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Note

Preparative fractionation of oligoriboguanylates by anion-exchange chromatography on TSK DEAE-5PW

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Hitherto, one of the major impediments to the large scale synthesis of oligonucleotides has been posed by the plethora of difficulties ascribable to guanosine. Last but not least of these is the inevitable self-aggregation of its nucleotides in aqueous solution. Thus, purification of guanosine-rich oligomers is generally carried out in the presence of chaotropic reagents such as urea and formamide. This classic approach, however, creates serious problems of its own. By the very virtue of their strong hydrogen-bonding ability, these reagents not only succeed in breaking up the aggregate's structures, they also show considerable affinity towards the bases themselves. Consequently, they will remain as the final contaminants. Their separation in turn poses quite a dilemma, since a complete removal, in principle, requires the guanylate oligomers to be in the non-aggregated form. Furthermore, as the chain length increases so does the aggregate's stability. In our experience, removal of urea at room temperature by DEAE-Sephadex ion-exchange chromatography is not complete, as witnessed by IR spectroscopy[1]. Recently Schott *et al.*[2] seem to have had more success with this method, albeit at an high cost in product yield[2].

An elegant method introduced by Wells *et al.*[3] successfully bypasses these problems. An alkaline buffer environment of pH 12 unstacks the nucleotides. Separation by an ion-exchange/reversed-phase mechanism then takes place on a polychlorofluoroethylene type (RPC5, etc.) matrix functionalized with a quaternary amine (Adogen 464). The gels available for this purpose, however, show various rather inconvenient drawbacks[4–6].

While attempting to reproduce the excellent chromatographic resolution in the guanylate homooligomer series as described by Lohrmann and Orgel[7], we tested a commercial material TSK DEAE-5PW whose performance easily outdoes that of the RPC types. The material, an hydrophillic resin manufactured by Toyo Soda of Japan. has been employed as a medium for the chromatography of proteins[8] at elevated pH.

EXPERIMENTAL

Materials

Poly-G and poly-C were obtained from Sigma (Munich, F.R.G.), dT_8 and the dT_{4-22} "ladder" from Gibco-BRL (Karlsruhe, F.R.G.), all other nucleotides and

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T4-kinase from Boehringer (Mannheim, F.R.G.). [γ^{35}S]ATP for nucleotide labelling was obtained from Amersham (Braunschweig, F.R.G.). High-performance liquid chromatography (HPLC)-grade water was supplied by a Milli-Q purifier from Waters-Millipore (Eschborn, F.R.G.). All other reagents were of analytical purity from Merck (Darmstadt, F.R.G.). A 2 *M* triethylammonium bicarbonate (TEAB) stock solution was prepared by passing carbon dioxide through a 2 *M* mixture of triethylamine and water until the pH had dropped to a value of 8. Except for TEAB, all eluent solutions were passed through 0.2-μm membrane filters from Sartorius (Göttingen, F.R.G.) under reduced pressure. Sephadex G-10 was obtained from Pharmacia (Freiburg, F.R.G.), Fractogel TSK DEAE-65(S) (25–50 μm particles) from Merck. The columns for liquid chromatography consisted of glass tubes equipped with flanged piston-type adapters. The TSK DEAE-5PW 75 mm × 7 mm HPLC column was obtained from Labotron (Zurich, Switzerland).

Medium pressure liquid chromatography (MPLC) column

This column was built by the workshop of our institutes. Heraeus Quarzschmelze (Hanau, F.R.G.) supplied the glass. Reichelt Chemie Technik (Heidelberg, F.R.G.) the small parts. A 500 mm × 7 mm I.D. × 15 mm O.D. quartz-glass tube served as the column material. The glass tube was encased in a semi-open metal cartridge with threading at both ends and sealed with PTFE rings. This construction easily withstood pressures of 50 bar. The column was capped at the ends by fritted delrin adapters affording connection with standard 1/16-in. PTFE tubing. For packing the column, the upper adapter and seal were removed, a PTFE ring was placed on the glass surface and a metal funnel fastened to the upper end. A thick slurry of the gel, in our case Fractogel, in isopropanol was introduced at the top. The funnel was sealed by a fritless delrin adapter followed by the pumping through of 100 ml isopropanol (flow-rate, 3.5 ml/min; maximum pressure, 50 bar), 200 ml of methanol and finally a methanol–water gradient reaching 100% water within 1 h.

