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Chromatography of Ribonuclease T_1 Digests of RNA on the DEAE-Cellulose in 7 M Urea

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Studies on the chromatography of oligonucleotides and ribonuclease T_1 digests of RNA on DEAE-cellulose in the presence of 7 M urea showed that the oligonucleotides were separated according to their purine-pyrimidine ratio as well as their net charge.

Tomlinson and Tener (1962) reported that pancreatic ribonuclease digests of soluble ribonucleic acid (s-RNA)[1] could be fractionated in 7 M urea on DEAE-cellulose according to the degree of polymerization (or net charge) and independent of the base composition. The method has also been applied to the determination of end groups in s-RNA (Bell *et al.*, 1963) and the separation of guanine-rich oligonucleotides (Lipsett and Heppel, 1963). This report concerns the extension of DEAE-cellulose chromatography in the presence of 7 M urea to ribonuclease T_1 (Sato and Egami, 1957) digests of high molecular weight RNA. The behavior of various oligonucleotides of known base sequence was also examined in this system. Our results show that this method of fractionation of oligonucleotides depends not only on their net charge, but also on their purine-pyrimidine ratio.

Experimental Procedure

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 Beckman Model G pH meter was used for pH determinations.

RNA.—High molecular weight RNA from yeast was prepared by the method of Crestfield *et al.* (1955). RNA from tobacco mosaic virus (TMV-RNA) was a gift from Dr. C. A. Knight, Virus Laboratory, University of California at Berkeley.

Enzymatic Digests of RNA and Isolation of Oligonucleotides.—Pancreatic ribonuclease (2 × crystallized from alcohol) was obtained from Worthington Biochemical Corporation, Freehold, N. J. Ribonuclease T_1 (Sato and Egami, 1957) was prepared as described by Rushizky and Sober (1962a). Alkaline phosphatase from *E. coli* was a gift from Father Donald J. Plocke, Peter Bent Brigham Hospital, Boston, Massachusetts, and was used to dephosphorylate (Khorana and Vizsolyi, 1961) 3′-phosphate-terminal oligonucleotides.

The preparation of enzymatic digests of RNA with pancreatic ribonuclease and ribonuclease T_1, as well as the isolation and characterization of di- and trinucleotides by mapping, paper chromatography, or column chromatography followed by desalting, has been published (Rushizky and Knight, 1960; Rushizky and Sober, 1962a,b).

The mono- and oligonucleotides present in the eluted A_{260} peaks were characterized by their absorbance ratios (250/260, 280/260, and 290/260 mμ) at pH 2 and 7, by mapping, and by determination of base ratios after alkaline digestion (1 N KOH for 24 hours at 23°) followed by paper electrophoresis in 0.02 M ammonium acetate, pH 3.8 on Whatman No. 3 paper.

Adsorbent.—DEAE-cellulose (E. H. Sargent Co., Whatman Floc DE-50, lot 76–81, 1.0 meq of N per g) of 100–230 mesh was used throughout these experiments. The adsorbent was washed as described previously (Peterson and Sober, 1956) and suspended in starting buffer (containing 7 M urea), and the pH was adjusted with dilute acetic or hydrochloric acid. The adsorbent was then washed twice with 2–3 volumes of starting buffer (Table I). The DEAE-cellulose (9 g of dry adsorbent for a 1.3 × 50–cm column) was then packed with gradually increasing pressure to 10 lb/sq in. and washed with starting buffer until the pH and conductivity of the effluent solution were the same as those of the influent solution. The adsorbent was washed and repacked between each run.

Chromatography.—The samples (in a volume of 2–3 ml) were applied by gravity flow and washed into the column with two 1-ml portions of starting buffer. Linear gradients were used as indicated in Table I. Constant flow rates were obtained with a kinetic clamp pump.

