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BEHAVIOUR OF SINGLE-STRANDED OLIGODEOXYRIBONUCLEOTIDES ON A DEAE-5PW ANION-EXCHANGE COLUMN

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SUMMARY

The separation of large, single-stranded oligodeoxyribonucleotides, prepared by chemical synthesis, was carried out on a DEAE-5PW anion-exchange column with eluents containing only volatile salts. It was found that gradient elution is essential and that a high resolution can be achieved at elevated temperatures (*ca.* 50°C). Also, neutral pH of the eluents was found to be desirable for the separation. Under optimum conditions, a linear relationship between the elution volumes and the number of bases in the substrates was observed.

INTRODUCTION

Because of the recent rapid development of gene manipulation, the preparation of various DNA fragments carrying genetic information has become extremely important in biochemistry. Such DNA fragments, oligodeoxyribonucleotides, are commonly obtained by chemical synthesis[1–3], enzymic digestion of DNA[4] and reverse transcription from mRNA[5]. Traditionally, the purification and identification of such oligodeoxyribonucleotides have been carried out by open-column chromatography[6,7], gel electrophoresis[8] or thin-layer chromatography[9,10]. However, there has not been a satisfactory method for purification when the samples to be purified are single-stranded oligodeoxyribonucleotides, synthesized chemically. Contamination of a sample having a finely designed base sequence with impurities that have sequences similar to or almost identical with that of the sample often prevents complete ligation

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of the sample from building up a targeted large DNA. Also, by use of a DNA sample containing undesirable DNAs, proper *E. coli* transformation is made less efficient.

High-performance liquid chromatography (HPLC), by means of which a number of biologically important materials have been isolated, has not been effectively utilized in this field. In particular, there have been few applications of HPLC to larger, single-stranded DNA fragments[11,12]. Even then, satisfactory practical procedures were not reported. The samples studied were very simple, the column material was toxic to cells and the salts used in the mobile phases were all difficult to remove from the fractions after the separation.

In this study, we investigated the separation of large single-stranded oligodeoxyribonucleotides by anion-exchange chromatography with a newly developed DEAE-5PW column. The eluent used was a solution containing only salts that can be removed from the fractions by lyophilization.

EXPERIMENTAL

The oligodeoxyribonucleotides used were synthesized chemically by the liquid-phase triester method described previously[2,13,14]. The sequences of the samples are listed in Table I. Ammonium acetate and ammonium formate of analytical-reagent grade were purchased from Nakarai Chemicals (Kyoto, Japan). Water was purified by a Milli R/Q water purifier (Millipore, Bedford, MA, U.S.A.).

TABLE I

BASE SEQUENCES OF THE OLIGODEOXYRIBONUCLEOTIDES USED AND THEIR ABBREVIATIONS

Substrate	*No. of bases*	*Abbreviation*
dCATGGT	6	6b
dTCAAATC	7	7b
dCGGATTTGA	9	9b
dCGACCCGGGT	10	10b
dCATCTTCATGGC	12	12b
–	16	16b
dCCIAAITCCATCCAICCITAIGC	23	23b
dCCIAAITCCATCCAICCCATITAITC	26	26b

The high-performance liquid chromatograph was a Model HLC-803D (Toyo Soda, Tokyo, Japan), equipped with a GE-4 gradient system (Toyo Soda) and a thermostatically controlled column oven. The column effluents were monitored at 260 nm with a UV-8 Model II variable-wavelength UV detector (Toyo Soda). A commercially available anion-exchange column (7.5 cm × 7.5 mm I.D.) of DEAE-5PW (Toyo Soda) was utilized.

Eluents were degassed immediately prior to use by a combination of sonication and evacuation. The injection volume of the samples was 20 μl. The concentration of the samples was made as low as possible and therefore the highest sensitivity of the detector was used.

RESULTS AND DISCUSSION

As a preliminary study, separation was carried out under isocratic conditions. The solutes used were 7b, 10b, 16b and 26b (Table I). The ammonium acetate concentrations in the eluent were 1 and 0.9 *M*. This slight difference in concentration produced great differences in the elution volumes of the solutes, as shown in Fig. 1. The elution volume of 26b, which was eluted most slowly in the chromatogram shown in Fig. 1A, was 7.6 ml when the salt concentration was 1 *M* and 23.4 ml at 0.9 *M* (see Fig. 1B). On the other hand, 7b appeared at almost the same elution position at both salt concentrations. From these results, gradient elution was found to be essential for improved separation.

