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Note

Resolution of RNA by paired-ion reversed-phase high-performance liquid chromatography

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High-performance liquid chromatography (HPLC) has developed into a rapid and efficient method for the resolution and the preparative isolation of a large number of biologically and pharmacologically important molecules. For the most part, analyses of nucleic acids have been limited to purines and pyrimidines[1], nucleosides[2,3], nucleotides[4], and short oligonucleotides[5,6]. Recently, the resolution of large DNA restriction endonuclease digestion fragments by HPLC has been reported using columns containing RPC-5[7] and Kel-F resins[8]. To data, no information has appeared concerning the separation of mRNA size molecules on commercially available columns. We report here preliminary experiments for the partial resolution of tRNA from globin mRNA by paired ion reverse-phase HPLC, using commercially available C_{18} columns in the presence of the tetrabutylammonium counter ion.

EXPERIMENTAL

Yeast transfer RNA (type X) and rabbit globin mRNA were purchased from Sigma and Bethesda Research Labs., respectively. Bacterial RNA was isolated from a culture of *E. coli* strain K-12 in logarithmic growth phase as described previously[9].

Radiolabeling of 1-μg aliquots of RNA with cytidine 3′5′-[5′-^{32}P] bisphosphate (specific activity 2000–3000 Ci per mmol from Amersham) and RNA ligase (P-L Biochemicals) was performed essentially according to the method described by Bruce and Uhlenbeck[10]. After incubation for 16 h at 4°C, the samples were extracted three times each with phenol[11] and diethyl ether.

Chromatography was performed on a Waters Assoc. instrument equipped with two Model 6000 solvent delivery systems, a Model 660 solvent programmer, a Model U6K sample injector, and two μBondapak C_{18} columns (30 × 0.4 cm, each) in series. The pumps were programmed to produce a linear gradient (solvent program number 6) from methanol to water with each solvent containing 5 m*M* PIC A reagent (tetrabutylammonium phosphate, Waters Assoc.).

Samples containing 5–25 μl (0.1–0.5 μg) of radiolabeled RNA were applied to the columns at a flow-rate of 1 ml/min, with an operating pressure of 55–69 bars. A 40 ml methanol to water gradient was generated immediately after injection, and 1-ml fractions were collected. The amount of radioactivity in each fraction was measured directly by Cerenkov radiation.

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Aliquots of 200 μl were removed from fractions to be analyzed by polyacrylamide gel electrophoresis and dried prior to resuspension in a solution containing 10 mM Na_2EDTA, 50% (v/v) formamide, and 0.01% (w/v) bromophenol blue and xylene cyanol FF dyes. The samples were then heated to 68°C for 2 min and layered atop a 3.5% polyacrylamide gel (14 cm × 18 cm × 0.15 cm) containing 7 M urea, using a buffer system described previously[12]. Following electrophoresis for 2 h at 200 V, the gels were dried prior to autoradiography.

RESULTS

Purified tRNA (80 nucleotides) and a population of molecules in which rabbit globin mRNA ($\approx$ 650 nucleotides) predominates were radiolabeled separately with