Results

Chromatography of Enzymatic Digests of RNA.—When a ribonuclease T_1 digest of yeast RNA was chromatographed at pH 7.5 on DEAE-cellulose in 7 M urea, the results shown in Figure 1A were obtained. It was found that the elution profile was not as simple or unambiguous as that reported by Tomlinson and Tener (1962) for pancreatic ribonuclease digests of

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[1] Abbreviations used in this work: RNA, ribonucleic acid; TMV-RNA, tobacco mosaic virus ribonucleic acid; DEAE-cellulose, diethylaminoethylcellulose; Tris, tris-(hydroxymethyl)aminomethane.

TABLE I
CHROMATOGRAPHY OF OLIGONUCLEOTIDES ON DEAE-CELLULOSE IN 7 M UREA[a]

Figure	Compounds Chromatographed	Sample (A_{260})	Flow Rate (ml/hr)	Total Gradient Volume[b] (ml)	Starting Buffer		Limit Buffer[c]	
					Molarity	*p*H	Molarity	*p*H
1A	Yeast RNA digest, ribonuclease T_1	622	26	2400	0.01 M Sodium acetate	7.5	1.0 M Sodium acetate	7.5
1B	TMV-RNA digest, ribonuclease T_1	664	26	2400	0.01 M Sodium acetate	7.5	1.0 M Sodium acetate	7.5
2	Yeast RNA digest, ribonuclease T_1	2000	70[a]	5000	0.01 M Ammonium carbonate	8.6	0.31 M Ammonium carbonate	8.6
3	Yeast RNA digest, pancreatic ribonuclease	660	26	2400	0.01 M Sodium acetate	7.5	1.0 M Sodium acetate	7.5
4A	CpUpGp, UpCpGp ApApGp	242	26	2400	0.01 M Sodium acetate	7.5	1.0 M Sodium acetate	7.5
4B	CpUpGp, UpCpGp ApUpGp	154	31	2400	0.01 M Sodium acetate	7.5	0.5 M Sodium acetate	7.5
4C	CpUpGp, UpCpGp, mixture of trinucleotides (PuPuPy)	181	34	2400	0.005 M Tris-Cl	7.8	0.005 M Tris-Cl 0.3 M NaCl	7.8
5A	CpGp, UpGp, ApGp	390	32	2400	0.005 M Tris-Cl	7.8	0.005 M Tris-Cl 0.3 M NaCl	7.8
5B	CpG, UpG, ApG	230	33	2400	0.005 M Tris-Cl	7.8	0.005 M Tris-Cl 0.3 M NaCl	7.8
6A	GpCp, CpGp, UpGp	159	32	2400	0.005 M Tris-Cl	7.8	0.005 M Tris-Cl 0.3 M NaCl	7.8
6B	CpGp, GpCp, UpGp ApGp	308	32	2400	0.05 M Tris-Cl	7.8	0.05 M Tris-Cl 0.3 M NaCl	7.8
7	Cp, Ap, CpCp, mixture of (PuPy)	400	31	2400	0.05 M Tris-Cl	7.8	0.05 M Tris-Cl 0.3 M NaCl	7.8

[a] In all cases the column dimensions were 1.3 × 50 cm (inside diameter × height) except in Figure 2 which was 2.2 × 75 cm. [b] The dotted line represents the input gradient for Figures 1 and 3–7; and the effluent gradient for Figure 2. [c] (Peterson and Sober, 1959).

RNA. Thus, peak 2 (dinucleotides) appeared as a double and peak 3 as a multiple peak, while the remainder of the material was eluted as a broad band.[2] The chromatogram obtained with a ribonuclease T_1 digest of high molecular weight TMV-RNA was similar to that of yeast RNA (Fig. 1B). However, when an identical ribonuclease T_1 digest of yeast RNA was fractionated on DEAE-cellulose in 7 M urea but at *p*H 8.6 in an ammonium carbonate system, the dinucleotides and several trinucleotides were clearly resolved from each other (Fig. 2). By contrast, a pancreatic ribonuclease digest of the same yeast RNA yielded a regular series of peaks when chromatographed on DEAE-cellulose in 7 M urea at *p*H 7.5 as described by Tomlinson and Tener (1962). In confirmation of their results, the peaks numbered 1 through 6 (Fig. 3) were shown to contain the mono- to hexanucleotides, respectively. Peaks 1 through 4 were identified by two-dimensional mapping and peaks 5 and 6 by base ratios.

