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GEL FILTRATION CHROMATOGRAPHY OF POLYDEOXYNUCLEOTIDES USING AGAROSE COLUMNS

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SUMMARY

Gel filtration characteristics of polydeoxynucleotides passing through agarose columns have been determined using different temperatures, eluents and pore sizes. These conditions affected the degree of separation and range of usefulness. In the practical case of purification after synthesis, there were found ways to minimize unwanted hydrogen-bonding cochromatography. The polydeoxynucleotides were inferred to chromatograph as elongated structures because of their great elution dissimilarity to globular proteins of the same molecular weight and marked similarity to double-stranded polydeoxynucleotides.

INTRODUCTION

Gel filtration chromatography has been effectively used for purification of polydeoxynucleotides resulting from chemical and enzymatic synthesis. Sephadex G-50 (dextran) was employed by NARANG *et al.*[1] to give a rapid first stage for separation of products resulting from chemical polymerization of deoxynucleotide trimers. HOHN AND SCHALLER[2] made a study of such columns for separation and chain-length determination of chemically synthesized oligodeoxynucleotides. Bio-Gels P-60 (ref. 3) and P-100 (ref. 4) (polyacrylamide) were utilized for fractionation and size estimation of enzymatically synthesized polydeoxynucleotides. More recently, such polymers have been chromatographed on Bio-Gels A-0.5m and A-5m (agarose) for which HAYES *et al.*[5] obtained elution volume calibration curves of single-stranded polymers ranging from 11–866 nucleotide units in length. This report deals with these agarose columns and the effects of eluent molarity both on product separation according to size and on the extent of cochromatography of thymidylate and deoxyadenylate polymers. The effects of temperature and use of 8 *M* urea[6] were also studied. It was of interest also to relate the chromatographic characteristics of globular proteins with polydeoxynucleotides and to draw inferences from this as to the molecular configurations of the latter.

MATERIALS AND METHODS

The oligodeoxynucleotides $[^3H](dT)_3$, $[^3H](dT)_{10}$, $[^3H](dT)_{14}$ and $[^3H](dT)_{15}$ were synthesized according to KHORANA AND VIZSOLYI[7]. The procedure of HAYES *et al.*[3] was used to prepare the polydeoxynucleotides $d(T)_6(A)_{\overline{50}}$, $d(T)_3(A)_{\overline{140}}$, $(dA)_{\overline{310}}$, $(dC)_{\overline{101}}$ and $d(T)_6(C^{Ac})_{\overline{49}}$. The double-stranded polydeoxynucleotide $d(T)_6(A)_{\overline{50}} \cdot d(T)_{\overline{50}}(A)_6$ was synthesized using $d(T)_6(A)_{\overline{50}}$ as template with $(dT)_6$, dTTP, dATP and calf thymus DNA polymerase[8]. $[^{14}C]$-Urea was prepared by MURRAY AND RONZIO[9]. Human γ-globulin was obtained from Merck, Sharp & Dohme, α-chymotrypsinogen from Mann Research Laboratories, horse liver alcohol dehydrogenase from Worthington Biochemical Corporation, and bovine serum albumin from Calbiochem.

Absorbance readings were taken using a Unicam SP-800 ultraviolet spectrophotometer. Radioactivity determinations were done with a Beckman CPM-100 liquid scintillation counter using glass vials containing 0.5 ml of aqueous sample dissolved in 10 ml of 6.8 g/l of 2,5-diphenyloxazole and 0.21 g/l of 2,2′-*p*-phenylene-bis(5-phenyloxazole) in toluene that was mixed with 13 % by volume of Colosolve Solubilizer CS-3 (Sentol Associates). Internal standards were $[^3H]$-water and $[^{14}C]$-urea.

