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SEPARATION OF OLIGONUCLEOTIDES ON ION-EXCHANGE DERIVATIVES OF SPHERON

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SUMMARY

Diphenylamine–formic acid hydrolyzate of *Bacillus subtilis* DNA (a mixture of pyrimidine oligodeoxyribonucleotides) was chromatographed using an ionic strength gradient on seven ion exchangers: five DEAE-Spherons 300 of various capacities, two types of BD-Spheron 300 and DEAE-Sephadex A-25. The suitability of ion-exchange derivatives of Spheron for the chromatography of oligonucleotides is discussed. The separation of mono- to undeca-deoxyribonucleotides was achieved.

INTRODUCTION

The most common ion-exchange sorbents used in the chromatography of oligonucleotides are DEAE-cellulose and DEAE-Sephadex, but TEAE-cellulose or QAE-Sephadex are also used[1,2]. During a study of the application of ion-exchange derivatives of macroreticular glycol-methacrylate gels of Spheron™ to high-performance liquid chromatography (HPLC) of biopolymers and their fragments[3], we found in a preliminary experiment (*cf.*, Fig. 11, ref. 3) that these rigid beads are also suitable for chromatography of oligonucleotides. The aim of the present short study is a comparison of the chromatography of a mixture of pyrimidine oligodeoxyribonucleotides (prepared[4] by partial specific hydrolysis of DNA from *Bacillus subtilis*) on columns of DEAE-Spherons 300 of various capacities, on columns of BD-Spherons 300 (*i.e.*, benzoylated or benzylated DEAE-Spherons of various types) and on DEAE-Sephadex A-25. In earlier papers a more detailed chemical characterization of Spherons P-100, P-300 and P-1000 (ref. 5) and the application of ion-exchange derivatives prepared from Spheron P-300 to rapid chromatography of technical enzymes[6] and sugars[7] has been described. DEAE-Spheron 300 is characterized in greater detail elsewhere[8]. The BD-Spherons have not yet been described.

EXPERIMENTAL

Materials

DEAE-Spherons 300 (particle size 20–40 μm) were prepared in our laboratory from Spheron P-300 (Lachema, Brno, Czechoslovakia) using the methods described elsewhere[3,8]. The derivatives are summarized in Table I. BD-Spherons 300 were prepared in our laboratory by benzoylation or benzylation of DEAE-Spheron 300. $B_{OH}D$-Spheron 300 was prepared on reaction of DEAE-Spheron 300 (capacity 1.78 mequiv./g) with benzoyl chloride in ethyl acetate catalyzed by pyridine. B_ND-Spheron 300 was prepared from DEAE-Spheron 300 (capacity 2.04 mequiv./g) by alkylation with benzyl chloride in absolute ethanol. DEAE-Sephadex A-25 was a product of Pharmacia, Uppsala, Sweden.

The partial specific diphenylamine–formic acid hydrolyzate of *Bacillus subtilis* DNA was prepared as follows. *Bacillus subtilis* DNA was isolated using the method of Marmur[9]. The DNA hydrolyzate was prepared according to the modified[4] Burton method[10]. The DNA solution was dialyzed against distilled water, then the DNA concentration was adjusted to 0.5–1 mg/ml and two volumes of 3% (w/v) diphenylamine in 98% formic acid were added. The mixture was incubated at 30° for *ca.* 20 h, then diluted with 0.5 volumes of distilled water and extracted with diethyl ether in a continuous liquid–liquid extractor. The aqueous phase was then dried in a vacuum evaporator at 50°, the residue dissolved in distilled water and dried twice more. After the last evaporation the residue was dissolved in the starting buffer A, and chromatographed (*cf.*, Figs. 1–3).

Chromatographic methods

The Spheron ion exchangers were suspended in water before chromatography, then deaerated, purified by sedimentation, regenerated with 2 *M* sodium chloride and cycled several times by washing successively with 2 *M* hydrochloric acid, water, 2 *M* sodium hydroxide and water, as has been described[3]. They were then equilibrated with the starting buffer A (for composition see below) and the suspension was introduced into a column and sedimented during the solvent flow using occasional pressure pulses of 10–25 atm. until the required height was achieved. DEAE-Sephadex was treated as recommended by the producer.