The differences observed with the two enzymatic digests of the same yeast RNA preparation suggested that some factor(s) other than net charge affects the separation of oligonucleotides on DEAE-cellulose in the presence of 7 M urea. Consequently, the fractionation behavior of various oligonucleotides with known base sequences was investigated using the same chromatography method.

[2] The two peaks eluted before peak 1 (guanylic acid) contained phenol, and guanosine 2′,3′-cyclic phosphate plus a degradation product from phenol, respectively.

Chromatography of Trinucleotides.—Under conditions which do not resolve any of the trinucleotides in a pancreatic ribonuclease digest, the compounds CpUpGp and UpCpGp emerge in a single peak but are completely separated from ApApGp (Fig. 4A). Figure 4B shows that CpUpGp and UpCpGp were also separated from ApUpGp upon a decrease of the salt gradient. In addition, ApUpGp and UpApGp were not resolved when subjected to the same chromatography conditions. Using the sodium chloride–0.005 M Tris-Cl, 7 M urea, *p*H 7.8, gradient recently described by Bell *et al.* (1963), an unresolved mixture of trinucleotides (designated PuPuPy) isolated from a pancreatic ribonuclease digest of RNA showed a stronger affinity for DEAE-cellulose and was separated from a mixture of CpUpGp and UpCpGp (Fig. 4C).

Chromatography of Dinucleotides.—Analogously to the trinucleotides, ApGp was separated from UpGp and CpGp (Fig. 5A). After dephosphorylation, some separation of these three dinucleotides was still observed (Fig. 5B). However, under the same conditions, GpCp, CpGp, and UpGp were eluted in a single peak (Fig. 6A). With 0.05 M Tris-Cl, a mixture of dinucleotides from a ribonuclease T_1 digest and GpCp gave the chromatogram shown in Figure 6B. The separation was similar to that in Figure 5A, although, as would be expected from the higher buffer concentration, a smaller volume was required for elution. CpCp (prepared according to Reddi, 1959) had a lower affinity for DEAE-cellulose in 7 M urea (Fig. 7) than a mixture of dinucleotides containing 1 purine and 1 pryimidine (PuPy) obtained from a pancreatic ribonu-

