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SEPARATION OF MACROMOLECULAR RNAs BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The reversed-phase high-performance liquid chromatography of synthetic and natural RNAs was studied. The results show that base composition alone cannot explain the retention characteristics of natural RNAs; secondary structures are probably involved. However, under the conditions described, the separation was independent of the sizes of the molecules. 18S and 28S ribosomal RNAs were eluted after tRNAs but before 9S globin mRNA. Furthermore, globin mRNA was resolved into two species, one containing and the other lacking poly(A). When applied to the separation of HeLa cell poly(A)-containing RNA, an heterogeneous pattern was obtained. Analysis of peptides synthesized by the mRNA showed that the separation is independent of size.

INTRODUCTION

Conventional methods of fractionating RNAs are based on centrifugation[1–4] and electrophoresis[5,6] which separate according to size. The need for methods that separate RNAs on the basis of criteria such as base composition is evident.

In recent years high-performance liquid chromatography (HPLC) has been shown to be a powerful technique for the purification of oligonucleotides. Ion-exchange[7,8] and reversed-phase chromatography[9,10] have been used. More recently, Nguyen *et al.*[11] described the separation of RNAs by paired-ion reversed-phase HPLC. However, these methods apply only to small RNAs and the separation depends on the lengths of the molecules.

We have recently shown that macromolecular synthetic RNA homopolymers can be separated by reversed-phase chromatography regardless of their lengths[12]. In the present paper we show that the separation of natural RNAs can also be obtained independently of their size.

MATERIALS AND METHODS

Materials

All chemicals used were analytical grade except acetonitrile (Touzart and Ma-

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tignon) which was HPLC grade. tRNA from *Escherichia coli* was obtained from Boehringer.

Chromatography was performed with a Varian 5000, which gives a fairly good gradient, at 0.2 ml/min and a detector set at 254 nm; Octadecylsilane columns, 30 × 4 mm, (MCH 10, Varian) were used throughout this work.

RNA preparation

Poly(A)$^+$ RNA, 18S and 28S ribosomal RNAs were prepared from HeLa cells. Polyribosomes were prepared by centrifuging cytoplasm for 2 h at 200,000 *g* in a Beckman Ti 50.2 rotor. RNAs were extracted by the phenol–chloroform method, as described by Perry *et al.*[13]. The ribosomal RNAs were separated by centrifugation in a 15–30% sucrose gradient in buffer A [10 m*M* Tris–HCl pH 7.4, 1 m*M* ethylenediaminetetraacetic acid (EDTA), 100 m*M* sodium chloride, 0.5% sodium dodecyl sulphate (SDS)] in a SW 27 rotor at 127,000 *g* and 20°C for 18 h.

Poly(A)$^+$ RNA was prepared as described by Aviv and Leder[14]. Crude rabbit globin mRNA was prepared according Schreier and Staehelin[15]. Besides globin mRNA, this fraction (called "crude globin mRNA fraction") contains 18S RNA and fragments of 28S rRNA and globin mRNA.

SnRNAs were purified as previously described[16].

HPLC separation of RNAs

RNAs were dissolved in water, adjusted to pH 6.6 with 0.1 *M* ammonium acetate and applied to a MCH 10 column equilibrated with 0.1 *M* ammonium acetate buffer (pH = 6.6) (solution A). Elution was performed with an increasing linear gradient of solution B (50% acetonitrile in water), to 40% in 200 min. The flow-rate was 0.2 ml/min.

Other methods employed

mRNA translation *in vitro* and analysis of synthesized peptides by polyacrylamide gel electrophoresis were performed as previously described[17]. Electrophoresis of RNAs in agarose gel was carried out as described by Locker[18].

Radioactive Poly(U) was hybridized as described by Lee and Engelhardt[19].

TABLER I

COMPARISON BETWEEN THE RETENTION TIMES AND THE SIZES OF SOME NATURAL RNAS

RNAs were purified as described in Materials and Methods, then applied to a C_{18} column (MCH 10) and eluted as described.

