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# Separation of *Escherichia coli* Ribosomal Ribonucleic Acids by Reversed-Phase Chromatography*

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ABSTRACT: Reversed-phase chromatography has been applied to the separation and purification of 16S and 23S rRNAs from *Escherichia coli* on a 50-mg scale. The column consisted of methyltrialkyl($C_8$–$C_{10}$)ammonium chloride in 1,1,1,3-tetrachlorotetrafluoropropane or other similar solvents, supported on dimethyldichlorosilane-treated diatomaceous earth, and was developed by sodium chloride gradient elution. The products were characterized and the purity verified by ultracentrifugation, polyacrylamide gradient gel electrophoresis, base composition, and end-group analysis. The 16S RNA had only adenosine as the 3′-terminal nucleoside, and had a molecular weight of about 530,000. RNA preparations from rat spleen and Novikoff hepatoma cells were resolved into several component peaks in similar chromatographic runs. Smaller RNAs elute before rRNAs and can also be resolved.

Three techniques commonly used for separating rRNAs are ultracentrifugation (McConkey, 1967), gel electrophoresis (Dingman and Peacock, 1968; Loening, 1967; Eliceiri and Green, 1970), and chromatography on methylated albumin kieselguhr columns (Murakami, 1967; Osawa and Sibatani, 1967; Mandell and Hershey, 1960). Fractionation of rRNAs as the cetyltrimethylammonium salts has has also been reported (Young, 1968).

We have applied reversed-phase chromatographic systems, which have proven useful for tRNA separations (Kelmers *et al.*, 1965, 1970; Weiss and Kelmers, 1967; Weiss *et al.*, 1968), to the separation of 16S and 23S rRNAs from *Escherichia coli*. The system employs a column material consisting of methyltrialkylammonium chloride extractant dissolved in an organic solvent and supported on an inert support. The RNAs are eluted from the column with a buffered NaCl gradient. Reversed-phase chromatography gives as good or better resolution of a wide range of RNAs as the other techniques, and its scale-up capability has already been demonstrated for the separation of tRNAs (Weeren *et al.*, 1970). This technique provides a simple, inexpensive method for separating a variety of RNAs on both an analytical and preparative scale.

## Materials and Methods

*Materials.* Adogen 464, methyltrialkyl($C_8$–$C_{10}$)ammonium chloride, was obtained from Ashland Chemical Co., Columbus, Ohio. The equivalent weight of the material used was 487, determined by titration with $HClO_4$ in the presence of mercuric acetate (Fritz, 1952; Pifer and Wollish, 1952). Trioctylpropylammonium bromide was obtained from Eastman Organic Chemicals. Dimethyldilaurylammonium chloride (Aliquat 204) was obtained from General Mills. 1,1,1,3-Tetrachlorotetrafluoropropane was obtained from DuPont as Freon 214, and also from Peninsular Chemresearch, Gainesville, Fla. Urea (Ultra Pure) was obtained from Mann Research Laboratories. Acid-washed, dimethyldichlorosilane-treated Chromosorb W (100–120 mesh) was obtained from Johns-Manville Products Co. Another support material, a fluorohalocarbon resin, Plaskon CTFE 2300 powder, was obtained from Allied Chemical Co. The powder was sieved through stainless steel screens to obtain an 80–120 mesh material which gave better flow properties.

Polynucleotides and calf thymus DNA were obtained from General Biochemicals. Novikoff hepatoma 4–8S nuclear RNA was kindly provided by Dr. Harris Busch

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of the Baylor College of Medicine, and rat spleen RNA was given to us by Dr. Carl Wust of the University of Tennessee.

*Preparation of rRNA.* Gram quantities of rRNA were prepared from either *E. coli* B or Q13, a nuclease-deficient strain. After growth, the cells were ruptured with a Gaulin press, and the cell debris was removed by centrifugation. The ribosomes were recovered from the supernatant solution by centrifugation at 100,000*g*.

Total rRNA was prepared from the ribosomes by a phenol extraction procedure based on the method of Stanley and Bock (1965). The ribosomes were suspended in a 0.1 M sodium acetate solution (pH 6.0), containing 0.001 M EDTA, 0.5–2.0% sodium dodecyl sulfate (Stutz and Rawson, 1968), and 0.1% Macaloid, and stirred for about 5 min at 20°. After cooling to 5°, the solution was stirred with an equal volume of 88% phenol, previously equilibrated with the buffer solution. The extraction was repeated, and total RNA was precipitated from the aqueous buffer phase by the addition of three volumes of cold (−20°) ethanol. The solid was redissolved in 0.1 M sodium acetate, and the high molecular weight rRNA was precipitated from 2.0 M sodium acetate, leaving most of the lower molecular weight material (tRNA and 5S rRNA) in solution. The precipitate was washed with ethanol, partially dried, and stored at −20° as a moist solid.

