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# HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY OF OLIGORIBONUCLEOTIDES USING LINEAR AND HYPERBOLIC SALT GRADIENTS

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## SUMMARY

A method for separation and chain length determination of oligo- and polynucleotides by high-performance anion-exchange chromatography was developed, which allows resolution of individual fragments according to their chain length $n$, up to $n \approx 10$ by linear gradient of sodium chloride and up to $n \approx 30$ by an hyperbolic gradient of this salt. The hyperbolic relationship between $n$ and the salt concentration at which elution of the fragment occurs allows determination of the degree of polymerization of oligo- and polynucleotides with unknown $n$. The method proposed can be used also for estimation of the effective charge of nucleic acids with complex structure.

## INTRODUCTION

Fractionation of poly- and oligonucleotides is often used in studies of the synthesis, degradation and structure of nucleic acids, gene engineering experiments, etc.[1–9]. Modern fractionation methods based on high-performance liquid chromatography (HPLC) allow the reduction of separation times and achieve good resolution[7–10].These newly developed methods are mainly used for the fractionation of restriction fragments[2,4,7–10]. However, the separation of homopolynucleotides with different chain lengths may be useful for the determination of the lengths of primers and products of DNA-polymerases[5], and for chemically synthesized homopolynucleotides[11]. The present work describes the use of the high-performance ion-exchange chromatography on a Mono Q column (Pharmacia) for the rapid fractionation and chain length determination of oligo- and polyribonucleotides.

## EXPERIMENTAL

Separation was made with a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden) and a Mono Q HR 5/5 anion-exchange column (5 cm

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× 0.5 cm I.D.). Elution was performed with a gradient of NaCl in 25 m*M* Tris–HCl (pH 7.1 at 20°C), 1 m*M* $MgCl_2$. All chemicals were of analytical grade (Reakhim, U.S.S.R.), flow-rates were from 0.1 to 1.5 ml/min. Two kinds of gradient (total volume 30 ml) were used: linear (0 – 1 *M*) and hyperbolic $[NaCl] = 0.61 \cdot V_{el} / (6.1 + V_{el})$ where $V_{el}$ is the eluent volume. The absorbance of the eluate was monitored at 254 nm. The dependence between $n$ and $[NaCl]_{el}$ was linearized in double reciprocal coordinates.

Preparations of polyA and polyU were from Reanal (Budapest, Hungary): 4.6–5.6 S and 3.4–6.0 S respectively. Polynucleotides were hydrolyzed in 0.3 *M* KOH at 37°C. The polymerization degrees of polyA and polyU were determined by the end-phosphorus method as described by Seamen[12].

## RESULTS

Typical elution patterns of polyA- and polyU-hydrolyzates are presented in Fig. 1. Fragments up to $n = 10$ are completely resolved, but peaks of oligonucleotides with higher chain lengths are also clearly visible. Analysis of the relationship between the eluting salt concentration and $n$ shows that it can be linearized in double reciprocal coordinates ($1/[NaCl]_{el}$ *vs.* $1/n$) and therefore is hyperbolic (Fig. 2).