The chromatography of the partial specific hydrolyzate of DNA used for comparison of the efficiency of ion exchangers 1–5 (Table I) was carried out as follows. A 10-mg amount of a mixture of oligonucleotides was dissolved in 2 ml of the starting buffer A (0.01 *M* sodium acetate, pH 5.3) and chromatographed on a column (23 × 0.8 cm) of ion exchanger at 25° and 50 ml/h through-flow. The buffer was pumped onto the column of an amino acid analyzer with a Proportional Programmed Micropump (Developmental Workshops, Czechoslovak Academy of Sciences, Prague, Czechoslovakia). First, undesirable reaction products were eluted with *ca.* 150 ml of buffer A, then a linear concentration gradient of sodium chloride, 0–0.4 *M*, in the same buffer was applied (final composition of the buffer = B; total volume of the gradient = 1.2 l) and finally the column was washed with 1 *M* sodium chloride (solution C). The effluent was estimated by continuous measurement of absorbance at 254 nm using a Uvicord (LKB, Stockholm, Sweden). When chromatographies on ion exchangers 5–8 were compared (Table I, Figs. 1–3), 2 ml

of a solution containing 20 mg of a mixture of oligonucleotides in the starting buffer A were applied onto the column. Other conditions were identical.

RESULTS AND DISCUSSION

The macroporous rigid and hydrophilic Spheron ion exchangers were developed for the HPLC of biopolymers and their higher-molecular-weight fragments. They proved valuable in the chromatography of enzymes[6] and other proteins[3], but also in the separation of higher-molecular-weight polypeptide fragments of proteins[11]. In these experiments the ion exchangers with maximum capacity proved most effective[12]. Preliminary experiments on the chromatography of nucleic acids, oligonucleotides and adenosine phosphates were also carried out on these ion exchangers (*cf.*, ref. 3). It was thus found that the DEAE-derivatives of Spheron can also be used with these substances. The most suitable type of functional group and the optimum degree of substitution of Spheron for the chromatography of nucleic acids is still not known. However, in the experiments aimed at the separation of adenosine phosphates, it was found that the best results were achieved on ion exchangers of maximum capacity. Therefore the question remained as to what degree of substitution and which functional group is suitable for the chromatography of oligonucleotides.

The experiments are summarized in Table I and some are illustrated by Figs. 1–3. From these it follows that, for the chromatography of pyrimidine oligodeoxyribonucleotides, the ion exchangers with the maximum degree of substitution give the best separations, *i.e.*, of ion exchangers 1–5 that of 2.05 mequiv./g capacity was the most suitable. Only the best separation is shown (Fig. 1). The results of individual chromatograms, evaluated on the basis of visual inspection, are given in the penultimate column of Table I. In contrast to chromatograms of other types of substances on DEAE-Sperons of various capacities (*e.g.*, sugars[7], amino acids, peptides and proteins[8,12]), Spheron ion exchangers of the lowest capacity (No. 1 in Table I) are still capable of a relatively good separation of oligonucleotides, com-

TABLE I

SEPARATIONS OF OLIGODEOXYRIBONUCLEOTIDES ON ION-EXCHANGE DERIVATIVES OF SPHERON P-300 AND ON DEAE-SEPHADEX

The mixture of deoxyribonucleotides used was a specific partial diphenylamine–formic acid hydrolyzate of DNA from *Bacillus subtilis*.

Ion exchanger			*Separated peaks (number of nucleotides in the oligonucleotide)*	*Fig.*
No.	*Name*	*Capacity (mequiv./g)*		
1	DEAE-Spheron 300	0.26	1 ÷ 8	—
2	DEAE-Spheron 300	0.63	1 ÷ 8	—
3	DEAE-Spheron 300	1.23	1 ÷ 9	—
4	DEAE-Spheron 300	1.63	1 ÷ 9	—
5	DEAE-Spheron 300	2.05	1 ÷ 11	1
6	B_{OH}D-Spheron 300*	1.80	1 ÷ 11	2
7	B_{N}D-Spheron 300**	2.00	1 ÷ 9	—
8	DEAE-Sephadex A-25	3.50	1 ÷ 8	3

* DEAE-Spheron 300 substituted by benzoylation, predominantly on hydroxy groups.
** DEAE-Spheron 300 substituted by benzylation, predominantly on nitrogen.