*Analytical Methods.* The RNA in the chromatographic fractions was precipitated with ethanol and redissolved in 0.01 M Tris-HCl (pH 7.0) containing 0.001 M $MgCl_2$, then stored at −20°.

Nucleotide composition and end-group assays of the RNAs were obtained by anion-exchange chromatography after alkaline hydrolysis. RNA samples were hydrolyzed for 20 hr at 38° in 0.33 M KOH and then neutralized with 2 M $HClO_4$ (Steiner and Beers, 1961; Davidson and Smellie, 1952). After centrifugation, the supernatant was chromatographed on a high-pressure anion-exchange column (Scott *et al.*, 1970) using an ammonium acetate gradient. Base composition and molecular weights were calculated from peak areas, determined by integration, and molar extinction coefficients, determined under elution conditions. Base composition was also determined by nucleoside content. RNA samples were converted into nucleosides either by hydrolysis with 1 M KOH and alkaline phosphatase or a combination of venom phosphodiesterase and alkaline phosphatase (Uziel *et al.*, 1968; Burtis, 1970). Nucleosides were separated by cation-exchange chromatography (Katz and Comb, 1963; Uziel *et al.*, 1968). As standards, reference nucleotides and nucleosides were chromatographed under the same conditions as the RNA hydrolysates.

*Column Packing Preparation and Chromatography.* The packing was prepared by adding, dropwise, 50 ml of a 0.1 M solution of Adogen 464 in Freon 214, or other diluents, to 100 g of Chromosorb W, with constant mixing to achieve even distribution. The resulting moist packing was slurried in a solution of the same composition as the eluting solution; *e.g.*, 0.05 M Tris-HCl (pH 7.4), 0.01 M $MgCl_2$, 0.35 M NaCl, and 3.0 M urea. The slurry was poured into a column, also containing the solution, and allowed to settle. The column was equilibrated by pumping at least a column volume of the solution through the column. The column was jacketed, and ethylene glycol from a controlled temperature bath was circulated through the jacket to obtain constant temperatures of 5, 25, or 35°. The rRNA sample was applied to the top of the packing in 2–5 ml of the starting gradient solution.

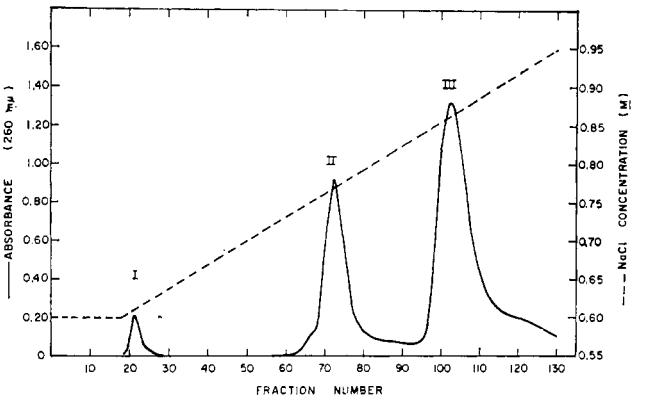


FIGURE 1: Chromatogram from reversed-phase chromatography of *E. coli* Q13 rRNA on Adogen 464–Freon 214–Chromosorb W. Column 1 × 205 cm; temperature 25°; eluent 0.05 M Tris-HCl (pH 7.2), 0.01 M $MgCl_2$, and NaCl as shown; flow rate 0.75 ml/min.

The desired gradient was generated with a nine-chamber box (Phoenix Precision Instruments) connected to the column through a pump (Beckman Accu-Flow). Constant flow rates between 0.5 and 1.0 ml per min were maintained. The column effluent was monitored at 260 mμ (Beckman DB-G Spectrophotometer), and 6–9-ml fractions were collected.