	Retention time (min)	*Molecular weight (Dalton)*	*Base number*
28S	133	$1.5 \cdot 10^6$	4850
18S	127	$0.6 \cdot 10^6$	1750
tRNAs	80–110	$(2–3) \cdot 10^4$	75–85
glob-poly(A)$^+$ in mRNA	170	$2.2 \cdot 10^5$	589
snRNAs	110–120	$(3–6) \cdot 10^4$	106–196
Poly(U)	106	$5 \cdot 10^5$–$2 \cdot 10^6$	$(1.5–6) \cdot 10^3$
Poly(A)	173	$5 \cdot 10^5$–$2 \cdot 10^6$	$(1.5–6) \cdot 10^3$

RESULTS

Retention times and the size of the RNA macromolecules

Natural RNAs are retained on an octadecylsilane column equilibrated with 0.1 *M* ammonium acetate and can be eluted by increasing the amount of acetonitrile. Table I summarizes the retention times of different RNAs using a linear gradient (0–20%) of acetonitrile (see Materials and Methods). *E. coli* tRNA is eluted first, while globin mRNA is most strongly retained. 28S rRNA is eluted before globin messenger RNA, showing that size is not a critical parameter in the retention behaviour. Among the synthetic homopolymers, poly(U) is the least retained, while poly(A) is the most strongly retained. However, attempts to correlate the retention time with base composition were not successful. It seems that other parameters, such as secondary structure, have to taken into account[20].

Separation of E. coli tRNAs

When chromatographed on an octadecylsilane column, *E. coli* tRNAs were eluted as heterogeneous material (Fig. 1). There are two possibilities to explain this result. The heterogeneity is an artefact, or reflects a partial separation of the different RNAs. In order to test these possibilities we pooled different fractions and rechromatographed the corresponding RNA under the same conditions. The results, presented in Fig. 1, clearly show that the heterogeneity results from a true separation of the tRNA molecules.

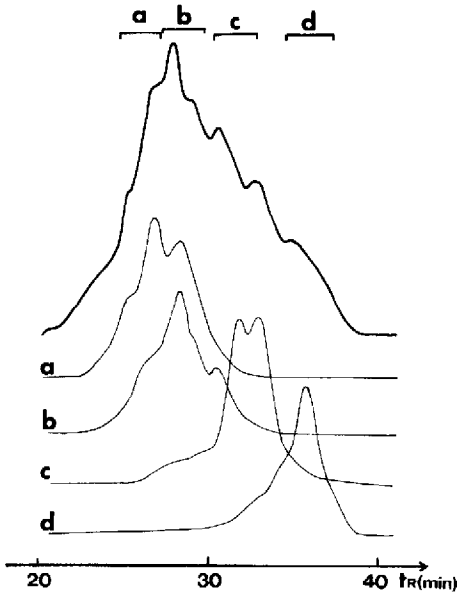

Fig. 1. Separation of *E. coli* tRNAs by reversed-phase chromatography. tRNAs were applied to the C_{18} column (MCH 10, Varian) equilibrated with 0.1 *M* ammonium acetate, pH 6.6, and eluted by increasing the acetonitrile–water (1:1) to 20% in 50 min at 1 ml/min (thick curve). Different fractions (a, b, c, d) were collected, precipitated overnight by two volumes of ethanol and chromatographed under the same conditions (thin curves).