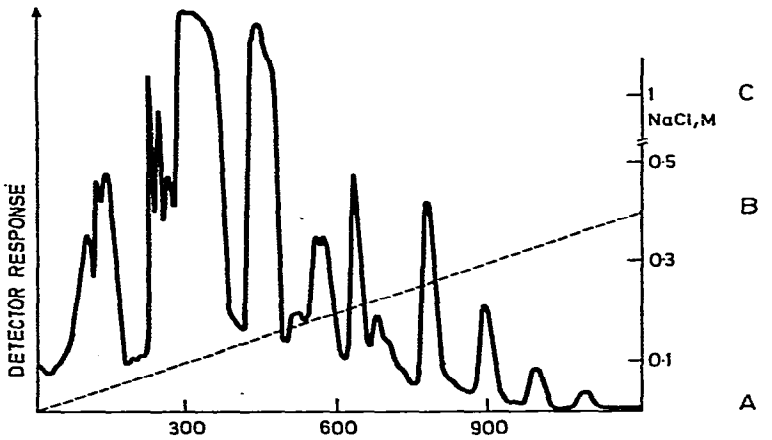

Fig. 1. Chromatography of partial specific diphenylamine–formic acid hydrolyzate of DNA from *Bacillus subtilis* on a column (23 × 0.8 cm) of DEAE-Spheron 300 (ion exchanger 5, Table I). The hydrolyzate (20 mg) was dissolved in 2 ml of the starting buffer A and applied onto the column and the unwanted by-products of the reaction were first washed with 150 ml of buffer A. Then a linear gradient of 600 ml of buffer A and 600 ml of buffer B (start of the chromatogram) was applied. Further elution with solution C did not wash out any components. Flow-rate, 50 ml/h; temperature, 25°; detection, continuous recording of the absorbance of the effluent at 254 nm, using a flow-through cell; chart speed, 40 mm/h. Abscissa, absorbance and NaCl concentration in the effluent; ordinate effluent volume. Buffers: A = 0.01 *M* sodium acetate, pH 5.3; B = buffer A, 0.4 *M* in NaCl; C = 1 *M* NaCl.

parable with DEAE-Sephadex (No. 8, Table I). Other types of compounds were hardly separated at all on Spheron ion exchangers with very low capacities. This can be explained tentatively by the polyplicity of the strongly acidic phosphate group in oligonucleotides and the possibilities of their multiple interaction with thinly distributed basic functional groups. The same notion could probably also be applied to nucleic acids in which the high degree of substitution of the anion exchanger or the strongly basic groups can lead to decomposition of the chromatographed biopolymers.

The value of 2 mequiv/g is not the highest attainable degree of substitution of the Spheron matrix. Anion exchangers with capacity 2.2 mequiv./g can be prepared, and the experimentally attainable limit would be *ca.* 2.4 mequiv./g (ref. 8). However, because of the relatively weak dependence of the separation efficiency on the capacity, it is not necessary for the purposes of the separation of oligonucleotides to endeavour to get the maximum degree of substitution, the achievement of which considerably complicates the preparation of these ion exchangers[8].

The Spheron ion exchangers are hydrophilic in principle, but much weaker than the derivatives of cellulose or polydextran. A certain degree of hydrophobicity of the matrix does not result in denaturation of biopolymers, and permits the performance of hydrophobic chromatography on the unsubstituted starting Spheron[13–16]. However, we have shown[8] that the hydrophobicity of Spheron decreases with an increasing degree of ionogenic substitution, which can be simply explained by the effect of the increase of the number of strongly polar functional groups. Therefore we considered it useful, for the experiments on the chromatography of oligo-

nucleotides, to hydrophobize the matrix of the ion exchangers of higher capacity or their functional groups, by introduction of benzoyl or benzyl groups. Using the ion exchanger 6 (B_{OH}D-Spheron 300, Table I), we separated the mixture of pyrimidine oligodeoxyribonucleotides into eleven main peaks (Fig. 2), but the general quality of the chromatogram was not improved and decreased yields of individual oligonucleotides were obtained. Either a part of the oligonucleotides was damaged, or they were sorbed irreversibly on the excessively hydrophobized segments of the matrix. The type of benzoylation used has an additive effect: introduction of a hydrophobic group simultaneously decreases the hydrophilicity of the matrix by substitution of the polar hydroxyl groups. The alkylation of nitrogen (B_ND-Spheron 300, Table I, No. 7) was unsuccessful. The separation is less effective, the shape of the peaks deformed and the permeability of the column also decreased (the latter diminished the flow to 40–45 ml/h). An excessive increase in ionic interaction during chromatography caused by quaternization of the functional group is also possible.

