remaining traces of phenol were extracted with ether. The solution was then lyophilized to remove the volatile buffer. The *B. subtilis* ribonuclease digest was obtained by treating yeast RNA in 0.1 M ammonium carbonate, *p*H 8.6, for 6 hours at 37° with 8 units of enzyme per mg of RNA. Under these conditions, the mono- and oligonucleotides in the digest terminate in the 2′,3′-cyclic-terminal-phosphate form (Rushizky *et al.*, 1963). Enzyme and buffer were then removed as described above.

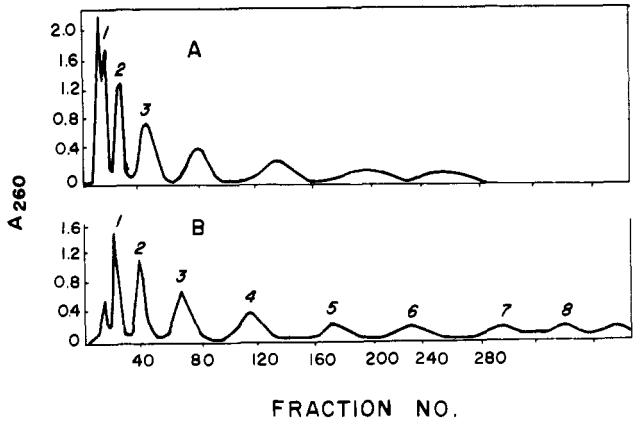

FIG. 1.—Chromatography of *B. subtilis* ribonuclease and ribonuclease T_1 digests of RNA (2.2 × 60-cm columns). (A) *B. subtilis* ribonuclease digest of yeast RNA (100 mg) in a volume of 7 ml was applied to a 2.2 × 60-cm column of DEAE-Sephadex A-25 equilibrated with 0.14 M NaCl–7 M urea–0.02 M Tris-Cl, *p*H 7.6. The column was then connected to a 7000-ml linear gradient of 0.14–0.28 M NaCl in the above buffer and developed (at 23°) at a flow rate of 100 ml/hour, 20 ml/fraction. The run was stopped at fraction no. 280. (B) Ribonuclease T_1 digest of MS 2-RNA (82 mg) in a volume of 2 ml were applied to a 2.2 × 60-cm column of DEAE-Sephadex A-25 in the same buffer as above. The column was then connected to a 9000-ml linear gradient of 0.14–0.32 M NaCl in the above buffer and developed (at 23°) at a flow rate of 100 ml/hour, 20 ml/fraction. In both A and B, the peaks are numbered according to the chain lengths of compounds present. Thus, peaks 1–3 refer to mono-, di-, and trinucleotide fractions.

Column Chromatography.—DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden) with a rated capacity of 1 meq N/g, was freed of fines by repeated decantation of material that did not settle about 30 cm in water (in a 12-liter jar of 15 cm diameter) in 5 minutes. The adsorbent was washed as described for CM-cellulose (Peterson and Sober, 1956), and suspended in the 7 M urea starting buffer and packed into columns by gravity flow. The exchanger in the columns was then equilibrated until the *p*H and conductivity of the effluent solution were the same as those of the influent solution. The flow rate of a 2.2 × 60-cm column (with a 20-cm hydrostatic head) was about 100–120 ml/hour. Somewhat faster flow rates were obtained with 4 × 50-cm columns.

The starting buffer systems contained 7 M urea and either 0.02 M Tris-HCl (*p*H 7.6) plus 0.14 M NaCl or dilute HCl at *p*H 2.7. The apparent *p*H values were determined in urea without dilution. Linear gradients of NaCl were used as indicated below.

The samples were introduced in a volume of 2–5 ml of buffer (about 100 and 500 mg of RNA digest for the small and larger columns, respectively) and washed into the adsorbent with two 1–5 ml portions of buffer. Constant flow rates were maintained with a kinetic clamp pump (Sigmamotor, Middleport, N.Y.). No differences were found when the runs were performed at room temperature or at 5°. After each experiment the exchanger was extruded and washed, and the columns were repacked.