Oligomer preparation

Hydrolysis. A 25-mg charge of poly-G (poly-C) was dissolved to near completion in 4.5 ml (3.5 ml) water, 0.5 ml (1.5 ml) 1 *M* potassium hydroxide were added and kept at 37°C for 20 min to 2 h according to the desired chain-length distribution.

TABLE I

GRADIENT FOR OLIGORIBOGUANYLATE FRACTIONATION VIA MPLC

Time (min)	*%A*
0	100
10	100
20	75
32	55
80	35
80	20
80	10
80	0

TABLE II

GRADIENT FOR OLIGOCYTIDYLATE FRACTIONATION VIA MPLC

Time (min)	*%C*
0	100
10	100
40	80
60	60
60	40
60	20
60	0

Treating the hydrolysate with *ca.* 60 μl 7 *M* perchloric acid to a pH of 1.5–2 and incubating at 37°C for another 25 min with subsequent neutralization removed cyclic phosphates[9], leaving a mixture of the 2'- and 3'-isomers. The reaction mixture was passed through an 0.2-μm membrane filter and diluted to 100 ml prior to applying it to the MPLC column.

Fractionation. Chromatography of oligoguanylates employed the following eluents: A, 2 *M* Tris(hydroxymethyl)methylamine hydrogen perchlorate, 15 m*M* potassium hydroxide, pH 11.5; B, A + 1.5 *M* potassium chloride, 5 m*M* potassium hydroxide, pH 11.5. Fractions were immediately neutralized with perchloric acid. Table I shows a typical gradient for a preparative-scale (25 mg) fractionation of $(Gp)_n$ on the MPLC column at 3 ml/min.

Chromatography of Oligo-C took place at neutral pH and at lower ionic strengths. A gradient is shown in Table II. Eluents: C, 10 m*M* Tris · HCl pH 7.5; D, A + 0.5 *M* potassium chloride.

Removal of homologue contaminants. Fractions containing a specific oligomer were pooled, diluted five-fold, applied to the 75 mm × 7 mm DEAE-5PW column and chromatographed with a gradient of 100% B over 3 h at a flow-rate of 1 ml/min. Gradients were optimized individually, $(Gp)_6$, for example, was eluted with 0.5–1 *M* salt (33–67% B) at pH 11.5.

Preliminary desalting. The eluate from the preceding step was diluted five-fold and applied to a 35 mm × 30 mm LC column of Fractogel then washed with 100 ml water. Removal of salt was accomplished for chain lengths of $\geqslant 4$ by washing with 150 ml 0.1 *M* TEAB (trimers: 0.02 *M* TEAB). Nucleotides $(Gp)_6$ and $(Cp)_8$ were eluted with 0.5 *M* TEAB. For intermediate chain lengths, 1 *M* TEAB and from the dodecamer upward, 2 *M* TEAB was used. Dimers were desalted by a linear gradient of 0–0.2 *M* TEAB in 200 ml. TEAB was removed by drying the eluate with a rotary evaporator, redissolving in water and redrying three times.

Final desalting. The once-desalted nucleotide (*ca.* 20 O.D. units at E_{max}) was dissolved in 4 × 0.2 ml water, applied to a 500 mm × 30 mm LC column of Sephadex G-10 and eluted with 0.05 *M* TEAB at a rate of 100 ml/h. Pretreatment of the gel was according to standard procedures as described by the manufacturer, plus an additional rinsing of the packed column with 5 l of eluent. In operation, rinses of 50 ml sufficed between fractions, extensive washing taking place overnight at 25 ml/h. TEAB was removed by rotary evaporation.

Identification

The first peak of the chromatogram was shown to be identical with GMP or CMP by its elution behaviour on Fractogel. The second peak of the cytidylate fractionation was first treated with alkaline phophatase, then subjected to hydrolysis by RNAse, A, resulting in a 1:1 mixture of cytidine and 3'-CMP as determined by comparing peak areas from chromatography on Fractogel. Alkaline phosphatase was removed before the RNAse treatment by gel filtration on Sephadex G-10. The octamers were identified through comparison with dT_8 and an oligo-dT "ladder" in polyacrylamide gel electrophoresis (PAGE) (0.3-mm gel) in the presence of 8 *M* urea after labelling at the 5'-end with ^{35}S by T4-kinase according to Maniatis *et al.*[10].