Bio-Gel A-0.5m and A-5m 100–200 mesh agarose beads were obtained from Bio-Rad Laboratories and packed into 8 cm^2 internal cross-section LKB Instruments columns fitted with adjustable end plungers. All columns used in this study had packed bed volumes of 772 ml: V_o = 270 ml (A-0.5m), 277 ml (A-5m). Each column was run horizontally (due to space limitations) with a Canalco Model SA ultraviolet monitor at the outlet, followed by a Sigmamotor peristaltic pump to limit the flow rate to 20–30 ml/h and not contribute bubbles to the monitor. Collection was in a Buchler fraction collector set to advance every half hour. The eluent was aqueous triethylammonium bicarbonate (pH 8.3) for the immensely practical reason that this is a volatile buffer[10]. In one case 0.2 *M* lithium chloride was used.

EXPERIMENTAL RESULTS

Hydrogen-bonding cochromatography

It was found early in previous work[5] that $[^3H](dT)_{11}$ would partially cochromatograph with both $d(T)_6(A)_{\overline{62}}$ and $d(T)_6(A)_{\overline{330}}$ when a mixture of all three was run through A-5m at 4° in 0.05 *M* triethylammonium bicarbonate. After this observation, all calibration work was done using only one polydeoxynucleotide at a time.

Identical mixtures of 0.5 mono-μmole of $[^3H](dT)_{15}$ and 0.8 mono-μmole of $d(T)_3(A)_{\overline{140}}$ were chromatographed on A-5m at 21°, varying the molarity of the triethylammonium bicarbonate (Table I). In two runs (footnoted b and c), the eluent was changed to 8 *M* urea just before the mixture was applied and then continued but only for such a time that the $(dT)_{15}$ peak would emerge ahead of the last applied urea. The 8 *M* urea was labeled with $[^{14}C]$-urea to facilitate identification of the end of urea elution. Two different extents of 8 *M* urea application are shown in Table I. During the course of this study, it was noted that the peak separation diminished sharply at the lowest molarities used.

Effect of eluent molarity on calibration

Each of the three polydeoxynucleotides $(dA)_{\overline{310}}$, $d(T)_3(A)_{\overline{140}}$ and $(dT)_{15}$ was

TABLE I

EFFECT OF TEMPERATURE, ELUENT MOLARITY AND UREA TOWARD ELIMINATION OF COCHROMATOGRAPHY OF $(dT)_{15}$ WITH $d(T)_3(A)_{\overline{140}}$ ON BIO-GEL A-5m AT 21°

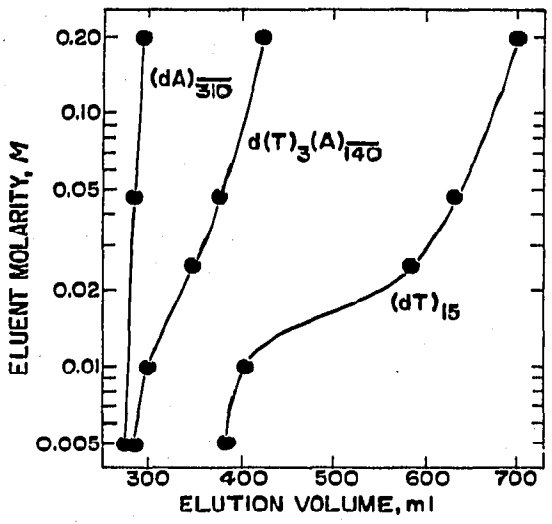
Eluent concentration (M)	*$(dT)_{15}$ in first peak[a] (% of total $(dT)_{15}$)*
0.2	66.1
0.2[b]	19.6
0.2[c]	4.0
0.05	10.6
0.05[d]	56.1
0.025	0.72
0.010	<0.01

[a] Sample applied: 0.8 mono-μmole $d(T)_3(A)_{\overline{140}}$ and 0.5 mono-μmole $(dT)_{15}$.
[b] 8 *M* urea for 0.38 of run.
[c] 8 *M* urea for 0.85 of run.
[d] 4°, for comparison.

chromatographed on Bio-Gel A-5m at 21° using triethylammonium bicarbonate from 0.005–0.20 *M* as eluent. The data for elution volume at the product peak *vs.* molarity are plotted in Fig. 1 with each curve representing one of the polydeoxynucleotides. The elution position of $(dA)_{\overline{310}}$ is very near the exclusion volume.