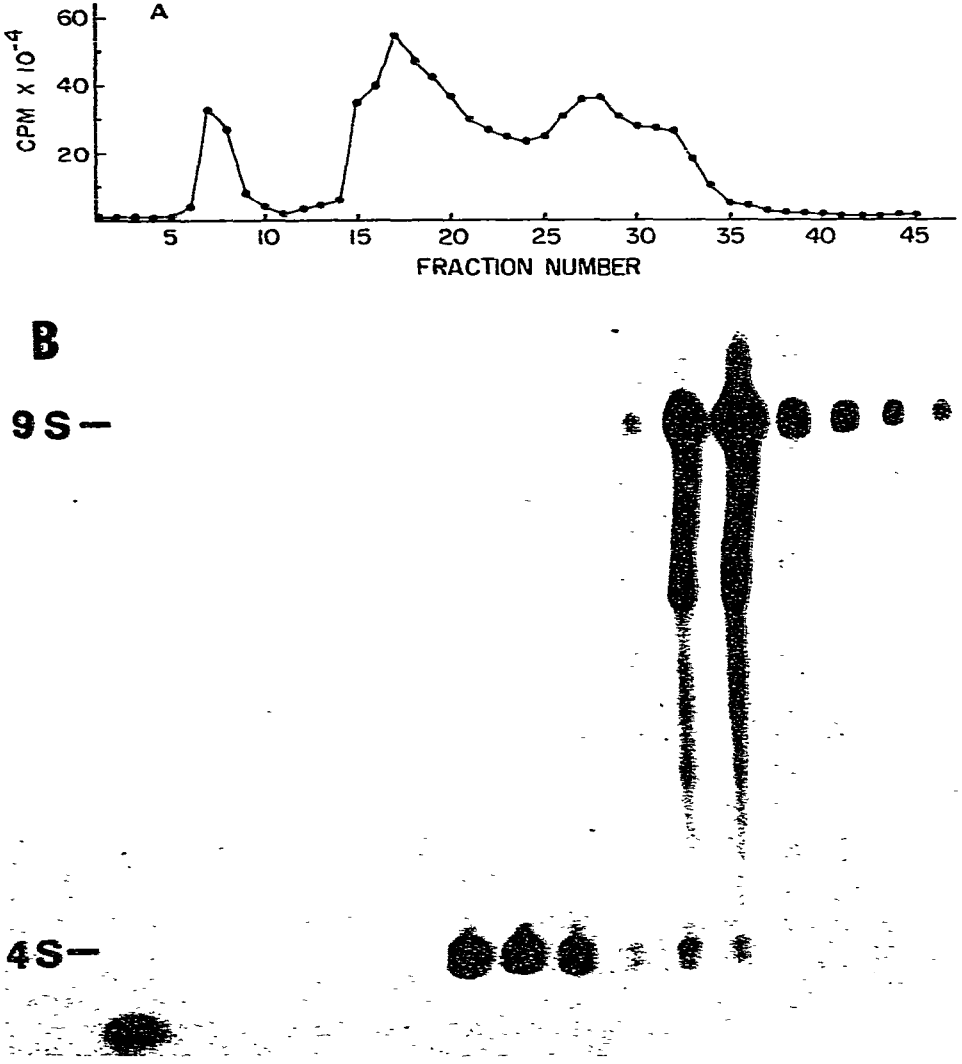

Fig. 1. Elution profile of radiolabeled 4S and 9S RNA following HPLC (panel A) and polyacrylamide gel analysis of fractions 15 through 32 (panel B).

^{32}pCp. Aliquots containing $2 \cdot 10^4$ acid-insoluble cpm from each were pooled and analyzed by HPLC in the presence of tetrabutylammonium phosphate on two C_{18} columns as detailed in the Experimental section. The distribution of radioactivity in the eluted fractions is shown in panel A of Fig. 1, and the electrophoretic mobility of the radioactivity in 200 μl of fractions 15 through 32 is shown in panel B. The peak of radioactivity in the early fractions 6–9 represents authentic unreacted ^{32}pCp as characterized by thin-layer chromatography on polyethyleneimine. The radioactivity which appears in fractions 16–19 is an anomaly. Its amount varies with the lot and "age" of ^{32}pCp used. This material migrates more slowly on polyethyleneimine than authentic ^{32}pCp, suggesting that it is a class of ^{32}P-containing molecules with more than two phosphate groups. Since these molecules are relatively small, they are not

