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Isotachophoretic Analysis of Sequence-Isomeric Nucleotides in the Presence of Sequence-Selective Intercalator

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A new chromatographic technique for separating oligonucleotide sequence isomers is described. Nucleotide isomers are chromatographed in the presence of organic reagent which binds to polynucleotides or DNA sequence-selectively. The separation between CpG and GpC was achieved in an isotachophoresis mode by using quinacrine as "intercalator" under neutral pH conditions.

The importance of separation and analysis of oligonucleotides is increasing rapidly in gene technology and related fields. Electrophoresis and high-performance liquid chromatography (HPLC) in their "ordinary" modes have heretofore widely been used to separate the mixture of polynucleotides or fragments from nucleic acid. The mechanism of their separation is mainly characterized by the difference in chain-length (degree of polymerization, molecular weight) of polynucleotides or by the difference in the kind of nucleoside bases contained.

On the other hand, an important feature of polynucleotides is that it can carry a genetic information. In this sense, it is important to attain an efficient separation between so-called sequence isomers of polynucleotide, in which the same kinds of nucleoside bases are contained in the same number but in a different ordering. Several chromatographic separations of oligonucleotide sequence isomers (di-to-hexanucleotides) have been reported by using ion exchange,[1] reversed-phase partition[2] and ion-pair partition modes.[3] However, the separation conditions are rather empirical, not giving general directions to the aimed separation nor allowing appropriate predictions as regard to the ordering of elution for particular isomeric mixture. Obviously, different approach is required for tne chromatographic separation of sequence isomers.

In the present communication, we propose a use of so-called selective DNA-binding (or DNA-recognizing) molecules in improving the separation of oligonucleotide sequence isomers. We would like to show the usefulness of this technique in the isotachophoretic separation of ribodinucleoside monophosphates isomers.

The separation by isotachophoresis (IP) is based on the difference in the effective ionic mobility of the analyte ions, and the separation is not possible when the analyte ions have the same mobility. However, the separation becomes possible if one uses ligands which selectively bind to some of the ions bringing about a change in mobility.[4] Quinacrine is known to interact with GC-rich DNA

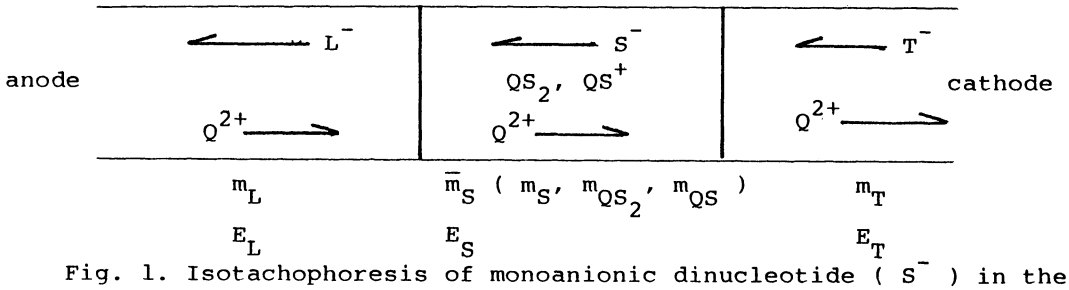
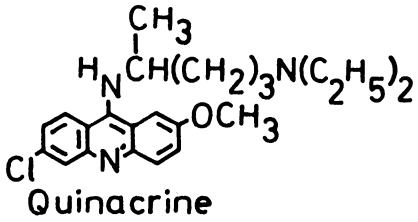


Fig. 1. Isotachophoresis of monoanionic dinucleotide (S^-) in the presence of intercalating ligand, dicationic quinacrine (Q^{2+}).

$$PU = (E_S/E_L - 1)/(E_T/E_L - 1) = (m_L/\bar{m}_S - 1)/(m_L/m_T - 1) \quad (1)$$



preferentially.[5] Theoretical calculation also predict that intercalating molecules prefer the interaction with pyrimidine-(3'-5')-purine sequence.[6] Therefore, it seems pertinent, in modeling and verifying the proposed sequence specific chromatography, to employ quinacrine as a CG-sequence selective ligand.

In our isotachophoresis, an aqueous methanol (1:1, v/v) containing 5 mM (1 M = 1 mol dm^{-3}) hydrochloric acid, 7.5 mM imidazole and a specified concentration of quinacrine was used as a leading solution. A terminating solution was 10 mM N-hydroxyethylpiperazine-N'-2-hydroxypropane-3-sulfonic acid (HEPPSO, Good's buffer). A Shimadzu IP-2A isotachophoretic analyzer fitted with a pre-analyzing column (1 mm i.d. x 40 mm), an analyzing column (0.5 mm x 150 mm), and an electric potential gradient detector was operated at 125 - 200μA (for 4-10 min) and then at 50-100μA (for IP data collection). Ribodinucleoside monophosphates were obtained from Sigma Chemical Co. and used as received. All the measurements were made at 25°C.

Since ribodinucleoside monophosphates are present predominantly in monoanionic forms and quinacrine in dicationic form under our conditions (pH being near neutrality), the present isotachophoresis system can be schematically illustrated by Fig. 1. The letter symbols L, S, and T indicate leading, sample and terminating zones respectively or respective ionic species. Q^{2+} stands for dicationic quinacrine, other cationic species being not shown for simplification. The symbols m and E are ionic mobility and electric potential gradient, respectively. $\bar{m}$ represents averaged or effective ionic mobility under particular isotachophoresis conditions. The isotachophoretic data were analyzed according to the PU value defined in Eq. 1.