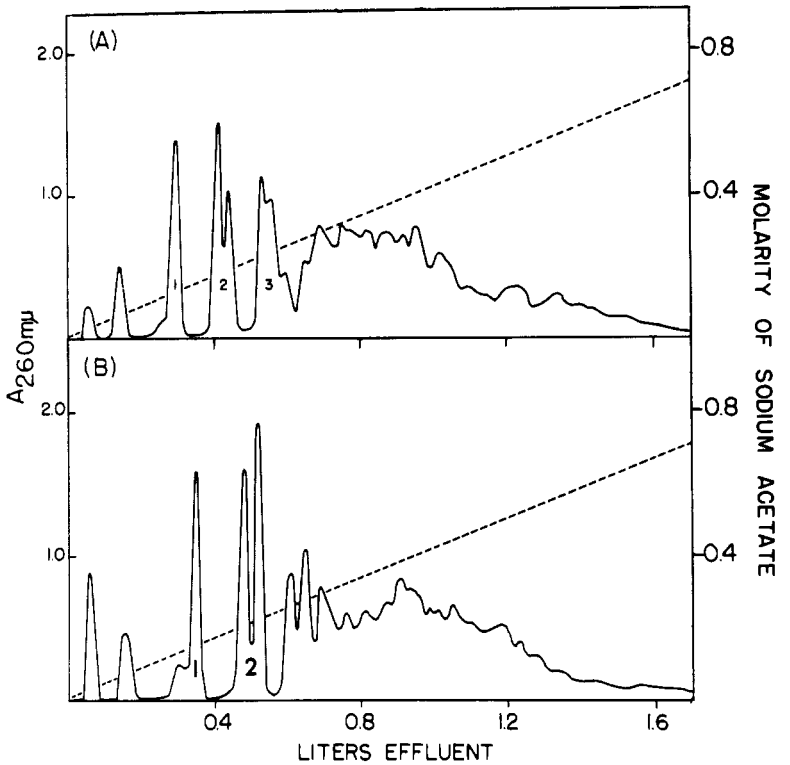

FIG. 1.—Chromatography of a ribonuclease T_1 digest of (A) yeast RNA and (B) TMV-RNA at *p*H 7.5 with a sodium acetate gradient. The dotted line represents the input gradient. See Table I for experimental data.

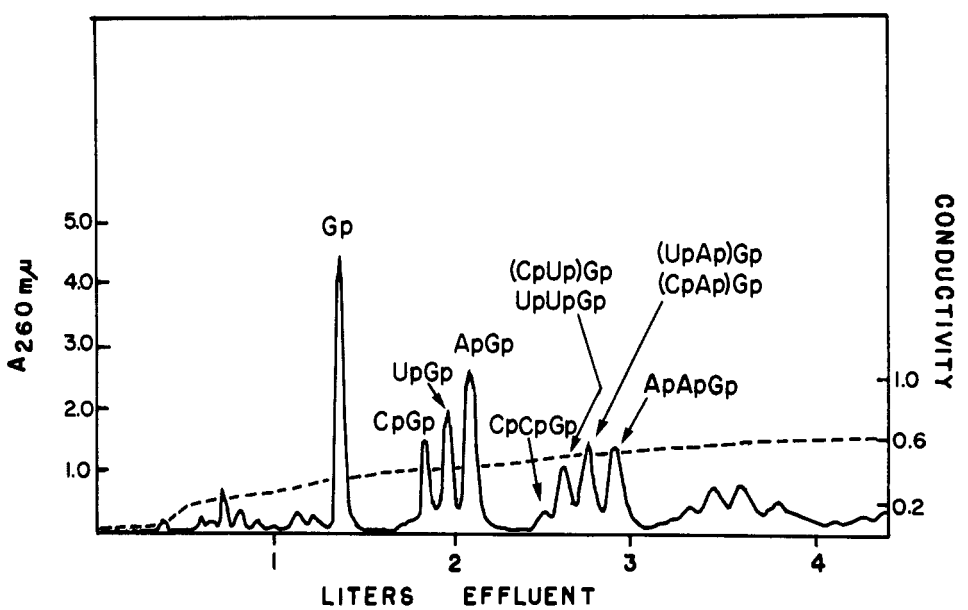

FIG. 2.—Chromatography of a ribonuclease T_1 digest of yeast RNA as in Fig. 1A, but at *p*H 8.6 with an ammonium carbonate gradient. The dotted line represents the effluent gradient as measured by conductivity. See Table I for experimental data.

clease digest of yeast RNA. Cp and Ap were also separated from each other under the same conditions.

DISCUSSION

The fractionation of oligonucleotides from enzymatic digests of RNA is of importance for the elucidation of nucleotide sequences in nucleic acids. Using DEAE-cellulose in the presence of 7 M urea, Tomlinson and Tener (1962) concluded that the separation of mixed polynucleotides depended only on their degree of polymerization (or net charge) and was independent of their base composition.