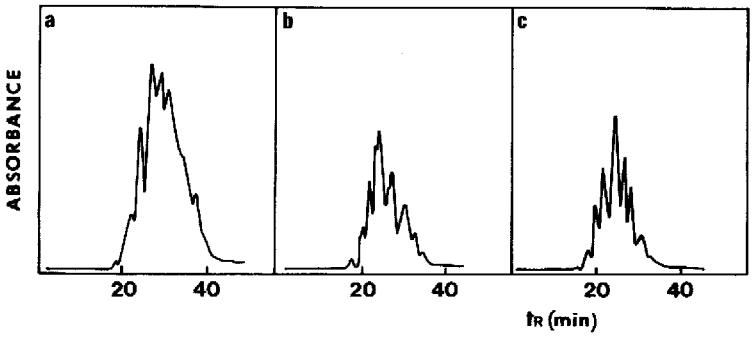

Fig. 2. Artefact appearing at high flow-rate. tRNAs were chromatographed as described in Fig. 1, but with the following flow-rates: a, 1 ml/min; b, 2 ml/min; c, 2.5 ml/min.

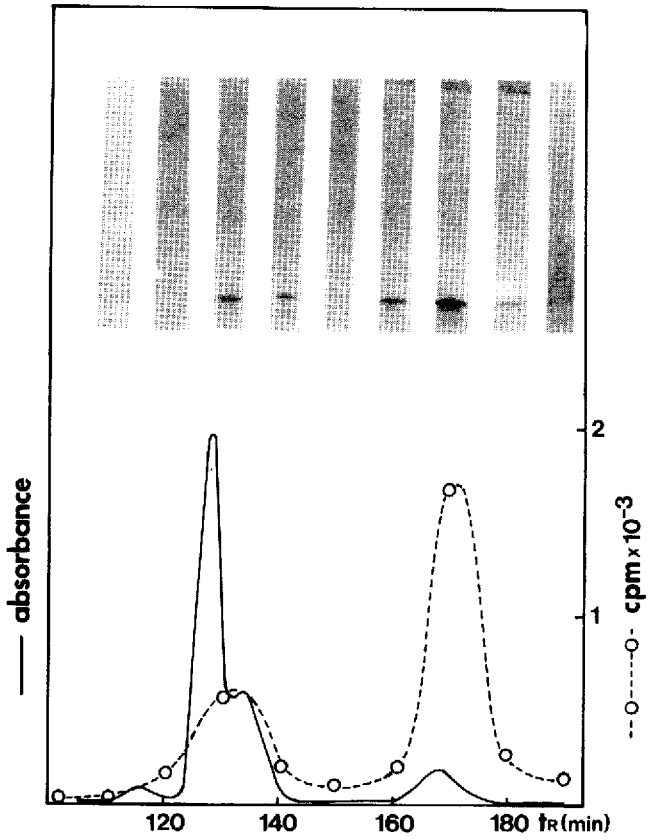

Fig. 3. Chromatography of crude globin mRNA. Crude globin mRNA fraction was prepared and chromatographed as described in Materials and Methods. Fractions were collected, RNA was precipitated and translated in a cell-free system. Aliquots were precipitated with trichloroacetic acid and counted for radioactivity[17]. ——, O.D. at 254 nm; O- - -O, activity of the *in vitro* system. The fractions were analysed by SDS gel electrophoresis. Autoradiograms of the gel are presented above each fraction.

It must be pointed out that such a separation is possible only when the flow-rate is less than 1 ml/min (Fig. 2A). Above this value a phenomenon, which we have previously described for synthetic homoribopolymers[12] and called "polypeak phenomenon", appears. Each different species of RNA behaved as a series of overlapping components, and atypical elution profiles were obtained (Fig. 2B and C).

Purification of globin mRNA

A crude fraction of globin mRNA was prepared by centrifugation of reticulocyte RNA. Besides 9S globin mRNA, this fraction contained a small amount of tRNA, 18S rRNA and degraded 28S rRNA (see Materials and Methods and ref. 14). When this fraction was chromatographed on C_{18} column, the optical density pattern at 254 nm revealed four peaks (Fig. 3). Agarose gel electrophoresis of the different fractions showed that the material eluted at 115 min was tRNA, at 127 min was mainly 18S RNA and at 167 min was 9S RNA (not shown). Material eluted at 133 min almost certainly represents pieces of 28S rRNA.