## Results and Discussion

Of the extractants tried, Adogen 464 gave better resolution of rRNAs and better reproducibility. Dimethyldilaurylammonium chloride extractant bound the rRNAs to the column but did not separate 16S and 23S rRNAs. Trioctylpropylammonium bromide extractant gave broad rRNA peaks which were not well separated. Also, trioctylpropylammonium bromide is slightly soluble in the aqueous phase, and consequently columns made with this extractant are less stable and not as reproducible as the Adogen 464 columns. The advantages and disadvantages of these extractants in reversed-phase chromatography have recently been summarized (Kelmers *et al.*, 1970). As solvents, carbon tetrachloride, chloroform, and 1,1,1,3-tetrachlorotetrafluoropropane gave equally good resolution of 16S and 23S *E. coli* rRNAs. While benzene and diisopropylbenzene also gave good rRNA separations, their aqueous solubility and ultraviolet absorption characteristics made them less acceptable as solvents. When benzene and diisopropylbenzene solvents were used, it was necessary to monitor the effluent at 280 mμ, and resolution of RNAs decreased as the solvent eventually washed off the column. The Plaskon support gave slightly sharper chromatographic peaks of *E. coli* rRNAs at 25° than Chromosorb W, but Plaskon was more difficult to pack into a column and gave slower flow rates, requiring an increase in pressure.

A typical chromatogram obtained by reversed-phase chromatography of a sample of *E. coli* Q13 rRNA is shown in Figure 1. Similar results were obtained using several other solvents, mentioned above, in place of Freon 214. The peak fractions were pooled, precipitated with ethanol, redissolved in dilute buffer solution, and analyzed by ultracentrifugation and polyacrylamide gradient gel electrophoresis. The small first peak, I, occurs near the breakthrough volume or void volume of the column under the conditions shown. Electrophoresis indicated this peak contained 4–5S material. Aminoacylation showed that part of the material

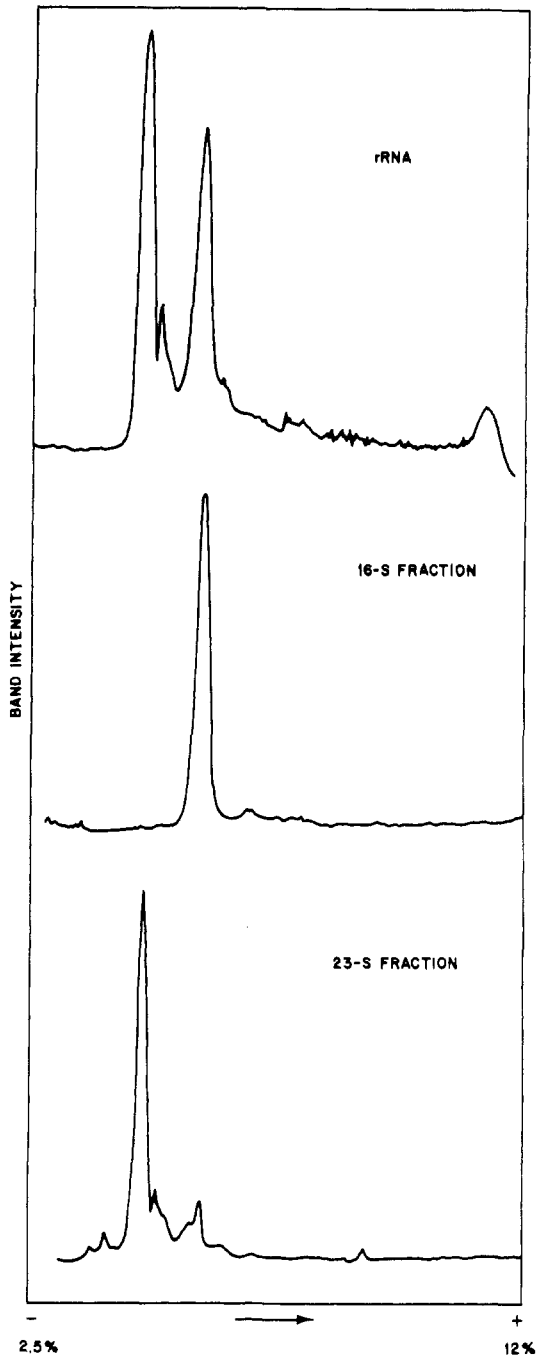


FIGURE 2: Polyacrylamide gradient gel electrophoresis of fractions obtained by reversed-phase chromatography of *E. coli* Q13 rRNA.