Our results show that in addition to net charge, the ratio of purine to pyrimidine residues of oligonucleotides also affects the binding on DEAE-cellulose in the presence of 7 M urea. For example, the trinucleotide containing 3 purine residues (ApApGp) has a stronger

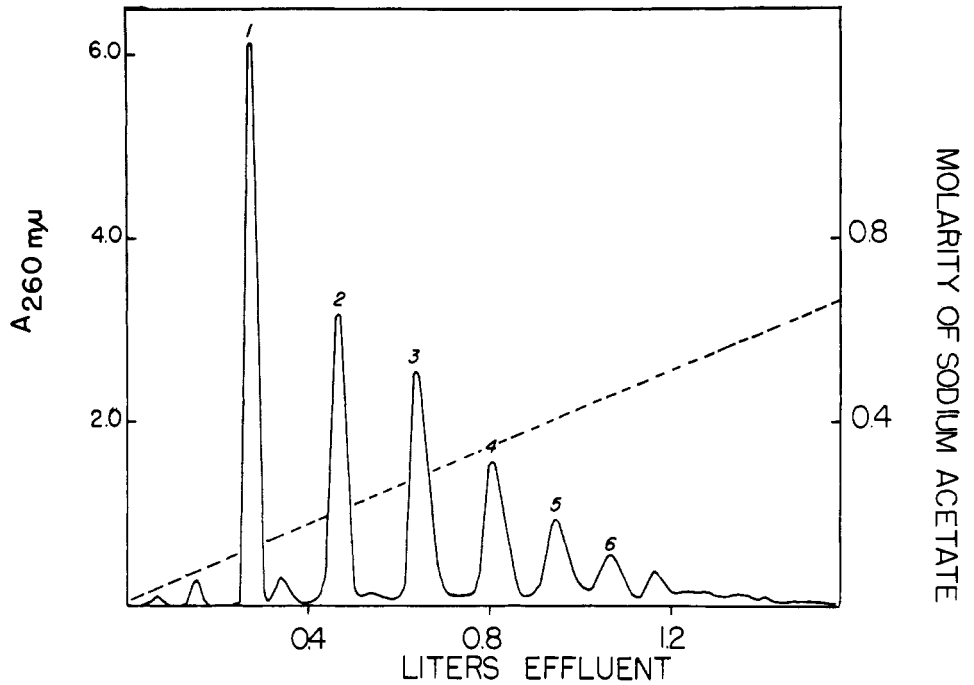

FIG. 3.—Chromatography of a pancreatic ribonuclease digest of yeast RNA as described in Table I

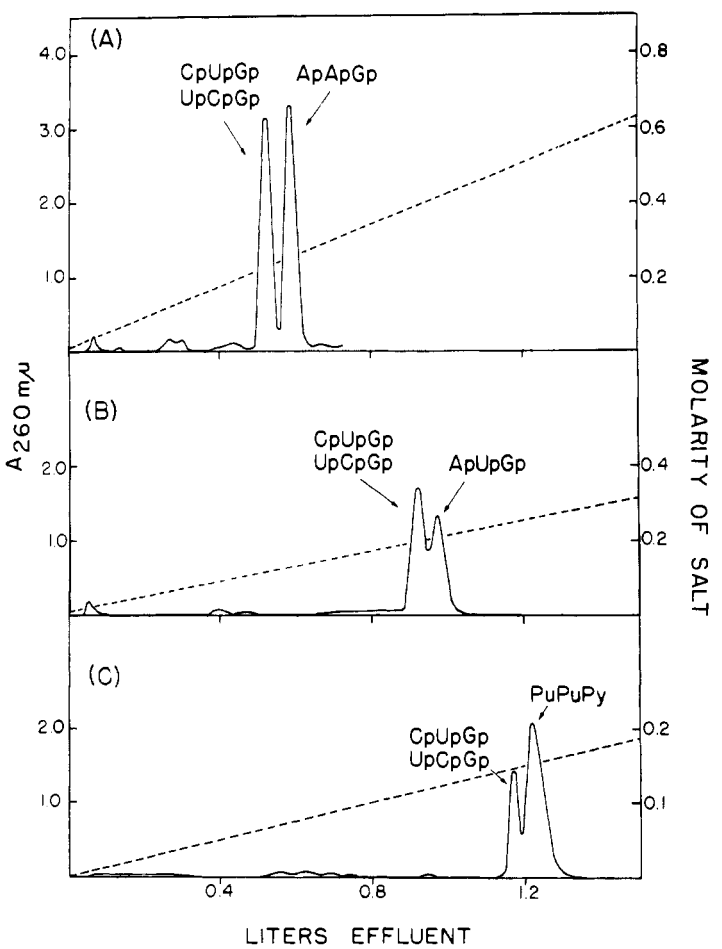

FIG. 4.—Chromatography of trinucleotides: (A) CpUpGp, UpCpGp, and ApApGp; (B) CpUpGp, UpCpGp, and ApUpGp; (C) CpUpGp, UpCpGp, and a mixture of trinucleotides (PuPuPy). See Table I for details.