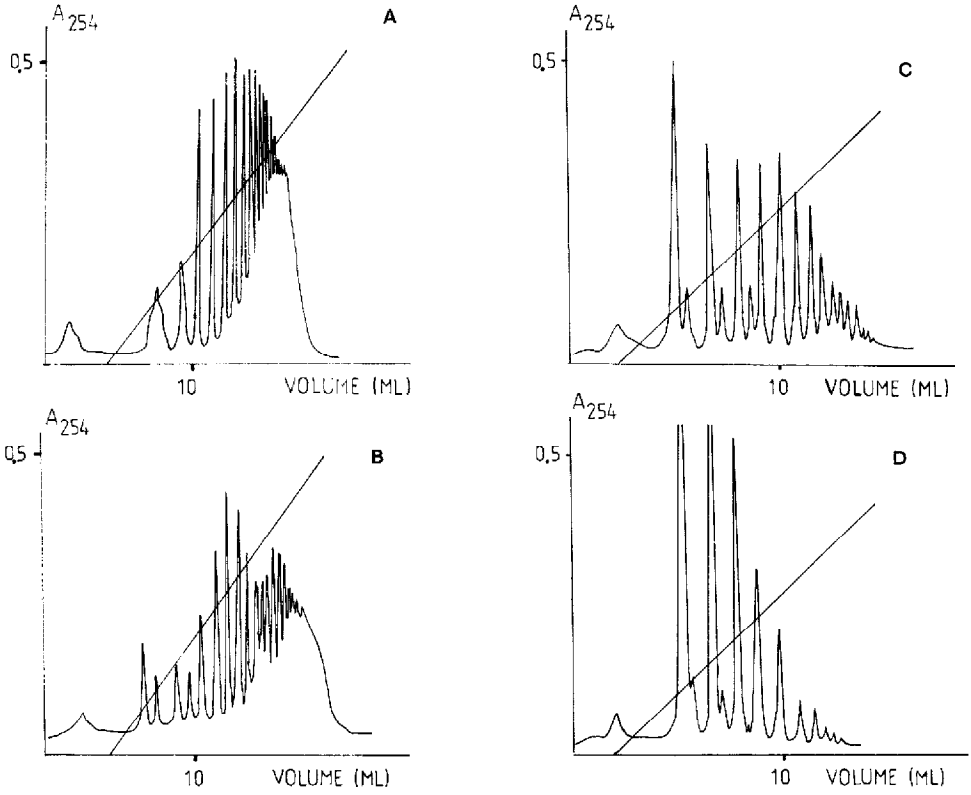


Fig. 1. Separation of hydrolytic fragments of: (A) polyA (hydrolysis time 45 min); (B) polyU (hydrolysis time 10 min); (C) polyA (hydrolysis time 3 h); (D) polyU (hydrolysis time 40 min). Flow-rate 0.1 ml/min.

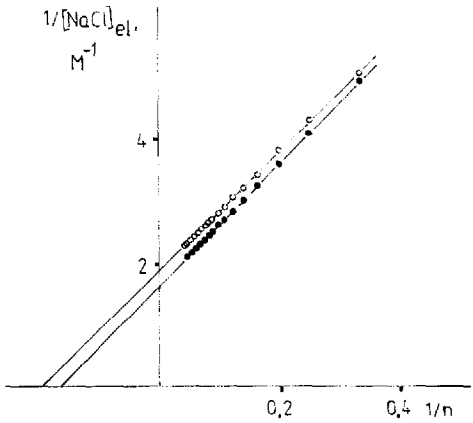


Fig. 2. Double reciprocal plots of the dependence between $n$ and $[NaCl]_{el}$ : $1/[NaCl]_{el} = a + b \cdot 1/n$. 45-min hydrolyzed polyA: $a = 1.89 \pm 0.01$; $b = 9.81 \pm 0.06$ (○). 10-min hydrolyzed polyU: $a = 1.64 \pm 0.01$; $b = 10.00 \pm 0.08$ (●).

We suggest that the relationship between $[NaCl]_{el}$ and $n$ may be presented in the analytical form $[NaCl]_{el} = [NaCl]_{lim} n/(n_{1/2} + n)$ where $[NaCl]_{lim}$ and $n_{1/2}$ are parameters of the hyperbola; $[NaCl]_{lim}$ is the salt concentration which elutes the polymer of infinite length, and $n$ is the polymerization degree of the oligonucleotide, eluted by $[NaCl] = [NaCl]_{lim}/2$. The dependences shown in Fig. 2 have $[NaCl]_{lim}$ and $n_{1/2}$ values of 0.61 $M$ and 6.1 for polyU and 0.53 $M$ and 5.2 for polyA.

Satellite peaks, which appear in the chromatograms of 10-min hydrolyzates of polyU are not taken into account, since the 40-min hydrolyzates showed no satellite peaks and they most probably correspond to the end-phosphate isomers[13].

The proposed equation allows one to determine (analytically or graphically) molecular masses of poly- and oligonucleotides by the use of $[NaCl]_{el}$ values. The polymerization degrees of polyA and polyU (Fig. 3) determined by this method are $4.7 \cdot 10^2$ and $3.5 \cdot 10^2$ respectively. Corresponding values obtained by the end-phosphorus method are $4.9 \cdot 10^2$ and $3.8 \cdot 10^2$. The sedimentation coefficients correspond to degrees of polymerization of about $(4–5) \cdot 10^2$.

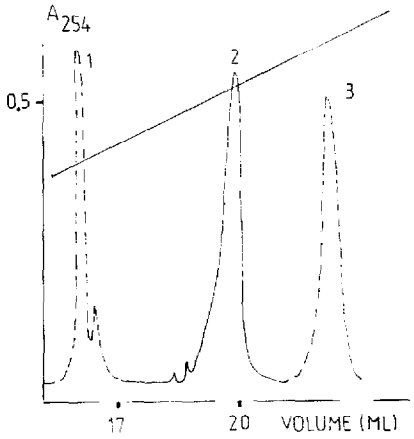


Fig. 3. Elution patterns of tRNA (1), polyA (2) and polyU (3). Flow-rate 0.1 ml/min.