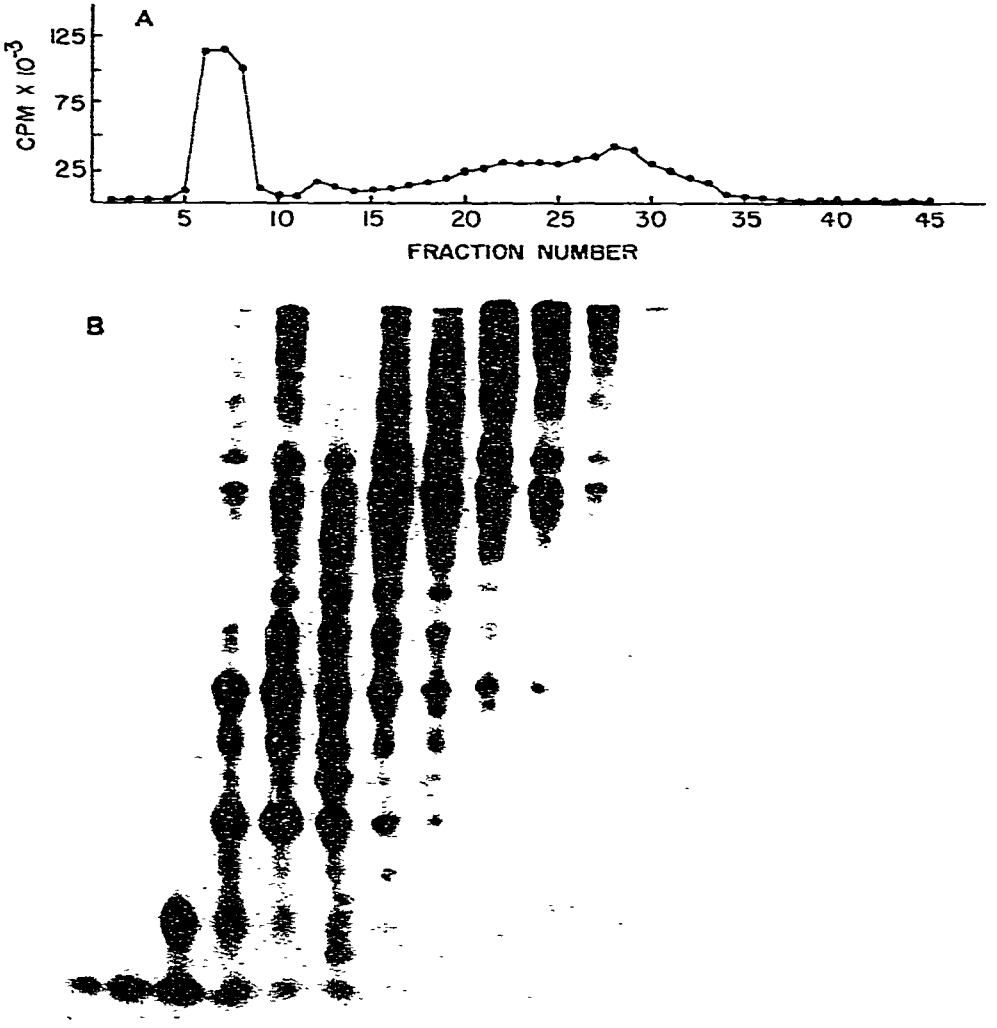

Fig. 2. Elution profile or radiolabeled *E. coli* RNA following HPLC (panel A) and polyacrylamide gel analysis of fractions 17 through 36 (panel B).

retained on 3.5% polyacrylamide gels under the conditions used here. What is apparent from the autoradiograph is that tRNA molecules elute prior to the larger globin mRNA molecules, predominating in fractions 21 and 26, respectively.

Another demonstration of the ability of this technique to separate RNA molecules on the basis of molecular weight is illustrated in Fig. 2. Here, instead of applying an artificial mixture of molecules from two different sources, RNA was isolated from a culture of *E. coli* growing in logarithmic phase, ^{32}pCp labeled and chromatographed as above. Aliquots containing 200 μl from fractions 17 through 36 (Fig. 2, panel A) were processed for electrophoresis, and the pattern is shown in panel B of Fig. 2. Clearly, a gradient of molecules of increasing molecular weight eluted with increasing time.

DISCUSSION

The probability is low that RNA molecules would interact with non-polar $Si(CH_2)_{17}CH_3$ residues of a C_{18} column. In an attempt to promote this interaction, the tetrabutylammonium counter ion was added to the mobile phase. If the counter ion were to affect the mobility of RNA by providing some non-polar character to these polar molecules, it might be expected that the degree of retention of RNA by the column would be somewhat proportional to the molecular weight, *i.e.*, smaller RNA molecules would elute more rapidly from the C_{18} columns than larger ones. Fig. 3 illustrates this concept.

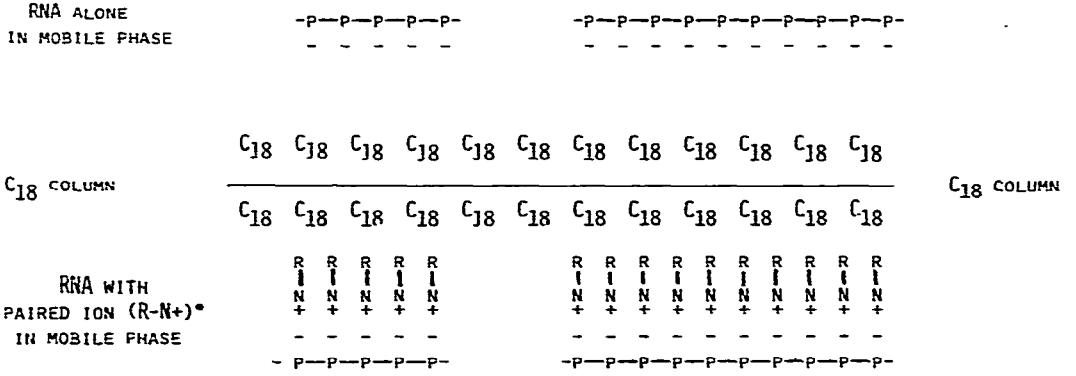

Fig. 3. Idealized view of RNA interaction with C_{18} column matrix in the absence and presence of tetrabutylammonium counter-ion. Above the C_{18} column two RNA molecules are shown; one has five phosphates and the second ten phosphates, each with a negative charge. Below the column these two molecules are shown paired with the counter ion containing the tetrabutyl group (R) which can interact with the C_{18} residues of the column. In this idealized view, the non-polar character contributed by the paired ion is proportional to the number of phosphates, and thus the molecule with five phosphates would be eluted from the column before the longer molecule.

The results presented here demonstrate that RNA molecules of molecular weights greater than tRNA can be fractionated by a simple technique employing commercially available HPLC columns and reagents. At the present state of development, the resolution observed is comparable to sucrose gradient centrifugation. However, the HPLC method offers some immediate advantages. First, comparable resolu-

tion is obtained in 40 min with HPLC as in a minimum of several hours by centrifugation. Second, because both the solvents and paired ion are volatile, subsequent steps, such as ethanol precipitation, are not necessary.

It should be emphasized that the resolution observed in this preliminary report may be increased in future work by manipulating a number of parameters. For example, there are as yet no data on the effects of different solvents, solute concentrations, or shapes of elution gradients. Such work is presently underway. If successful, the utility of this technique in isolating not only individual RNAs, but also individual restriction endonuclease fragments of double stranded DNA may become apparent.

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