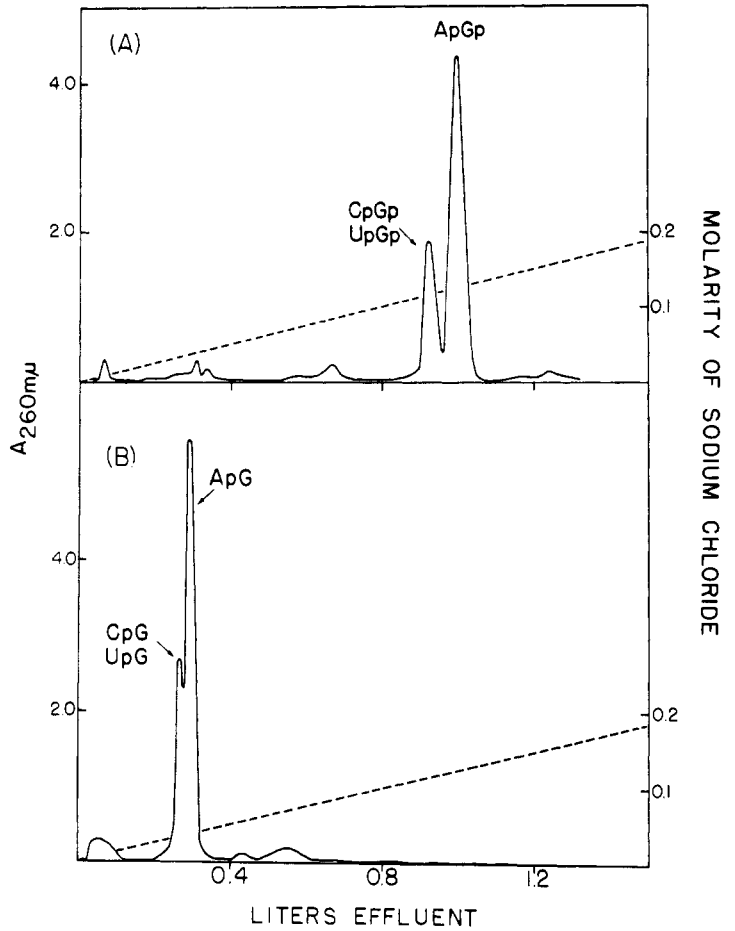

FIG. 5.—Fractionation of (A) dinucleotides CpGp, UpGp, and ApGp, and (B) dinucleoside monophosphates CpG, UpG, and ApG, as described in Table I.