The index of separation

The calibration of gel filtration columns is generally found to follow a linear relationship between the elution volume and the logarithm of the molecular weight[11]. This was found to be true for Bio-Gels A-5m and A-0.5m using single-stranded polydeoxynucleotides[5]. The equations for the linear portions of these plots are $\log n = 3.88 - 1.32\ (V_e/V_o)$ for A-5m and $\log n = 3.21 - 1.19\ (V_e/V_o)$ for A-0.5m, where n is the number of nucleotide units per molecule, V_e is the elution volume and V_o is the exclusion volume; the equations are in the form developed by DETERMANN AND MICHEL[12]. A practical number that can be used to describe the separation capability of such a column material independent of the size of the packed column is defined here as the index of separation, S, equal to the separation volume between the elution maxima of peaks of two substances differing in molecular weight by a factor of five on passing through 100 ml of packed bed volume. The slope constants in the above equations are inversely related to S. Using the data of Fig. 1 and some other calibration points over the molarity range, the curve in Fig. 2 has been derived. The value of S at 0.05 *M* on Bio-Gel A-5m at 21° is 23.4 ml (Fig. 2). The equation under these conditions is $\log n = 3.60 - 1.04\ (V_e/V_o)$. At this same molarity but at 4° Bio-Gel A-5m gave S = 19.3 ml, and Bio-Gel A-0.5m gave S = 20.6 ml using the data of HAYES *et al.*[5]. Curves describing the equations of HOHN AND SCHALLER[11] for chromatography of $(dA)_n$ oligomers in ammonium bicarbonate on Sephadex G-75 (3 cm diam. × 150 cm, V_t = 270 ml, V_o = 750 ml), $\ln K_d = 0.09 - 0.08n$ for 0.01 *M* and $\ln K_d = 0.13 - 0.044n$ for 1 *M*, are shown on Fig. 3; the $(dA)_n$ oligomer molecular weights (MW) were calculated as ammonium salts. Also plotted in Fig. 3 as the dashed line is the general equation of DETERMANN AND MICHEL[12] for globular proteins on Sephadex G-75 with no specification as to buffer, $\log MW = 5.624 - 0.752\ (V_e/V_o)$. Using the



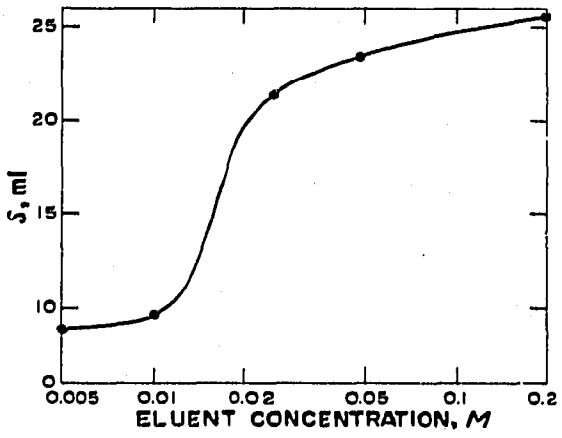
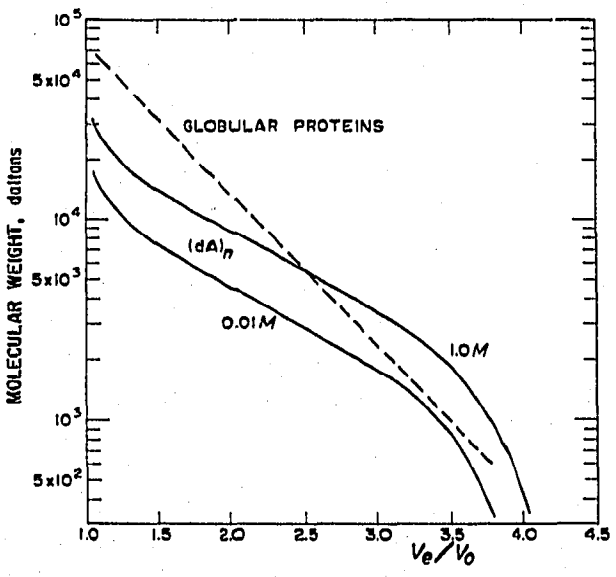


Fig. 1. Elution positions of (dA)$_{\overline{310}}$, d(T)$_3$(A)$_{\overline{140}}$ and (dT)$_{15}$ as related to changing eluent molarity (triethylammonium bicarbonate on Bio-Gel A-5m at 21°).