We have also tested the messenger activity by translation in cell-free protein-synthesizing system. Surprisingly, two peaks of activity appear (Fig. 3). They do not reflect a saturation problem of the cell-free system, because when twice as much RNA was used the radioactivity of synthesized peptides was doubled in all fractions (not shown). There are two possible explanations of this result. There exist either two different kinds of messenger RNA sedimenting around 9S or they represent two forms of globin mRNA. To test these hypotheses we analysed the proteins synthesized *in vitro*, using RNA separated by chromatography as a template. Fig. 3 clearly shows that only globin is synthesized. This shows that two different messengers were separated, both coding for globin.

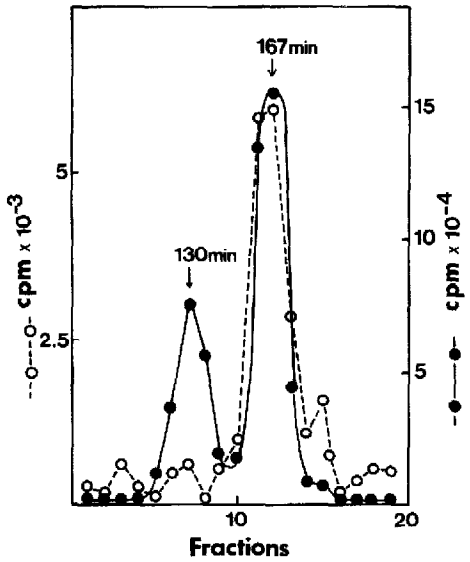

Fig. 4. Poly(A) content of the globin mRNAs, separated by reversed-phase chromatography. Crude globin mRNA was chromatographed and mRNA translated as in Fig. 3. For each fraction, RNA was translated in the *in vitro* system and hybridized to radioactive poly(U) (see Materials and Methods). ●——●, Activity in the cell-free system; O- - -O, poly(U) hybridized.

As certain fractions of reticulocyte RNA are known to have lost their poly(A) tail[21], we tested the poly(U) binding capacity of these two RNA fractions. Fig. 4 clearly shows that only the fractions eluted around 167 min contain poly(A). Thus, we have separated two kinds of globin mRNA; one is poly(A)$^+$ and the other is devoid of poly(A). In order to confirm this result we purified poly(A)$^+$ globin messenger RNA by chromatography on oligo dT cellulose[14]. This poly(A)$^+$ globin messenger RNA is eluted as a single peak around 170 min (result not shown).

Chromatography of total poly(A)$^+$ RNA from HeLa cells

Poly(A)$^+$ RNA was prepared as described in Materials and Methods. This fraction, when analysed by electrophoresis, exhibited some rRNA, mainly 18S rRNA.

The optical density pattern obtained after reversed-phase chromatography resembled the one obtained with globin mRNA (Fig. 5). The major peak (128 min) seen at 260 nm was identified as contaminant 18S rRNA. When analysed by translation in a cell-free system, a more heterogeneous pattern of activity appeared (Fig.