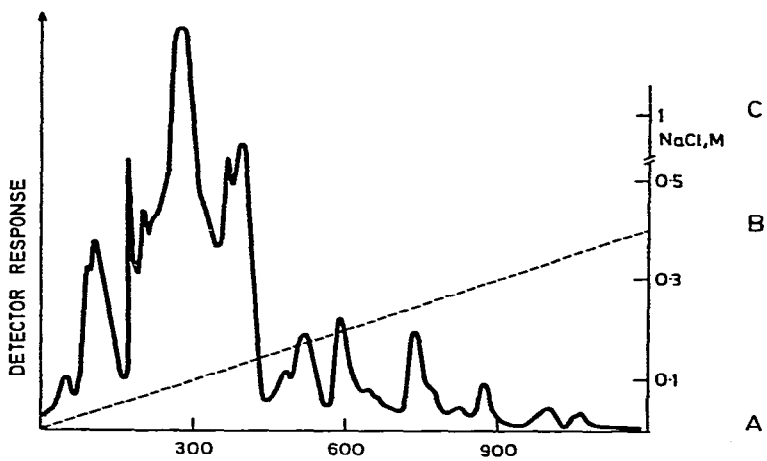

Fig. 2. Chromatography of the same hydrolyzate as in Fig. 1 on a column of B_{OH}D-Spheron 300 (ion exchanger 6, Table I). Other conditions as in Fig. 1.

For comparison with the results obtained on ion exchangers 5–7 (Table I), illustrated by Figs. 1 and 2, chromatography on the ion exchanger 8, DEAE-Sephadex (Fig. 3), was carried out. By comparison with Figs. 1 and 2, it is evident that the separation efficiency of Spheron ion exchangers is distinctly better under comparable conditions. These ion exchangers do not change their volume during elution with a gradient of ionic strength, and therefore they also permit regeneration and equilibration directly in the column. In addition, they permit a substantial increase in flow velocity because they are mechanically strong and can withstand high pressures. Their future development may be directed to further diminution of the particle size, which will permit a more rapid diffusion into their interior and a shortening of the time necessary for chromatography.

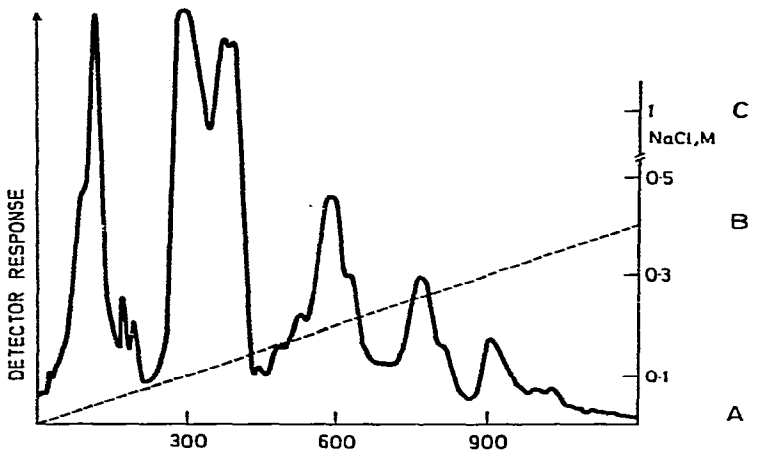

Fig. 3. Comparative chromatography of the same hydrolyzate as in Fig. 1 on a column (23 × 0.8 cm, starting dimensions) of DEAE-Sephadex (ion exchanger 8, Table I). Other conditions as in Fig. 1.

CONCLUSIONS*

DEAE-derivatives of Spheron 300 (particle size 20–40 μm, capacity *ca.* 2 mequiv./g) proved very suitable for chromatographic separation of pyrimidine oligodeoxyribonucleotides split specifically from the DNA of *Bacillus subtilis*. Their use permitted a good separation of mono- up to undeca-nucleotides. The quality of separation exceeded that of chromatography carried out under the same conditions on a DEAE-derivative of polydextran. During elution with an ionic strength gradient, the column volume did not change. Hydrophobization of DEAE-Spheron by benzoylation or benzylation did not increase the separation efficiency of these ion exchangers, and therefore it is not necessary for the purposes of the chromatography of oligonucleotides. The mild hydrophobicity of the matrix of these substantially hydrophilic ion exchangers is adequate for efficient separations.

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* *Note added in proof:* Svoboda and Kleinmann[17] used DEAE-Spheron 1000 successfully for preparative separations of nucleoside monophosphates and found the ion exchanger stable to radioactivity.

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