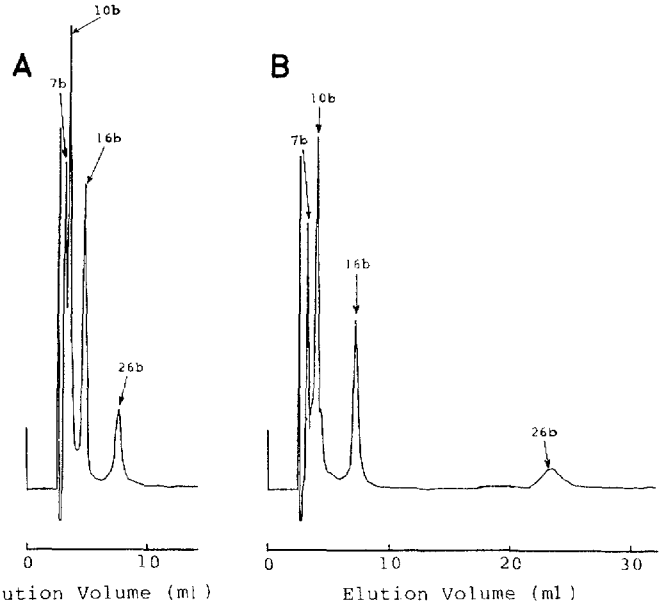


Fig. 1. Isocratic elution profile of single-stranded oligodeoxyribonucleotides. Conditions: column, DEAE-5PW; eluent, aqueous solution of ammonium acetate; concentration of eluent, (A) 1 *M* and (B) 0.9 *M*; flow-rate, 1 ml/min; temperature, 50°C.

The effect of differences in the salt on retention behaviour was also investigated. Fig. 2 shows a typical separation of a sample containing several types of single-stranded oligodeoxyribonucleotides, obtained by gradient elution with linearly increasing ammonium acetate concentrations. All of the solutes could be separated satisfactorily. In addition, it was found that the elution volumes of the solutes increased with an increase in the number of bases. These results indicate that by controlling the ammonium acetate gradient system, optimization of the separation of oligodeoxyribonucleotides can be achieved. The small humps on the baseline are probably due to the high sensitivity of the detector.

Another volatile salt, ammonium formate, which is frequently used in bio-

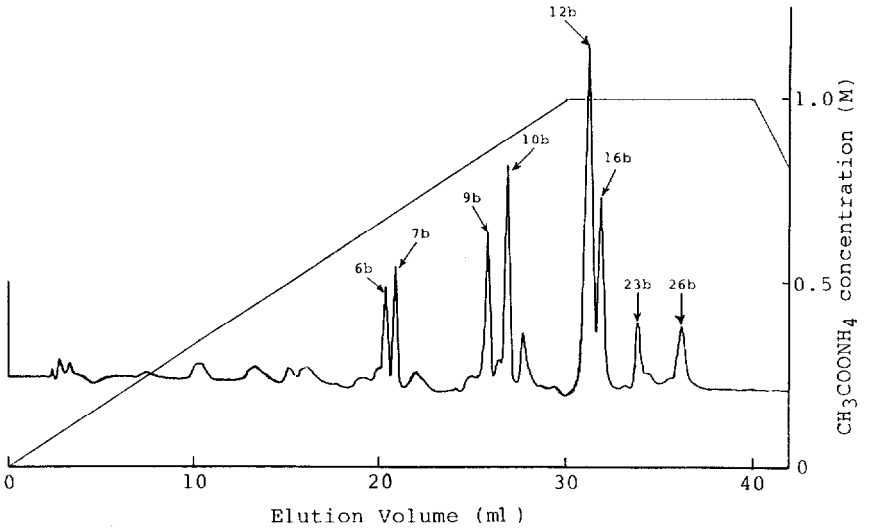


Fig. 2. Separation of single-stranded oligodeoxyribonucleotides. Eluent, linear gradient from 0.01 to 1 *M* ammonium acetate in 30 min and then isocratic elution (1 *M*) used after 30 min. Other conditions as in Fig. 1.

chemistry, was also used. When the same sample was chromatographed with that solvent, an elution profile similar to that shown in Fig. 2 was observed, as depicted in Fig. 3. This salt may possibly also be effective for the separation of these samples, but in this study all of the subsequent experiments were carried out with the use of ammonium acetate.