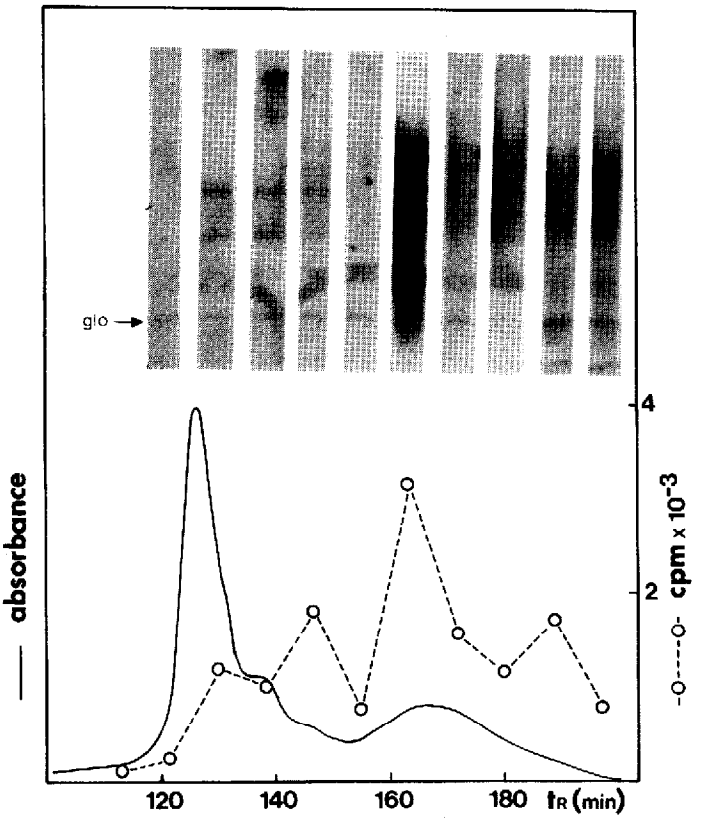

Fig. 5. Chromatography of HeLa poly(A)$^+$ RNA. HeLa poly(A)$^+$ RNA prepared as described in Materials and Methods was analysed by reversed-phase chromatography. Fractions were collected, precipitated and analysed by translation in a cell-free system. ——, O.D. at 260 nm; O- - -O, activity in the cell-free system. Synthesized peptides were analysed by SDS gel electrophoresis. Autoradiograms are presented above the corresponding fractions. glo → represents the endogenous activity of the cell-free system.

5). Most of the mRNA was found in a peak of activity eluted around 165 min. We analysed the protein synthesized *in vitro*. Fig. 5 shows that mRNAs were not separated according to their sizes because there is no relationship between the size of the peptide synthesized and the retention time of the corresponding mRNA.

DISCUSSION

The present work demonstrates that a separation of macromolecular RNAs, independently of their sizes, can be achieved by reversed-phase chromatography. HPLC allows more rapid separations than ultracentrifugation or electrophoresis.

Separation of oligo-RNAs by reversed-phase HPLC has been reported[9,10]. When the oligo-nucleotides are very small (less than 30 nucleotides), length is the main determinant of separation. We have previously developed a theory based on multiple point interaction to show that only macromolecules can be separated independently of their sizes[12].

Likewise, separation by paired-ion reversed-phase chromatography, reported by Nguyen *et al.*[11], is dependent only on the size of the RNA, because RNAs are polyphosphate ions.

Recently, Narihara *et al.*[22] described the fractionation of tRNA on siliconized porous glass, coated with trialkylmethylammonium chloride, but the separation was poor. This is probably because siliconized glass, coated with trialkylmethyl, is not so efficient as a commercially available octadecylsilane column. Pearson *et al.*[23] recently reported a good separation of tRNA on a weakly hydrophobic column with decreasing salt gradients. In our method an increasing amount of acetonitrile and decreasing salt concentration were used to elute RNAs. Pearson *et al.*[23] suggest that high concentrations of organic solvents should be avoided in chromatography. Organic solvents, such as ethanol, isopropanol or butanol, are currently used in the preparation of biogically active nucleic acids. In our experience, the mRNA was not damaged, as judged by its activity in a cell-free system.

When high flow-rate were used, purified RNAs did not behave as a single species. This is an artefact we have previously called "polypeak phenomenon"[12]. It is not due to either the detector or the gradient-forming device because it is never observed with small molecules (bases, naphthalene). It is a chracteristic of macromolecular RNAs that can be controlled by the flow-rate. When the flow-rate was lowered, the separation of two RNAs was always better. Thus, if an heterogeneous pattern is obtained, it can probably be improve by lowering the flow-rate. It must be pointed out that, using the program described in Materials and Methods, we have never observed the "polypeak phenomenon".

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