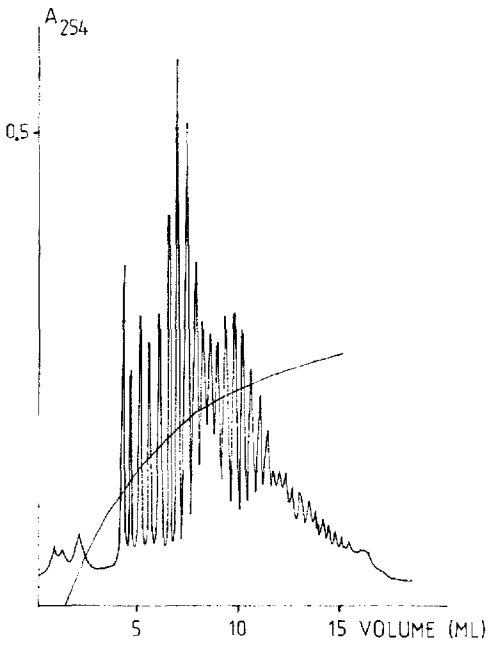


Fig. 4. Elution pattern of hydrolyzed polyU in the hyperbolic salt gradient. Flow-rate 0.25 ml/min.

The hyperbolic dependence between $[NaCl]_{el}$ and $n$ also allows one to improve the resolution of "long" oligonucleotides. For this purpose a gradient with an hyperbolic dependence between [NaCl] and volume was designed. The chromatographic pattern of a polyU hydrolyzate with this gradient is presented in Fig. 4. Practically complete resolution of the oligonucleotides up to $n \approx 30$ is achieved. However, in contrast to the expected dependence, the elution volumes are not proportional to $n$: short fragments are eluted later, whereas long ones appear earlier than was expected.

## DISCUSSION

The analytical expression of the dependence between $[NaCl]_{el}$ and $n$ allows one to determine chain lengths of oligo- and polynucleotides even beyond the mass-range of " hydrolytic standards". This method may be useful for experiments on the enzyme synthesis and degradation of nucleic acids, because it allows estimation of the concentration of each individual fragment in the mixture. High sensitivity and short time of analysis (for the mixture of $n \approx 10$, 2.5 h at flow-rate 0.1 ml/min and 15 min at 1 ml/min) make this procedure useful for kinetic experiments with the enzymes of nucleic acid metabolism.

This method may be applied not only to homopolynucleotides but also to nucleic acids of mixed composition and complex structure when their charges are in part inaccessible for the matrix of opposite charge (protein, ion exchanger). To verify this assumption the chromatography of total tRNA was developed in the system described (see Fig. 3). This estimation of the effective charge (*ca.* 20) is reasonable

taking into account the ternary structure of tRNA and number of bound magnesium ions[14].

The results shown in Figs. 2 and 3 demonstrate the main limitations of the proposed method. First, the hyperbolic relationship doesn't allow the determination of the chain length of polynucleotides with $n \gg 500$ with acceptable accuracy. Secondly, the difference in parameters for the $n$ *vs.* $[NaCl]_{el}$ dependence for polyA and polyU shows that these polynucleotides have different affinities for the ion exchanger (see also refs. 1, 13 and 15). In our experiments this difference leads to earlier elution of longer polyA then polyU molecules. This chromatographic behaviour may result from differences in the secondary structures and affinities for magnesium ions of these molecules. Addition to the eluent of compounds which destroy the secondary structure of nucleic acids may result in the "unification" of $n$ *vs.*$[NaCl]_{el}$ dependences.

The hyperbolic gradient designed for the given homooligoribonucleotides on the basis of $[NaCl]_{el}$ *vs.* $n$ dependence allows optimum resolution of both short and long fragments.

Long oligonucleotides ($n \gg n_{1/2}$) as shown above have lower $[NaCl]_{el}$ in the hyperbolic gradient than in a linear one. The probable cause of this effect is prolonged washing of the column by the buffer of "sub-eluting" ionic strength, insufficient for complete desorption of long fragments but quite close to it. Therefore, a practically isocratic elution which takes place under such conditions will distort the expected pattern.

Chromatography on Mono Q is also convenient for the semipreparative separation of oligonucleotide mixtures, because the capacity of this ion exchanger allows loads to 10 mg of mixture on the 1-ml column and the separation time in a linear gradient at a flow-rate of 1 ml/min is within 40 min. The flow-rate used in most of our experiments (0.1 ml/min) was chosen to ensure complete equilibration between phases, but the effect of an increase in flow-rate up to 1.0 ml/min on the resolution of fragments with $n \ll 20$ was negligible (data not shown). "Long" fragments can be separated in the hyperbolic gradient at flow-rates less than 0.5 ml/min. The use of Mono Q HR 16/10 column (gel volume 20 ml) allows scale-up of this separation (up to 200 mg).

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