Table 1 summarizes the effect of quinacrine on the PU values of three ribodinucleoside monophosphates. It is seen that quinacrine causes a general increase in PU value (decrease in effective mobility of the dinucleotides), the effect being much pronounced with CpG when compared with GpC and UpA. A different behavior of the two sequence isomers CpG and GpC toward quinacrine is of our concern. That the two isomers are clearly separated from each other is

Table 1. The effect of quinacrine on PU value of ribodinucleoside monophosphates (XpY)[a)]

Quinacrine	XpY	PU	ΔPU
0 mM	CpG	0.35	0
	GpC	0.35	0
	UpA	0.35	0
5 mM	CpG	0.45	0
	GpC	0.41	0.04
	UpA	0.39	0.06
10 mM	CpG	0.54	0
	GpC	0.48	0.06
	UpA	0.46	0.08

a) L, 5 mM HCl, 7.5 mM imidazole, 50% MeOH-H_2O.
T, 10 mM HEPPSO ; $\Delta PU = PU_{CpG} - PU_{XpY}$.

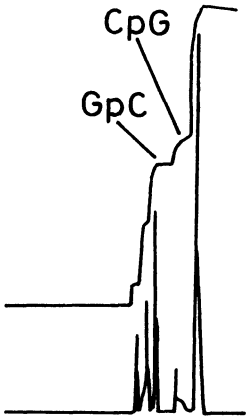


Fig. 2. Separation of CpG and GpC in the presence of 10 mM quinacrine.

demonstrated in the isotachopherogram shown in Fig. 2. In the absence of quinacrine, they had exactly the same mobility showing no indication of mutual separation.

The addition of aliphatic ammonium ions, triethylammonium and tetrabutylammonium ions, to leading solution was studied in order to evaluate the effect of cationic pairing ions on the mobility of the monoanionic ribodinucleoside monophosphates. As Table 2 shows, the addition of typical aliphatic ammonium ions caused mobility depressions which were similar in magnitude to those induced by the addition of quinacrine dihydrochloride. However, an important fact is that the aliphatic ammonium ions made no discriminations among isomeric dinucleotides nor among dinucleotides with different nucleoside-base composition. This indicates that a simple electrostatic association between the analyte ion and the added cationic ligand (ammonium ion) can not recognize the difference in nucleoside bases. This further indicates that the differentiation between sequence isomers CpG and GpC disclosed in Table 1 was caused by other type of interaction. The interaction is most probably that known as pseudo-intercalation,[7)] where the planar quinacrine molecule is placed into stack between the base-pairs formed from complementary dinucleotides (Fig. 3).

In general, the separation of nucleotides by isotachophoresis is accomplished by taking advantage of the difference in basicity of the nucleoside-bases involved, as studied by Kiso and co-workers.[8)] In fact, we successfully separated several two-component dinucleotide mixtures (e.g., GpC - GpA and CpG - UpA) by carrying out isotachophoresis at pH 4.0 rather than at pH 7.0. However, a pH-variation technique can not be expected to help separation of sequence isomers, since the same bases are contained in the same number in the analyte molecules.

A great volume of informations are continuedly being collected on the interactions of a certain family of low-molecular-weight compunds, either synthetic

Table 2. The effect of aliphatic ammonium ions on PU value of ribodinucleoside monophosphates

TEA[a]	XpY	PU	ΔPU	TBA[b]	XpY	PU	ΔPU
5 mM	CpG	0.48	0	10 mM	CpG	0.60	0
	GpC	0.47	0.01		GpC	0.61	-0.01
	UpA	0.47	0.01		UpA	0.61	-0.01
10 mM	CpG	0.58	0				
	GpC	0.58	0				
	UpA	0.57	0				

a) TEA; triethylamine hydrochloride.

b) TBA; tetrabutylammonium chloride.

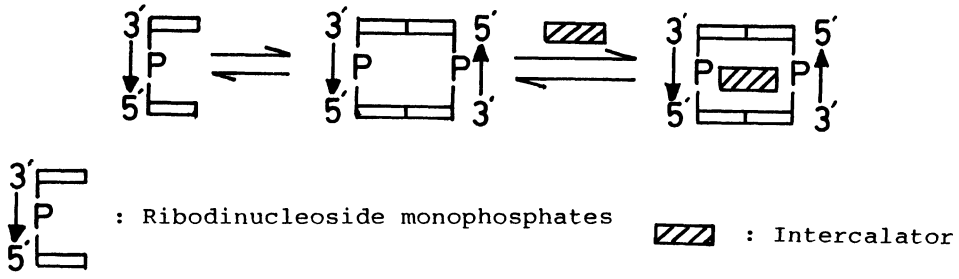


Fig. 3. Pseudo-intercalation mechanism.

or natural, with DNA or polynucleotides. All these informations are expected to help develop chromatographic systems to separate oligonucleotide sequence isomers or improve separation among non-isomeric oligonucleotides. At the same time, these chromatographic techniques should in turn be useful in studying the nature of interaction between such small molecules and oligonucleotides. Incidentally, there is no particular problem in the choice of modes of chromatography e. g., electrophoresis and HPLC. In fact, our preliminary study indicated that HPLC-column loaded with a cationic acridine derivative successfully resolved the mixture of sequence isomers CpG and GpC.

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