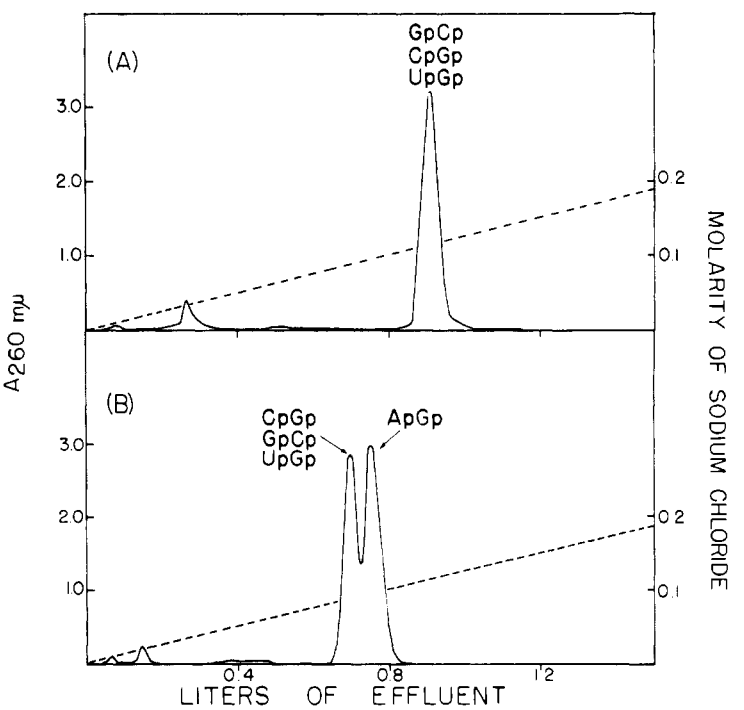

FIG. 6.—Fractionation of dinucleotides (A) GpCp, CpGp, and UpGp, and (B) CpGp, GpCp, UpGp, and ApGp, as described in Table I.

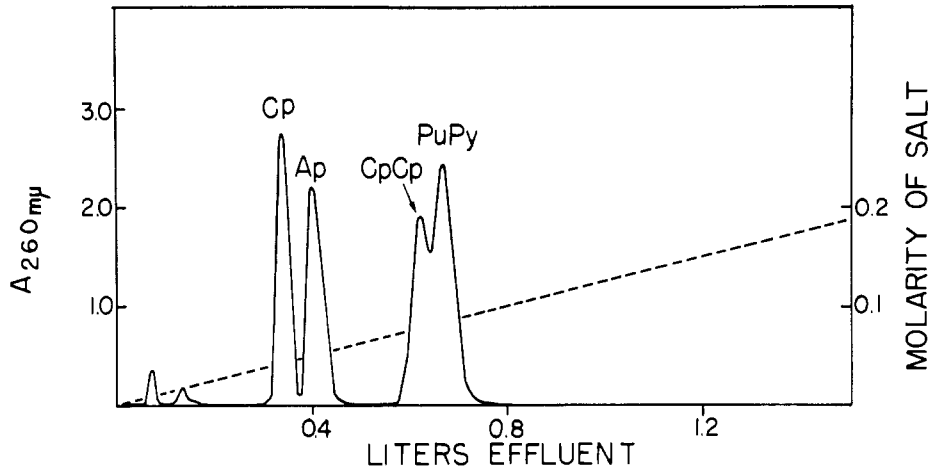

FIG. 7.—Fractionation of mono- and dinucleotides Ap, Cp, CpCp, and a mixture of dinucleotides (PuPy), as described in Table I.

affinity for the adsorbent than those with 2 pyrimidine and 1 purine residue such as CpUpGp and UpCpGp; while the trinucleotides consisting of 2 purine and 1 pyrimidine residue (ApUpGp, UpApGp) are intermediate in binding ability.

The behavior of the dinucleotides examined is consistent with the results obtained with trinucleotides. Thus, CpCp precedes UpGp and CpGp, which in turn emerge from the column before ApGp. The same pattern is observed with the dinucleoside monophosphates CpG, UpG, and ApG. However, raising the *p*H from 7.5 to 8.6 increases the affinity for DEAE-cellulose of Up over that of Cp (Staehelin *et al.*, 1959) so that CpGp precedes UpGp (Figs. 1, 2).

The base sequence in an oligonucleotide does not seem to affect the elution pattern in 7 M urea, since the pairs ApUpGp-UpApGp and GpCp-CpGp emerge from the column together, respectively. This agrees with our results and those previously reported by Tomlinson and Tener (1962) for pancreatic ribonuclease digests of RNA, where all trinucleotides are eluted in the same peak.

According to our findings, such oligonucleotides are eluted in the same chromatographic position not only because of their equal chain length or charge, but also because of their identical purine-pyrimidine ratios. This is in agreement with the results obtained by Lipsett and Heppel (1963) in the separation of guanine-rich oligonucleotides.

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