Fig. 2. Index of separation, S, of single-stranded polydeoxynucleotides as a function of eluent (triethylammonium bicarbonate) molarity using Bio-Gel A-5m at 21°.



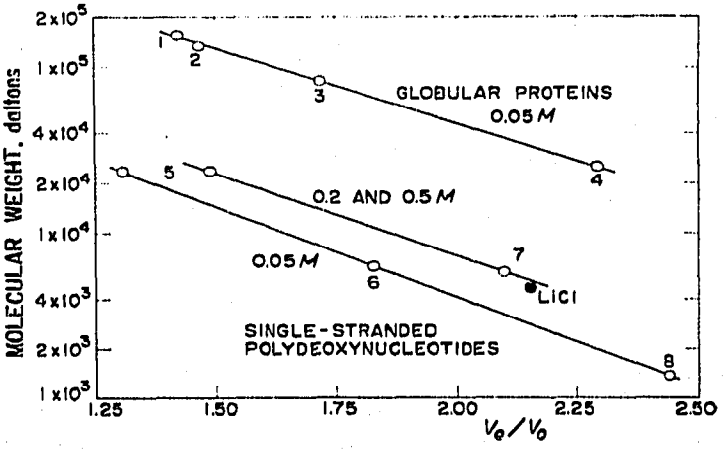


Fig. 3. Replots of data from HOHN AND SCHALLER[2] ((dA)$_n$ oligomers) and from DETERMANN AND MICHEL[12] (globular proteins); data handling is described in the text.

Fig. 4. Calibration curves for globular proteins and single-stranded polydeoxynucleotides on Bio-Gel A-0.5 m at 4° with triethylammonium bicarbonate (open circles) at indicated molarities and 0.2 M lithium chloride (dark circle) as eluents: (1) human γ-globulin; (2) bovine serum albumin dimer; (3) horse liver alcohol dehydrogenase; (4) α-chymotrypsinogen; (5) d(T)$_8$(A)$_{\overline{50}}$; (6) (dT)$_{15}$; (7) (dT)$_{14}$; and (8) (dT)$_8$.

central linear portions of the $(dA)_n$ curves, $S = 43$ ml at 0.01 M and 45 ml at 1 M; S for the protein line is 24 ml.

In Fig. 4 are linear portions of the calibration curves of globular proteins and single-stranded polydeoxynucleotides on Bio-Gel A-0.5m at 4° using triethylammonium bicarbonate. The polydeoxynucleotide molecular weights are those of triethylammonium salts. The lines can be characterized: 0.05 M polydeoxynucleotide, $S = 22.3$, log MW $= 5.81 - 1.10\,(V_e/V_o)$; 0.2 M and 0.5 M polydeoxynucleotide, $S = 24.5$, log MW $= 5.86 - 1.00\,(V_e/V_o)$; 0.05 M globular protein, $S = 26.8$, log MW $= 6.49 - 0.914\,(V_e/V_o)$. The isolated point in Fig. 4 marks the elution position of $(dT)_{14}$ as the lithium salt in 0.2 M lithium chloride.

Comparative chromatography of polydeoxynucleotides with possibly differing molecular configurations