Characterization of Mono- and Oligonucleotides Isolated by Column Chromatography.—Material in the peaks from ribonuclease T_1 digests of RNA was desalted and oligonucleotides up to the tetranucleotide level were identified by mapping (Rushizky and Sober 1962a,b,c). An aliquot of the tetramer peak was rechromatographed in the 7 M urea-HCl system at H*p* 2.7, and the isolated fractions were pooled, desalted,

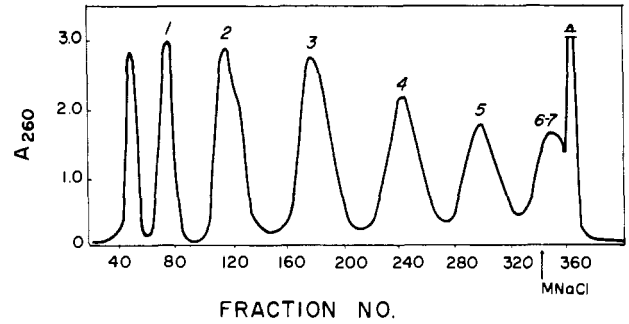

FIG. 2.—Chromatography of ribonuclease T_1 digest of RNA (4 × 50-cm columns). A ribonuclease T_1 digest (500 mg) of yeast RNA in a volume of 5 ml was applied to a 4 × 50-cm column of DEAE-Sephadex A-25 equilibrated with 0.14 M NaCl–7 M urea–0.02 M Tris-Cl, *p*H 7.6. The column was then connected to a 7000-ml linear gradient of 0.14–0.28 M NaCl in the same solution as above and developed at 4° at a flow rate of 100 ml/hour, 20 ml/fraction. At tube 345, the eluent was changed to 1 M NaCl–7 M urea–0.02 M Tris-Cl, *p*H 7.6, in order to finish the run. The peaks are numbered according to the chain length of the compounds present. The first peak contains guanosine 2′,3′-cyclic-terminal phosphate, the peak designated as peak 1 guanosine 3′-phosphate, peak 2 the dinucleotides ApGp, CpGp, and UpGp, peak 3 the trinucleotides, etc. The material in the last peak (after peaks 6–7) was not identified but the A_{260} reached 23.4.

and mapped together with a small amount of the original digest as marker. The individual fractions (see below) were also characterized by paper chromatography with solvent A followed by identification of the compounds by their absorption ratios at *p*H 2 and 7 (Rushizky and Sober, 1962b). Material from the pentamer fraction was similarly rechromatographed in the 7 M urea-HCl system and the isolated peaks were identified by their base ratios. The penta- to octanucleotide peaks were hydrolyzed to mononucleoside-3′-phosphates with ribonuclease T_2 (Rushizky and Sober, 1963) and their chain length was ascertained from the (Ap + Cp + Up)/Gp ratio.

The mono-, di-, and trinucleotide peaks from *B. subtilis* digests of RNA were similarly characterized with the modification that aliquots of each fraction were treated (before desalting) with 0.1 N HCl for 3 hours at room temperature in order to hydrolyze cyclic-terminal phosphate groups to the corresponding 3′-(2′-) stage. Both the cyclic- and acid-treated forms were distinguished by paper chromatography with solvent A (Rushizky *et al.*, 1963).

RESULTS

High-molecular-weight RNA from yeast and bacteriophage MS 2 was hydrolyzed by *B. subtilis* ribonuclease and ribonuclease T_1. The resulting digests containing oligonucleotides of mixed purine-pyrimidine ratios were then fractionated by column chromatography on DEAE-Sephadex A-25 in 7 M urea–0.02 M Tris-HCl, *p*H, 7.6, with linear gradients of NaCl. The compounds in the A_{260} peaks from both digests were then characterized by standard procedures including mapping. With the *B. subtilis* ribonuclease digest, peaks 1–3 were thus found to contain mono-, di-, and trinucleotides present in the cyclic-terminal-phosphate form (Fig. 1A). Peaks from the ribonuclease T_1 digest of MS 2-RNA were found to contain oligonucleotides as follows (Fig. 1B): The small peak preceding peak 1 contained guanosine 2′,3′-cyclic-terminal phosphate. Peaks 1–4 contained guanylic acid, di-, tri-, and tetranucleotides, respectively, as identified by

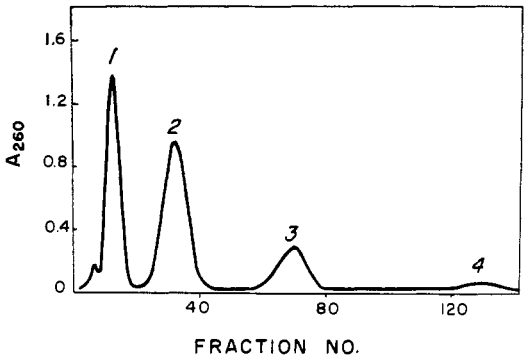

FIG. 3.—Chromatography of tetra- and pentanucleotide fractions of ribonuclease T_1 digest of RNA. Two ml (A_{260} = 500) of tetranucleotides from a ribonuclease T_1 digest of yeast RNA was applied to a 2.2 × 60-cm column equilibrated with DEAE-Sephadex A-25 in 7 M urea–dilute HCl (*p*H 2.7). The column was connected to a 4000-ml linear gradient of 0–0.2 M NaCl in the same solution and developed (at 23°) at a flow rate of 100 ml/hour, 20 ml/fraction. For an identification of the compounds in peaks 1–4, see text.