The effects of flow-rate on the retention behaviour of the solutes were examined, and the results are summarized in Table II. The gradient system used for the measurements was varied according to the flow-rates, as shown in the table. Changes in flow-rate made little difference to the retention positions and elution profile. Therefore, a flow-rate of 1 ml/min. which shortens the analysis time, was used.

The changes in the retention behaviour of the samples at different temperatures were studied. The relationship between the number of bases in the samples and their elution volumes observed at different temperatures is depicted in Fig. 4. The elution volumes were obtained by use of a linear gradient system (see Fig. 4). Good separation of oligodeoxyribonucleotides containing up to twelve bases was obtained at 35°C. At elevated temperatures, improvements in the separation could be achieved. In particular, the resolution of substrates with more than sixteen bases was improved. However, the slope of the curves for the samples with more than sixteen bases was still shallow under the applied linear gradient conditions. Therefore, in order to increase the retention of the larger oligodeoxyribonucleotides, isocratic elution was used after the application of a gradient with the same slope for 30 min. The results are shown in Fig. 5, where it is demonstrated that at higher temperatures a better resolution could be obtained under these conditions. This implies that the separation of single-stranded DNA fragments is strongly dependent on both temperature and the gradient system.

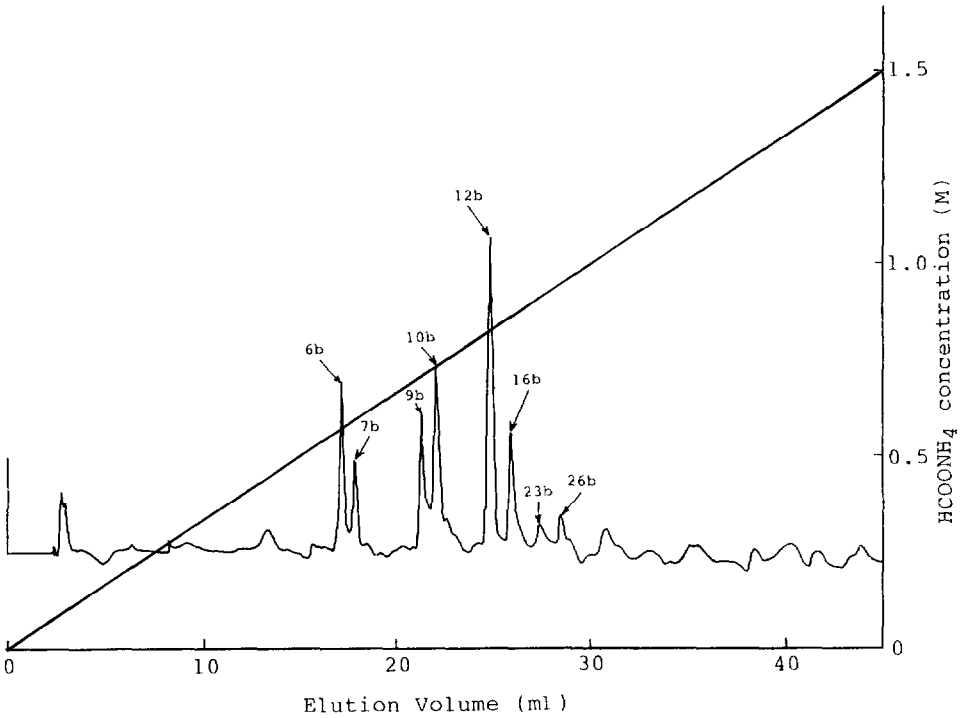


Fig. 3. Separation of single-stranded oligodeoxyribonucleotides. Eluent, linear gradient from 0.01 to 1.5 *M* ammonium formate in 45 min. Other conditions as in Fig. 1.

Fig. 6 summarizes the results from a more detailed study of the effect of temperature on the retention of the samples. Great variations in the elution volumes at different temperatures were observed for each sample. These results indicate that the elution volumes of each oligodeoxyribonucleotide, except 12b, will increase as the temperature is increased, and this effect is greater for samples with a larger number of bases. The unusual behaviour of 12b seen in Fig. 6 is still unexplained.