Bio-Gel A-5m at 4° equilibrated with 0.05 M triethylammonium bicarbonate was loaded with a mixture of 0.48 mono-μmole of $d(T)_6(A)_{\overline{50}}$ and 0.43 mono-μmole of $(dT)_{14}$, both of which were ^{3}H-labeled in the dT units. There emerged two peaks, the first of which contained, in addition to $d(T)_6(A)_{\overline{50}}$, 0.195 mono-$\mu$mole of $(dT)_{14}$ as determined by specific radioactivity; the second peak was the remainder of pure $(dT)_{14}$. The mixture in the first peak corresponded to 45 % of the dA units in $d(T)_6(A)_{\overline{50}}$ that were presumably hydrogen-bonded to dT units from $(dT)_{14}$, forming an average structure $d(T)_6(A)_{\overline{50}} \cdot 1.6(dT)_{14}$. The elution volume for the first peak is plotted as point 4 on Fig. 5. Similarly, a mixture of 0.48 mono-μmole of $(dT)_6(A)_{\overline{50}}$ and 4.3 mono-μmoles of $(dT)_{14}$ was chromatographed. Analysis of the first peak showed that 73 % of the dA units were hydrogen-bonded in an average structure $d(T)_6(A)_{\overline{50}} \cdot 2.6(dT)_{14}$; this is point 3 in Fig. 5.

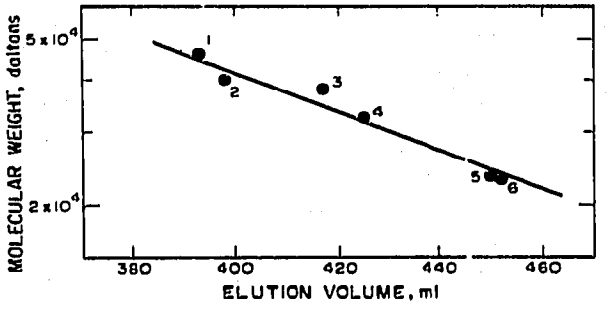


Fig. 5. Elution positions of: (1) $d(T)_6(A)_{\overline{50}} \cdot d(T)_{\overline{50}}(A)_6$; (3) $(dC)_{\overline{101}}$; (3) $d(T)_6(A)_{\overline{50}} \cdot 2.6\,(dT)_{14}$; (4) $d(T)_6(A)_{\overline{50}} \cdot 1.6\,(dT)_{14}$; (5) $d(T)_6(C^{Ac})_{\overline{49}}$; and (6) $d(T)_6(A)_{\overline{50}}$ using Bio-Gel A-5m at 4° and 0.05 M triethylammonium bicarbonate.

Also making up Fig. 4 are chromatography data for $d(T)_6(A)_{\overline{50}}$, point 6, and the completely replicated, double-stranded derivative $d(T)_6(A)_{\overline{50}} \cdot d(T)_{\overline{50}}(A)_6$, point 1. The remaining two chromatography points are $(dC)_{\overline{101}}$, point 2, whose ultraviolet absorption spectrum in the eluent showed it to be in the non-protonated coil configuration[13] ($\lambda_{max} = 269$ nm, pH 8.3), and $d(T)_6(C^{Ac})_{\overline{49}}$, point 5, of unknown molecular configuration. The line drawn to fit the points had the imposed restriction $S = 19.3$ characteristic for this column packing, eluent and operating temperature.

DISCUSSION

The gel filtration properties of oligodeoxynucleotides have been investigated[2,14,15] and found suitable for application as a useful adjunct to ion exchange and paper chromatography. Gel filtration chromatography of polydeoxynucleotides on Sephadex G-50 has been put to practical use by NARANG *et al.*[1], who accomplished rapid, partial purification in separating chemically synthesized polytrimers from condensing agent, organic solvent and unused trimer. Polydeoxynucleotides synthesized by enzymatic procedures[3–5,16] have been purified by use of dextran, polyacrylamide and agarose gels that effect isolation of polymer free from reaction buffer, unused deoxynucleoside 5′-triphosphate and oligodeoxynucleotide initiator that is required as a starting block onto which polymerization occurs. The products from such enzymatic chain-lengthening reactions are mixtures of polymer molecules with lengths distributed according to Poisson theory[4] around a mean length. It is this mean length in nucleotide units, n, accurately determinable by use of a radioactive initiator, that has been found for agarose columns[5] operating at 4° with 0.05 M triethylammonium bicarbonate to fit a straight line on a plot of log n *vs.* V_e (elution volume). Equations for this relationship are log $n = 3.88 - 1.32\ (V_e/V_o)$ for Bio-Gel A-5m and log $n = 3.21 - 1.19\ (V_e/V_o)$ for Bio-Gel A-0.5m. When the initiator size is not very different from that of the product, as in a limited synthesis or one step in the formation of a block copolymer, great resolution capability is asked of the gel column to separate product from that part of the initiator to which nothing added. Long columns, recycling chromatography and best techniques in packing and system plumbing all alleviate this problem. However, due to inter- and intramolecular interaction such as aggregation and hydrogen bonding, further improvements in chromatography and chemistry are needed. N-Acetylation of guanine, cytosine and adenine rings of the nucleotide units in both the polymerizing monomer 5′-triphosphate and the initiator has supplied a chemical solution[5] to some aggregation and hydrogen-bonding problems.