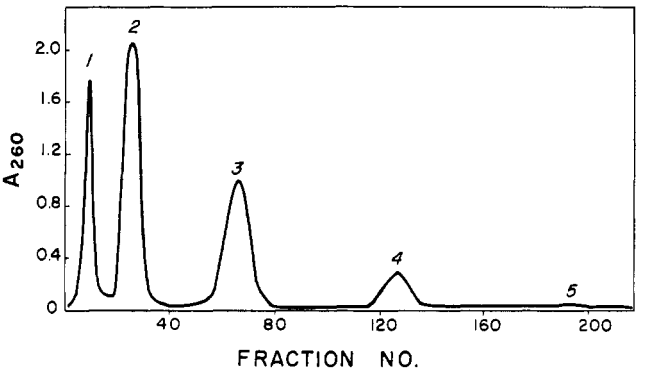

FIG. 4.—Rechromatography of pentanucleotide fractions of ribonuclease T_1 digest of RNA. One ml (A_{260} = 400) of pentanucleotides from a ribonuclease T_1 digest of yeast RNA was applied to a 2.2 × 60-cm column equilibrated with DEAE-Sephadex A-25 in 7 M urea–dilute HCl (*p*H 2.7). The column was connected to a 6000-ml linear gradient to 0.2 M NaCl in the same solution and developed at 4° at a flow rate of 100 ml/hour, 20 ml/fraction. For an identification of the compounds in the peaks see text.

mapping. The compounds in peaks 5–8, with (Ap + Cp + Up)/Gp ratios of 4.1, 4.9, 5.9, and 7.0, were penta-, hexa-, hepta-, and octanucleotides, respectively. Similar results were obtained whether 2.2 × 60-cm columns (Fig. 1) or 4 × 50-cm columns (Fig. 2) were used for the fractionation of 100 mg or 500 mg of oligonucleotides, respectively.

The tetra- and pentanucleotide fractions of the ribonuclease T_1 digest of yeast RNA (peaks 4 and 5, Fig. 2) were subfractionated by chromatography on DEAE-Sephadex A-25 in 7 M urea at *p*H 2.7. The material in each of the four tetramer peaks obtained (Fig. 3) was desalted and identified by mapping, by paper chromatography with solvent A and by determination of base ratios. Of the 27 possible tetranucleotides, 25 were identified as described previously (Rushizky and Sober, 1962b). The second chromatography step in 7 M urea at *p*H 2.7 was thus shown to group the tetramers into four fractions (see footnote (*a*), Table I) according to differences in net charge owing to the variation in base composition. The material in each of the four major peaks of the pentamer fractionation (Fig. 4) was desalted and characterized only by determination of base ratios. It was thus shown that this rechromatography, like mapping (see Fig. 3, Rushizky and Sober, 1962b), separated the tetra- and pentamers according to their net charge as shown in Table I.

TABLE I

BASE RATIOS OF TETRA- AND PENTANUCLEOTIDES

	Figure 3 (Tetranucleotides)[a]			Figure 4 (Pentanucleotides)[a]		
Peak	Ap + Cp	Up	Gp[b]	Ap + Cp	Up	Gp[b]
1	3.1	0	1.0	4.2	0.1	1.0
2	2.1	1.2	1.0	2.9	1.0	1.0
3	1.0	1.9	1.0	2.1	2.0	1.0
4	0	3.0	1.0	1.3	3.1	1.0
5[c]						

[a] Ratios reflect the following compositions obtained for the tetranucleotides (Rushizky and Sober, 1962b). Peak 1: ApApApGp, (ApApCp)Gp, (ApCpCp)Gp, CpCpCpGp. Peak 2: (ApCpUp)Gp, (CpCpUp)Gp, (ApApUp)Gp. Peak 3: (ApUpUp)Gp, (CpUpUp)Gp. Peak 4: UpUpUpGp. Similarly, the pentanucleotides show the analogous distribution. [b] The amount of Gp was set to 1.0 and the other values were multiplied accordingly. [c] The base ratio of the A_{260} material in peak 5 (Fig. 4) was not ascertained. From the absorption ratios at *p*H 2 and 7, it probably contains $(Up)_4Gp$.