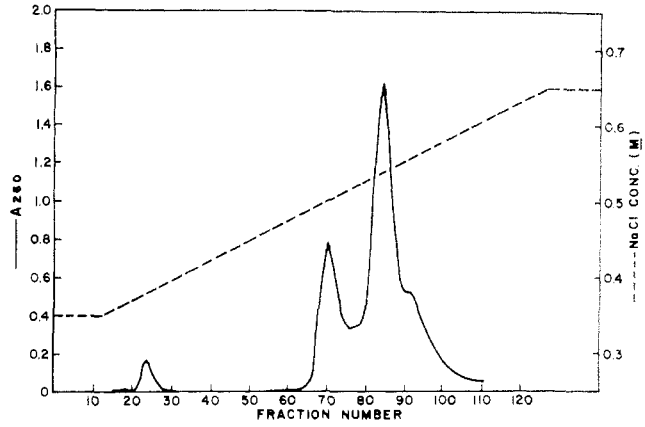


FIGURE 3: Chromatography of *E. coli* Q13 rRNA on Adogen 464–Freon 214–Chromosorb W. Column 1 × 240 cm; temperature 5°; eluent 0.05 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, 3.0 M urea, and NaCl as shown; flow rate 0.75 ml/min.

in peak I was tRNA. Peak II, the first major peak, contains 16S rRNA; peak III contains primarily 23S rRNA, identified by electrophoresis and sedimentation behavior. The shoulder on the rear of the 23S peak has not been conclusively identified, but the material behaves similarly to the 23S material on electrophoresis.

Figure 2 shows typical electrophoresis patterns obtained by scanning the gels dyed after electrophoresis. The direction of travel of the RNAs is from left to right over a continuous gradient ranging from 2.5 to 12% polyacrylamide. The 0.5 × 9.0 cm gels were run at a constant current of 4 mA/tube. After electrophoresis the gels were stained with Pyronin Y, and the stained gels were scanned. The top part of Figure 2 shows an rRNA sample before reversed-phase chromatography. The 16S fraction obtained by reversed-phase chromatography is shown in the center part of Figure 2; the 23S fraction is shown in the lower part of the figure.

With some RNA samples it may be desirable to operate the column at 5° in order to reduce the possibility of thermal or ribonuclease degradation of the RNA. The rRNAs are eluted at lower NaCl concentrations at the lower temperature (Figure 3). However, at 5° the eluent must be 3 M in urea to obtain good recovery (>80%) of the RNAs from the column. With 1 M urea or with no urea present, the RNA recovery is generally less than 40%. In the presence of 3 M urea, the RNAs tend to elute at a lower NaCl concentration. As a consequence of these effects, the 16S and 23S rRNAs are not as well separated at 5° with 3 M urea in the eluent (Figure 3) as they are at 25° where urea is not required (Figure 1). However, resolution is still sufficient to obtain good 16S and 23S rRNA separation. In each case, the solutions contained 0.01 M $MgCl_2$. As with tRNAs (Weiss and Kelmers, 1967); 0.01 M $MgCl_2$ in the eluent also shifts the rRNA elution range to lower NaCl concentration.

After a single chromatographic run, the 16S rRNA gives a single band on electrophoresis, but the 23S peak still contains a small amount of 16S material as seen in the lower part of Figure 2 and verified by ultracentrifugation. However, this 16S material can be removed by rechromatography (Figure 4). The upper part of Figure 4 shows a chromatogram from reversed-phase chromatography using a 2-l. NaCl gradient ranging from 0.35 to 0.65 M. The fractions containing the 23S rRNA, shown by the cross-hatching, were rechromatographed after precipitation with ethanol, using a 1-l. gradient ranging from 0.40 to 0.65 M NaCl to give a purer 23S product, which gave a single peak on electrophoresis.

End-group analysis of the 16S rRNA product by anion-exchange chromatography after alkaline hydrolysis indicated a molecular weight of 530,000, with adenosine as the only 3′-terminal nucleoside. This value of the molecular weight agrees well with values determined by physical methods (Stanley and Bock, 1965). However, more than one 3′-terminal nucleoside, and a correspondingly low value for the molecular weight of the 23S rRNA product were repeatedly obtained, even after rechromatography, although electrophoresis gave a single band indicating a pure 23S product. Lower molecular weights than expected for 23S rRNA when determined by

TABLE I: Base Composition of *E. coli* Q13 rRNAs.

| RNA | Hydrolysis Method | Nucleoside Content (Mole %)[a] | | | |
|---|---|---|---|---|---|
| | | U | G | A | C |
| 16S | KOH + alkaline phosphatase | 25 ± 2.4 | 32 ± 0.5 | 23 ± 0.9 | 20 ± 2.8 |
| | Alkaline phosphatase + venom phosphodiesterase | 20 + 0.5 | 31 ± 1.1 | 26 ± 1.1 | 23 ± 0.7 |
| 23S | KOH + alkaline phosphatase | 24 | 32 | 26 | 18 |
| | Alkaline phosphatase + venom phosphodiesterase | 19 | 31 | 26 | 24 |