In this report, unwanted cochromatography of $(dT)_{15}$ with the almost tenfold longer $d(T)_3(A)_{\overline{140}}$ on Bio-Gel A-5m was lessened (Table I) by using urea in the eluent for part of the run, raising the temperature of the chromatography or lowering the eluent molarity. The last change was found to have a deleterious effect on the separation capability of the column (Fig. 1).

In the general form of the elution parameter equation[12] log MW $= a + b\ (V_e/V_o)$, the slope constant, b, indicates separation capability; the smaller the value of b the greater the separation between the same two separable peaks. Another term that reflects the common practical situation of having either a certain favorite set of column dimensions or a limited space in which to place a column is S, defined here as the separation volume between maxima of the elution peaks of two substances differing in molecular weight by a factor of five on passing through 100 ml of packed bed volume. For polydeoxynucleotides on Bio-Gel agarose columns with 0.05 M triethylammonium bicarbonate, values of S are 20.6 ml at 4° on A-0.5m and on A-5m 19.3 ml at 4° and 23.4 ml at 21°. The way S varies with the molarity of triethylammonium bicarbonate is shown in Fig. 2 with a sharp change between 0.01–0.2 M. The above values of S with agarose columns suggest that increasing the temperature and decreasing the pore size may increase separation. Analysis by DETERMANN AND

MICHEL[12] of many authors' use of Sephadex columns for chromatography of globular proteins also showed separation increasing with decreasing pore size, with the slope constant b (inversely related to S) decreasing with decreasing pore size. The work of HOHN AND SCHALLER[2] with $(pA)_n$ oligomers on Sephadex G-75 (Fig. 3) showed essentially no increase in S on going from 0.01 M to 1.0 M; their data did, however, show the large average value of $S = 44$ ml.

It has been recognized for some time (Fig. 3) that the minimum molecular weight for exclusion from a gel established with proteins does not apply to polynucleotides: that polynucleotides act much larger than indicated by their molecular weights. This situation is documented here and graphically displayed in Fig. 4. The eluents are 0.05 M, 0.2 M and 0.5 M triethylammonium bicarbonate on Bio-Gel A-0.5m at 4°. The molecular weights of the single-stranded polydeoxynucleotides were calculated as triethylammonium salts, in which form they were applied. If these molecules were to move through the column as bare polyanions in a sea of cations, their points on Fig. 4 would have had to be plotted at 0.7 of the values shown.

An effect of increasing ionic strength on single-stranded polynucleotides is to decrease electrostatic stress resulting in coiling of the molecules[17]; this reduces their hydrodynamic volume[18]. The change in V_e/V_o on going from 0.05 M to 0.2 M in Fig. 4 may represent partial collapse of the polynucleotide molecules. To test whether the triethylammonium cation might be inefficient in promoting this collapse due to its large size, a corresponding test with the much smaller lithium cation was tried. It is shown by the single point in Fig. 4 that such a steric effect is inoperative. The possibility that the agarose used here might have contained significant negative charges on the gel beads could supply the mechanism (common ion repulsion) to explain why, after high ionic strength collapsing, the polydeoxynucleotide and protein calibration lines in Fig. 4 are still far apart.

Fig. 5 demonstrates that at 0.05 M triethylammonium bicarbonate the single-stranded polydeoxynucleotides do not fall on a different curve from partially or totally double-stranded polydeoxynucleotides. This suggests that at 0.05 M the single-stranded molecules are no more coiled than the rod-like double-stranded ones.

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