DISCUSSION

At present one of the major obstacles to the determination of nucleotide sequences is the lack of methods to prepare, isolate, and characterize oligonucleotides of mixed purine-pyrimidine ratios ranging in chain length from about 5 to 20. Procedures for obtaining such compounds should also be capable of fractionating oligonucleotides terminating in cyclic-terminal phosphates, since these often predominate in partial enzymatic digests of RNA and are likely to contain all four bases. The presence of all 4 bases aids the characterization of these oligonucleotides by permitting further hydrolysis with the two specific ribonucleases now available. By contrast, oligomers from ribonuclease T_1 digests can be further characterized only by treatment with the other specific enzyme, pancreatic ribonuclease.

If one deals with specific ribonuclease digests, then it becomes advantageous to employ homogeneous RNA as the substrate since the frequency of different large oligonucleotides of the same size decreases with their increasing chain length. Such compounds from nonhomogeneous RNA are more likely to be contaminated with oligomers of equal chain length derived from different RNA molecules or from the original chain ends of RNA. Hence the probability of having to separate all possible oligomers of the same chain length is considerably reduced when homogeneous RNA samples are used. In this respect, RNA from bacteriophages such as MS 2 (Davis *et al.*, 1961) and R17 (Enger *et al.*, 1963) provides suitable material.

It was thus the purpose of this investigation to develop a method for the fractionation of tetra- and higher oligonucleotides of different purine-pyrimidine ratios. Since eluting buffers containing 7 M urea (Cole, 1960) and DEAE-cellulose chromatography yielded very good results with pancreatic ribonuclease (Tomlinson and Tener, 1962, 1963) but not with ribonuclease T_1 digests of RNA (Bartos *et al.*, 1963), similar buffers were tried with DEAE-Sephadex A-25 (Holley *et al.*, 1961; Tomlinson and Tener, 1963).

The compounds in the *B. subtilis* ribonuclease digest of yeast RNA were found to be separated according

to chain length up to the trimer level. An inspection of the elution profiles (Fig. 1A) shows that the cyclic-terminal-phosphate compounds emerge earlier than do the 3′-phosphate oligonucleotides of equal chain length (Fig. 1B), as expected from the difference in their negative charges, and thus indicates that the fractionation occurs as predicted for tetra- and higher oligonucleotides. Since the enzyme has no specific point of cleavage such as ribonuclease T_1, the oligonucleotides in a partial digest contain all four bases in various proportions precluding a determination of chain length from base ratio alone. Chain-length determination after enzymatic dephosphorylation would require cleavage of the cyclic-terminal-phosphate linkages by acid hydrolysis, which would entail losses owing to insolubility of higher oligomers. It seems likely that the larger oligonucleotides terminate in the cyclic phosphate form since it has been shown that *B. subtilis* ribonuclease digests of yeast RNA contain appreciable amounts (up to 95%) of cyclic-terminal-phosphate compounds at an enzyme-substrate ratio 10,000 times higher than the one used here (Rushizky *et al.*, 1963). Thus, it appears that larger cyclic-terminal oligomers can be separated by this procedure even though independent determination of chain length by current methods would be difficult.

With MS 2-RNA, mixed oligomers have been fractionated thus far according to chain length from the di- to the octanucleotide level. Continuation of the elution indicates that the separation of higher oligomers will be feasible by this approach with larger initial load. Furthermore, the subfractionation of mixed oligonucleotides of equal chain length by rechromatography at *p*H 2.7 (Fig. 3) should facilitate the identification of higher oligomers, especially with compounds derived from homogeneous RNA.

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Inhibition of Trypsin by Copolymers of Glutamic Acid and Other Amino Acids*

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In contrast to polyglutamic acid, copolymers of glutamic acid with tyrosine, phenylalanine, or leucine are efficient inhibitors of trypsin both at low and at high ratios of polymer to enzyme. On the other hand, the copolymer of glutamic acid and alanine is less inhibitory than polyglutamic acid. The extent of inhibition depends on the time of preincubation of the polymers with the enzyme. Soluble complexes of enzyme and inhibitor were detected in the ultracentrifuge. The copolymer of tyrosine and glutamic acid inhibits the action of trypsin on both small and macromolecular substrates. Urea greatly lowered the inhibitory capacity of this copolymer. Both electrostatic and hydrophobic forces play a role in the interaction of the inhibitor with trypsin. The activity of chymotrypsin is slightly enhanced by polyglutamic acid and partially inhibited by a copolymer of glutamic acid and tyrosine.

Biologically important macromolecules frequently exist in nature as complexes with other macromolecules, and their biological role may often depend on such associations. Various studies have been reported in recent years (for review articles see Sela and Katchalski, 1959; Katchalski *et al.*, 1964) on the interaction of synthetic charged polymers, including basic and acidic polyamino acids, with enzymes. Such interactions often cause inhibition of the enzyme and may provide a model for investigating the mode of action of some naturally occurring macromolecular inhibitors.

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