[a] Values for 16 S are average values with the average deviation shown.

chemical means have been reported by other investigators (McIlreavy and Midgley, 1967). It is possible that internal breaks in the 23S rRNA molecule give rise to the excess end groups while the physical integrity of the molecule is retained. It is also possible that smaller RNAs are associated with the 23S rRNA, as has been shown to occur with 28S nucleolar RNA (Prestayko *et al.*, 1970). Both adenosine and uridine end groups in the 3′-terminal position have been reported for 16S and 23S rRNA (McIlreavy and Midgley, 1967; Fellner and Ebel, 1970; Nichols and Lane, 1967; Takanami, 1967).

The base compositions of 16S and 23S rRNAs from *E. coli* Q13 as determined by nucleoside content after hydrolysis with either KOH and alkaline phosphatase or venom phosphodiesterase and alkaline phosphatase are given in Table I. The two hydrolysis methods gave significantly different results. The higher values of uridine and lower values of cytidine when KOH was used may have been caused by deamination (Burtis, 1970). However, by either method, the major base compositions of the 16S and 23S rRNAs were similar. The data obtained agree favorably with previously published data on other strains of *E. coli* (Stanley and Bock, 1965; Bartolome and Orrego, 1970; Maruyama and Mizuno, 1970). Melting curves ($A_{260}$ *vs.* temperature, unpublished data) of the 16S and 23S rRNAs showed no significant differences. Differences in secondary structure based on infrared spectra have recently been reported (Hartman and Thomas, 1970).

Figures 5 and 6 demonstrate the applicability of this reversed-phase chromatographic system to the separation of other RNAs. Figure 5 is a chromatogram of a rat spleen rRNA sample showing several peaks absorbing ultraviolet at 260 mμ. Although the peaks were not analyzed further, rRNAs are expected to elute later from the column in the

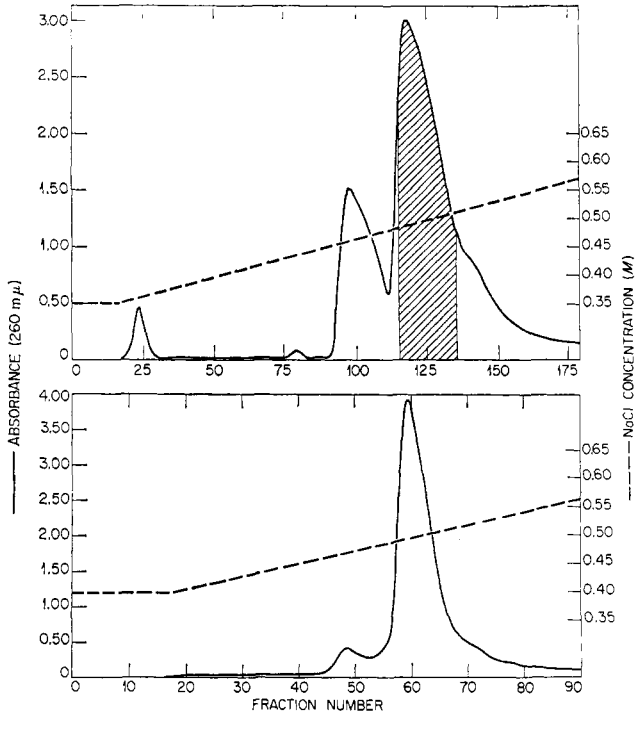


FIGURE 4: Purification of 23S rRNA fraction by rechromatography. Column 1 × 240 cm Adogen 464–Freon 214–Chromosorb W; temperature 5°; eluent 0.05 M Tris-HCl (pH 7.4), 0.01 M $MgCl_2$, 3.0 M urea, and NaCl as shown; flow rate 0.75 ml/min. (a) Chromatogram from reversed-phase chromatography of about 50 mg of *E. coli* Q13 rRNA. (b) Rechromatography of the 23S fractions, shown by the cross-hatching, after precipitation with ethanol.

