Separation of 5S RNA from Other Nucleic Acids by Polyamino Acid Kieselguhr Column Chromatography*

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ABSTRACT: A new simple method is described for the separation of nucleic acids. Kieselguhr columns were prepared with one of the polyamino acids: polylysine, polyarginine, or polyornithine. Nucleic acids bind to these columns and could be eluted with salt gradients. In the case of polylysine and polyarginine, additional pH gradients were necessary to optimize fractionation. These polyamino acid kieselguhr columns had several advantages as compared with other methods of nucleic acid fractionation: in that they could be quickly prepared, rapidly eluted, and reused several times without loss of resolution. The sequence of elution of nucleic acids varies from one polyamino acid to the other. On all polyamino acid columns examined the 5S rRNA was completely resolved from the tRNA.

In order to study the biological function of 5S RNA we became interested in methods for its isolation. Several complicated methods for the preparation of 5S RNA had been available. We concentrated on possible procedures for a rapid preparation and developed a convenient method for the isolation of 5S RNA by column chromatography on polyamino acid kieselguhr.

Material and Methods

Polyamino acids were purchased from Miles-Yeda Ltd. (Rehovoth, Israel). Poly-L-arginine sulfate was lot 35; the molecular weight of the parent compound was 21,900; both poly-L-ornithine (lot 29A, mol wt 15,800) and poly-L-lysine (mol wt 75,000–125,000) were used as hydrobromides. Other chain lengths have not been examined. Kieselguhr (Hyflo Super Cel, particle size 5–25 μ) was purchased from Serva (Heidelberg). Radioactive substances were donated by the Department de Biologie C. E. A. (Saclay). *Escherichia coli* tRNA was obtained from Schwarz BioResearch (Orangeburg). Levallorphane was generously provided by Hoffmann La Roche (Basel). ATP was obtained from Merck (Darmstadt), amino acids from Calbiochem, membrane filters from Sartorius Membranfiltergesellschaft (Göttingen), Alumina (bacteriological grade) from the Alcoa Co., and bovine serum albumin fraction V from Sigma (St. Louis).

Bacterial Strains and Media. E. coli K 12 wild type and K 12 F'lac meth$^-$ W3744 were gifts of Dr. P. H. Hofschneider. The cells were grown with constant shaking on 1% yeast extract (Difco) with phosphate buffer and glucose as carbon source. For labeling experiments, the cells were grown on the triethanolamine medium described by Simon and Van Praag (1964) with succinate as carbon source.

Preparation of Nucleic Acids. Total nucleic acids were isolated by phenol extraction (Schuster *et al.*, 1956; Kirby, 1956) from exponentially growing cells that had been washed and ground with alumina. The 5S RNA, as reference substance, was extracted as described by Comb and Zehavi-Willner (1967). DNA, tRNA, and 16S + 23S RNAs were purified by standard methods (Marmur, 1961; von Ehrenstein and Lipmann, 1961).

Fractionation of Nucleic Acids and Preparation of Polyamino Acid Kieselguhr Columns. Nucleic acid analysis was performed on MAK[1] according to Mandell and Hershey (1960). Polyamino acid kieselguhr columns were prepared as follows. Glass columns (35 × 2 cm; with fused-in fritted disk) were successively filled with (i) a covering layer of Whatman cellulose powder, (ii) a suspension of 8 g of kieselguhr mixed with the amount of polyamino acid specified in 50 ml of 0.4 M NaCl–0.06 M sodium phosphate (pH 6.7) (polyamino acid stock solution was 10 mg/ml in the same buffer except for polyArg: 2 mg/ml in 5% acetic acid), (iii) 2 g of kieselguhr in 10 ml of 0.4 M NaCl, and (iv) glass wool. Kieselguhr suspensions were heated before use and before adding the polyamino acid in order to remove air bubbles. The packed columns were washed with 150 ml of the initial elution buffer before the material was added in 50 ml of the same buffer. The elution gradients are described in Results. The rate of elution was up to 5 ml/min. This flow rate was maintained by 150-cm water pressure. Fractions of 10 ml were collected.

Labeling of RNA in Vivo. Parallel 100-ml cultures were grown to 0.1 OD_{550} unit/ml. One culture received [^{14}C]uracil (100 μCi) and the other culture received [^{3}H]uracil (1 mCi) 5 min after the addition of 600 μg/ml of levallorphane which inhibits 5S RNA synthesis (Röschenthaler *et al.*, 1969). Unlabeled uracil (10 μg/ml) was added to all cultures at this time. After further incubation at 37° for 15 min the bacteria were chilled by pouring onto frozen buffer (0.01 M Tris-HCl–0.01 M $MgCl_2$, pH 7.5) and harvested. Mixtures of control cells (^{14}C labeled) and treated cells (^{3}H labeled) were used for RNA extraction.