Apparatus

For HPLC/MPLC we employed a 300C HPLC pump and binary gradient former 250B from Gynkotek (Munich, F.R.G.). The UV detector was a Model 78, from Knauer (Bad Homburg, F.R.G.). Conductance was monitored by a type 410 meter equipped with a 50-μl cell from WTW (Weilheim, F.R.G.). For LC, pump Types 382 and 310 by ISCO/Colora (Lorch, Württemburg, F.R.G.) were used with Uvicord II detectors (LKB, Munich, F.R.G) set at 254 nm.

RESULTS

Our strategy for obtaining pure oligonucleotides was as follows. Alkaline hydrolysis of the polynucleotides resulted in a mixture of 2'-, 3'- and cyclic 2'-3'-phosphates. Treatment with acid removed the cyclic isomers. Application to a MPLC column filled with the 25–50 μm grade Fractogel and rectification with a gradient of potassium chloride at pH 11.5 for guanylates or pH 7.5 for cytidylates afforded the prepurified fractions. Re-chromatogrtaphy by HPLC with the 10-μm material removed the homologues still remaining after the first step. Desalting proceeded in two

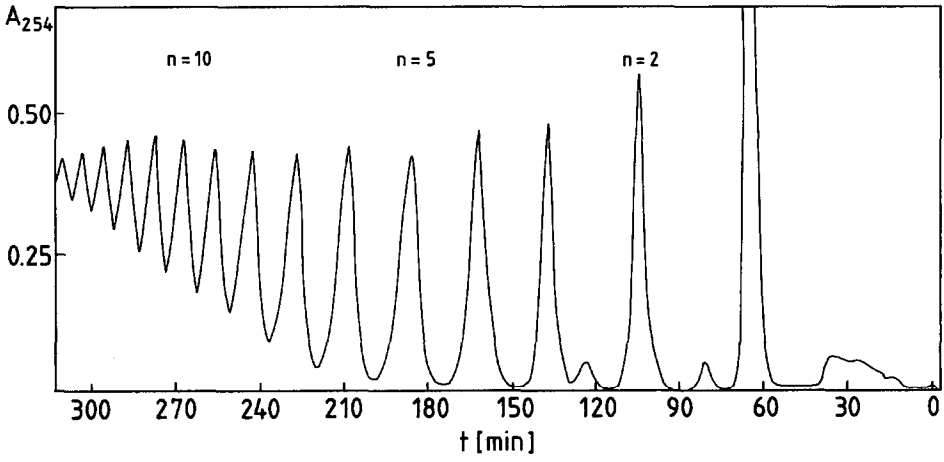

Fig. 1. MPLC fractionation of 25 mg $(Cp)_n$ on Fractogel TSK DEAE. Column: 500 mm × 7 mm. Flow-rate: 3 ml/min. Gradient: see Table II.

steps as well. The bulk of the salt was separated by ion exchange on a 35 mm × 30 mm LC column. The nucleotide was adsorbed on the gel, then washed and eluted by a step gradient of TEAB. Fractions once desalted still showed IR absorption bands due to residual bicarbonate. After a second gel-filtration step on Sephadex G-10, the product appeared salt free.

We obtained satisfactory results on the preprative scale using the inexpensive 25–50 μm grade Fractogel. A 500 mm × 7 mm (20 ml) bed-volume column permitted the rectification of over 25 mg hydrolysed poly-G or Poly-C. Baseline separation was achieved up to the pentamer (Fig. 1) in our MPLC separations. What is more, in the case of guanosine (Fig. 2), pH 11.5 is sufficiently alkaline for the elution buffer. The rate of hydrolysis is thus reduced compared to that at pH 12. Oligo-C was resolvable at neutral pH, as expected (Fig. 1).

The contamination of oligomer fractions by their homologues was removed via the 10-μm grade gel. A 75 mm × 7 mm (3.3 ml) HPLC column allowed the fractionation of 4 mg hydrolysate or the purification of 1 mg of a given oligonucleotide (Fig. 2). The finer particle size, of course, ensured better results, the resolution approaching that in the strictly analytical work by Lohrmann and Orgel[7]. Fig. 2 reveals that the fractions obtained by MPLC are indeed mixtures of the 2′ 3′phates and that the acid treatment has cleaved the cyclic phosphodiesters. The irregularities following the peaks at $n = 12$ in Fig. 2 are in all likelihood due to steps in the gradient. The changes in peak shape with differing gradients provide envidence in support of this explanation (not shown).