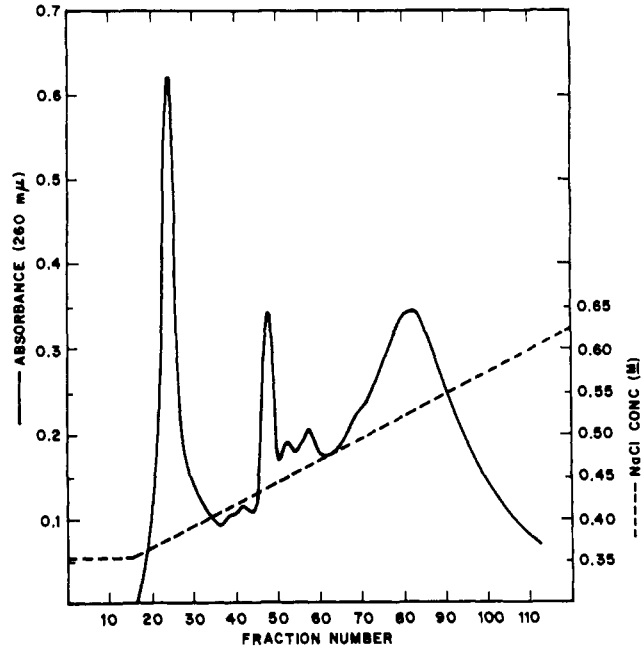


FIGURE 5: Chromatogram of rat spleen RNA. Column 1 × 240 cm Adogen 464–Freon 214–Chromosorb W; temperature 5°; eluent 0.05 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, 3.0 M urea, and NaCl as shown; flow rate 0.75 ml/min.

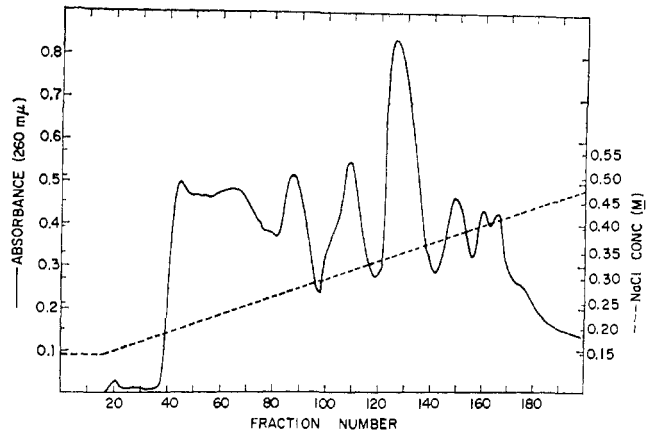


FIGURE 6: Chromatogram of 4–8S nuclear RNA from Novikoff hepatoma. Column 1 × 220 cm Adogen 464–Freon 214–Chromosorb W; temperature 5°; eluent 0.05 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, 3.0 M urea, and NaCl as shown; flow rate 0.75 ml/min.

region of fractions 65–100, and the first peak is probably 4–5S RNA. The 18S and 28S rRNAs did not separate under these conditions. It was also observed that 16S and 23S rRNAs from *E. coli* were not well separated when large amounts of low molecular weight RNAs were present in the sample.

Figure 6 shows a chromatogram obtained from a sample of 4–8S nuclear RNA from Novikoff hepatoma cells. At least eight 260-mμ-absorbing peaks were obtained using a 0.15–0.55 M NaCl gradient. These results compare favorably to the separation of nuclear RNAs obtained by DEAE-Sephadex chromatography (Moriyama *et al.*, 1970).

Other materials that have been successfully chromatographed on this column system include calf thymus DNA and polynucleotides. Polynucleotides such as poly(A) and poly(C) with molecular weights in the range 100,000 to 150,000 and double-stranded poly(I)·poly(C) with an average molecular weight of 500,000, elute at a lower NaCl concentration than 16S rRNA, with greater than 80% recovery. DNA behaves like the polynucleotides, eluting before 16S rRNA also. However, the recovery of DNA is low, about 50–60%, either at 25° or at 5° with 3 M urea present. The chromatograms indicated, by the broadness of the peaks and shoulders on some peaks, that these materials were polydisperse or contained more than a single component. The order of elution of DNA and 4S, 16S, and 23S RNAs on this reversed-phase chromatographic system is similar to that observed on methylated albumin kieselguhr columns (Mandell and Hershey, 1960; Midgley, 1965; Osawa and Sibatani, 1967).

Reversed-phase chromatography provides a simple and inexpensive method for separating a variety of RNAs on both an analytical and preparative scale, and should be equally applicable to the separation of oligonucleotides and mRNAs.

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