Results

Three basic polyamino acids were tested on kieselguhr carrier, one at a time: polyarginine, polyornithine, and

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[1] Abbreviation used is: MAK, methylated serum albumin coated kieselguhr.

polylysine. In order to apply these columns for our problem of 5S RNA isolation, we analyzed first the characteristics of these columns such as capacity, optimal elution gradients, and sequence of nucleic acids eluted.

The optimal concentration of polyamino acid (best resolution) for a given amount of kieselguhr and a given fractionation problem was first investigated. It was found that 6 mg of any of the polyamino acids (6 mg/column, added to 8 g of kieselguhr in the described procedure) suffice for optimal fractionation of 5 mg of total nucleic acids from *E. coli* as measured by optical density at 260 nm. This amount is sufficient to overcome eventual initial leaking of polyamino acid from the column. The columns are then stable for several reuses.

We used 0.06 M sodium phosphate buffered NaCl gradients[2] for elution. Gradients and sequence of elution differed from one polyamino acid to the other. In the case of polyornithine–kieselguhr the final NaCl solution had to be 3 M to elute all material. With polyarginine columns and a concentration gradient from 0.4 to 3.0 M NaCl (0.06 M sodium phosphate pH 6.7), only part of the nucleic acids were eluted. Raising the final NaCl concentration and the pH resulted in the elution of more ultraviolet-absorbing material but even at a final pH of 8.9 (0.1 M Tris-HCl), the nucleic acids were not completely eluted. A pH of about 10 was necessary to elute all material. After this treatment and equilibration with starting buffer the columns could be reused. No loss of resolution was observed after at least ten such experiments. Polylysine columns gave good resolution with NaCl gradients from 0.1 to 2.0 M with a pH gradient from 5.3 to 6.7 (DNA, then, was eluted at or just beyond 2.0 M NaCl).

All columns were run at room temperature with a flow rate of as high as 5 ml/min. Slower flow rates had no advantage. All three polyamino acid columns could be reused. There was no loss of resolution at the tenth time and we have not checked further.

In order to characterize the material being eluted in the different peaks, defined nucleic acids were purified and chromatographed on polyamino acid kieselguhr. On polyornithine, the 4S RNA was eluted before 16S RNA, 23S RNA, and DNA which were not completely separated. The sequence of elution on polylysine was the same but the DNA was resolved from the rRNAs and was well separated from them. The tRNA peak was especially broad on polyarginine. Some separation of tRNA species could be achieved on these columns.

A small peak could be observed on the optical density profiles: a small peak which was eluted after tRNA. This peak was 5S RNA. Isolated 5S RNA was eluted with the same NaCl concentration (see Figure 1), so did isolated unlabeled and labeled 5S RNA when chromatographed alone. An increase in optical density in the described location was noted after the addition of isolated 5S RNA to total nucleic acids with subsequent chromatography on polyarginine. The position of 5S RNA in the elution profile was also determined by chromatography of a nucleic acid mixture of *E. coli* labeled with [^{3}H]uracil and [^{14}C]uracil. The ^{3}H-labeled RNA contained little or no 5S RNA (see Methods).