The oligoguanylates susceptibility to alkaline cleavage becomes more serious as the chain length increases. Fortunately, hydrolysis does not occur to an appreciable extent as long as the nucleotides are adsorbed on the gel. This point is illustrated by the separations shown in Fig. 3. $(GP)_{13}$subjected to two-fold re-chromatography on

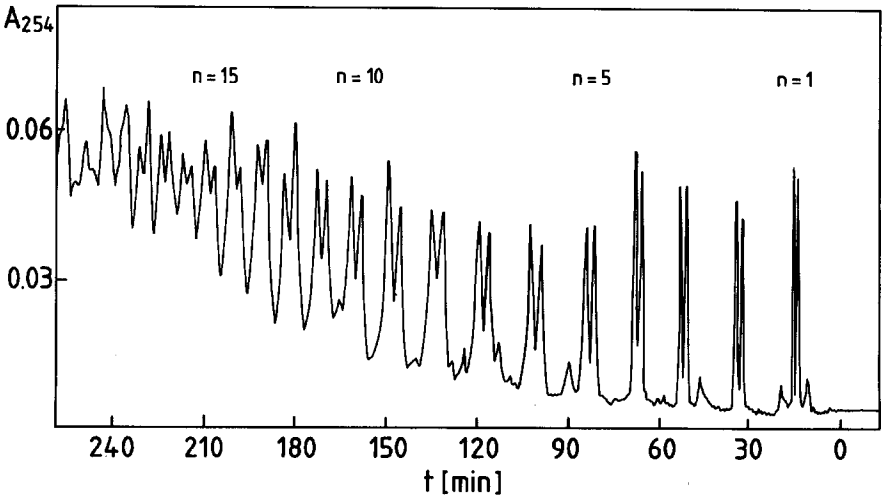

Fig. 2. HPLC fractionation of 0.5 mg $(Gp)_n$ on TSK DEAE-5PW (10 μm). Column: 75 mm × 7 mm. Flow-rate: 1 ml/min. Gradient: 100% A, 10 min; 75% A, 40 min; 25% A, 100 min; 0% A, 75 min. Fractions are 1:1 mixtures of 2′ and 3′-phosphate isomers, hence the double peaks in the chromatogram.

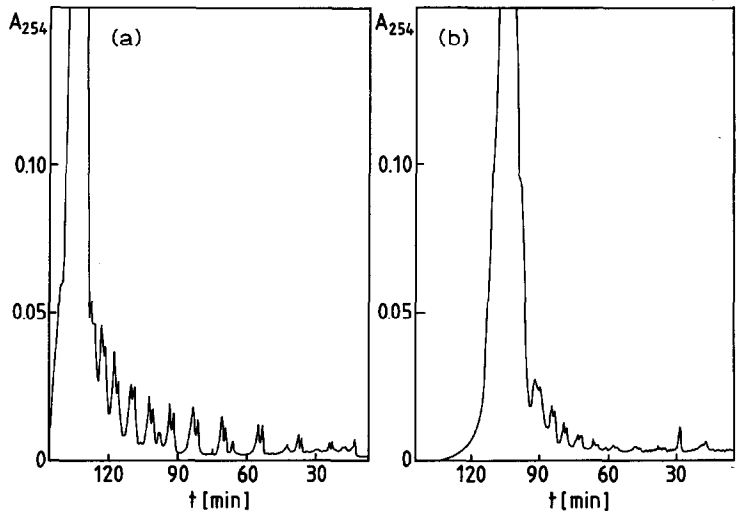

Fig. 3. First (a) and second (b) refractionation of *ca.* 1 mg $(Gp)_{13}$ by MPLC (see text). Column: 500 mm × 7 mm Fractogel TSK DEAE. Flow-rate: 3 ml/min. Gradient: 0–100% B in 180 min.

the 500 mm × 7 mm MPLC column (the HPLC column not being available at that date). The fraction size of the original rectification was 10 ml, the flow-rate 3 ml/min and the average time elapsed between collection and neutralization was 20 s. This amounted to a total time of 3.7 min from the elution of the nucleotide from the column up to the neutralization. Fig. 3a shows the resulting chain-length distribution as recorded during the first re-chromatography. In this case the fraction size was 5 ml, which lessened the corresponding time span to 2 min. The extent of hydrolysis was thus greatly reduced as is demonstrated by the chromatogram in Fig. 3b. Further reductions of the neutralization time should lead to additional improvements in product quality.

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