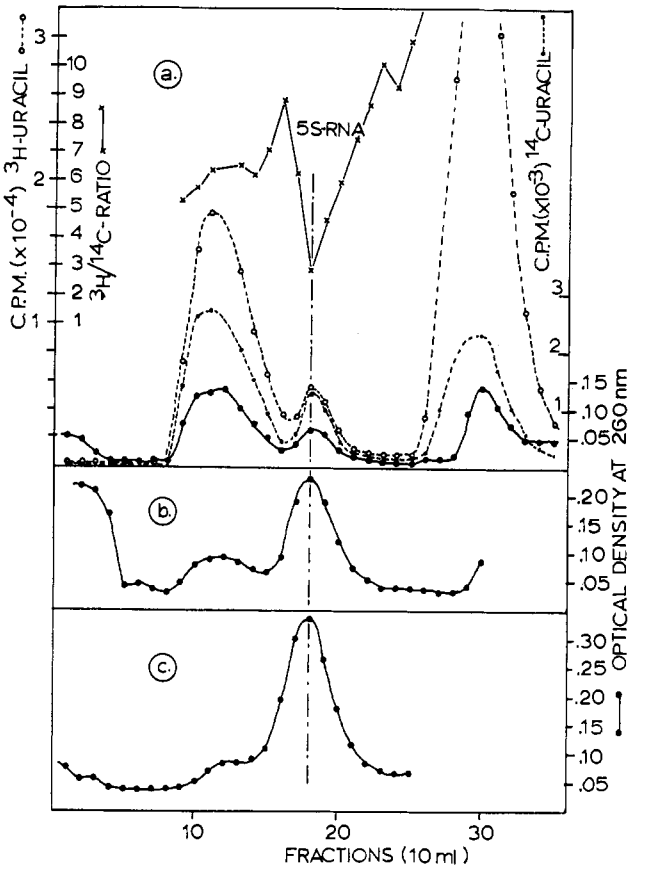

FIGURE 1: Separation of 5S RNA from the other nucleic acids on a polyarginine kieselguhr column. Nucleic acids were applied as described in Methods. Elution by 2 × 300 ml of 1.0–5.0 M NaCl (pH 6.7–8.9) linear gradient, constant flow rate of 5 ml/min. (a) RNA from a mixture of cells was chromatographed, the cells treated with levallorphane grown on [^{3}H]uracil, and control cells grown on [^{14}C]-uracil (see Methods). Total cellular nucleic acids from *E. coli* K 12 (4 mg) were added as carrier. Fractions of 10 ml were collected and optical density was determined both in flow through and fraction by fraction. The nucleic acids in the fractions were precipitated and radioactivity was determined. The last peak shown is DNA, the larger RNAs would be eluted later. The amount of 5S RNA still present in the ^{3}H-labeled RNA depends on time course and doses of levallorphane treatment of *E. coli*. (●—●) OD 260 nm; (●- - -●) ^{14}C cpm; (O- - -O) ^{3}H cpm; (×—×) ^{3}H:^{14}C ratio. (b) 4-mg total cellular nucleic acid from *E. coli* K12, but with 1.5 mg of purified 5S RNA added, OD 260 nm. (c) Half of the amount of nucleic acid as in part b, but with 2.5 mg of purified 5S RNA added, OD 260 nm.

On nucleic acid separation, the value with the lowest ^{3}H:^{14}C ratio marked the location of the 5S RNA. The fraction with the lowest ^{3}H:^{14}C ratio coincided with the position already found for 5S RNA (Figure 1a). This ratio was not further decreased on rechromatography.

Discussion

For the preparation of 5S RNA (Rosset *et al.*, 1964) several more complicated methods have been available. Principally two approaches had been exercised: 5S RNA was prepared either from purified 50S ribosomal subunits (Comb and Zehavi-Willner, 1967; Brownlee and Sanger, 1967) or by fractionation of total cellular RNA. The purifica-

[2] This buffer contained no magnesium. Preliminary results showed that the chromatographic behavior of nucleic acids completely changed at 0.01 M Mg.

tion of 50S ribosomal subunits is time consuming and requires extensive washings to remove tRNA. Fractionation of total RNA on Sephadex (Röschenthaler *et al.*, 1969) or polyacrylamide gel (unpublished data) yields 5S RNA, but reproducibility on Sephadex was low and both methods are tedious. Also 5S RNA cannot be resolved properly from tRNA on MAK columns.[3]

However, the separation of 5S RNA from the other nucleic acid species was possible by chromatography on columns of kieselguhr with polyamino acids. This method is relatively easy, rapid and highly reproducible, even in somewhat larger scale (65 × 3 cm was tested). The columns can be reused several times.

The best separation of 5S RNA was achieved on polyarginine columns. There, tRNA was eluted in a large peak with many shoulders which were separated from the 5S RNA peak which was eluted thereafter. The separation was clearly demonstrated by the isotopic labeling experiments. In these experiments RNA was chosen which contained the normal species and amounts of *E. coli* nucleic acids labeled with [^{14}C]uracil, and ^{3}H-labeled RNA from cells which had been treated with levallorphane. Levallorphane inhibits the synthesis of all rRNAs (Röschenthaler *et al.*, 1969). Therefore, little 5S RNA was labeled with tritium and, in the fractionation profile on polyarginine, the fraction with the lowest ^{3}H:^{14}C ratio would mark the position of 5S RNA. This fraction was indeed eluted with the optical density peak of 5S RNA and well separated from tRNA. The rapid isolation of 5S RNA by this method, thus, increases the chances for the determination of native 5S RNA levels.

Radioactive 5S RNA isolated by one passage on polyarginine did rechromatograph as only one peak. There is no amino acid accepting activity. It should be mentioned that polyamino acid kieselguhr columns can also be used for other RNA fractionation procedures.

After submission of this manuscript, Hatlen *et al.* (1969) achieved the separation of 5S RNA from tRNA and other nucleic acids on Sephadex G-100.

[3] Although on MAK elution profiles 5S RNA is noted as shoulder within the 4S RNA peak (Galibert *et al.*, 1965; Reich *et al.*, 1966), this shoulder does not seem to be correlated to the 5S RNA content only (Röschenthaler *et al.*, 1969).

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