In the same experiments, changes in temperature were also found affect the peak widths of the substrates studied. The peak widths at half-height, obtained from

TABLE II

EFFECT OF FLOW-RATE

Eluent: linear gradient from 0.01 to 1 *M* ammonium acetate in 60 min and then 1 *M* (A) in 30 min and finally 1 *M* (B). Other conditions as in Fig. 1.

Flow-rate (ml/min)	*Gradient programme*	*Elution volume (ml)*							
		6b	*7b*	*9b*	*10b*	*12b*	*16b*	*23b*	*26b*
0.5	A	19.6	20.4	25.1	26.5	30.5	31.3	32.8	35.3
1.0	B	20.4	20.9	25.8	26.9	31.2	31.9	33.9	36.2

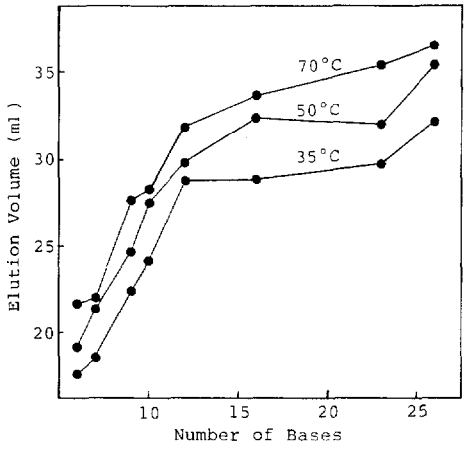


Fig. 4. Relationship between the number of bases and the elution volumes of oligodeoxyribonucleotides at different temperatures. Eluent, linear gradient from 0.01 to 1.5 *M* ammonium acetate in 45 min. Other conditions as in Fig. 1.

the chromatograms in Fig. 6, are listed in Table III. At higher temperatures, the value was small for samples with a smaller number of bases than 16b, whereas 23b and 26b produced wider peaks. From these results, measurement at 50°C can be considered to be suitable for all the nucleotides studied. Also, it was found that the effect of temperature was considerable when there were complementary components in the samples. A typical chromatographic change observed in such a case is depicted in Fig. 7, where 7b (dTCAAATC) and 9b (dCGGATTTGA) were selected as samples complementary with each other. In Fig. 7A, the separation of a mixture containing 7b and 10b (dCGACCCGGGT), obtained at 16°C, is shown. The relative peak-height ratio of 7b to 10b was 1.11. However, on addition of 9b to this mixture, the ratio

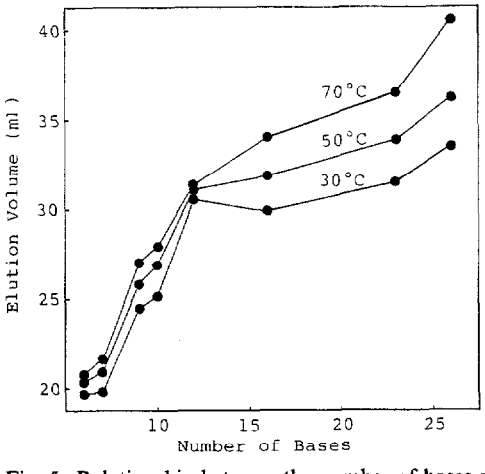


Fig. 5. Relationship between the number of bases and the elution volumes of oligodeoxyribonucleotides at different temperatures. Conditions as in Fig. 2.

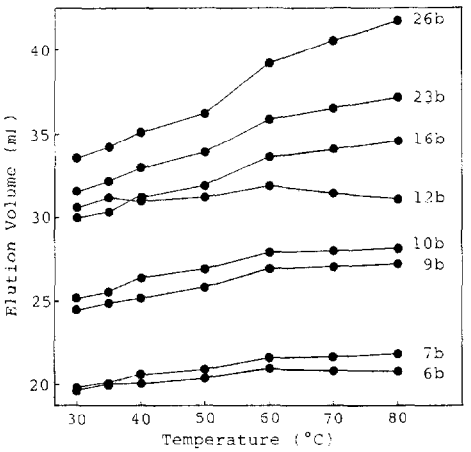


Fig. 6. Effect of temperature on the retention of single-stranded oligodeoxyribonucleotides. Other conditions as in Fig. 2.

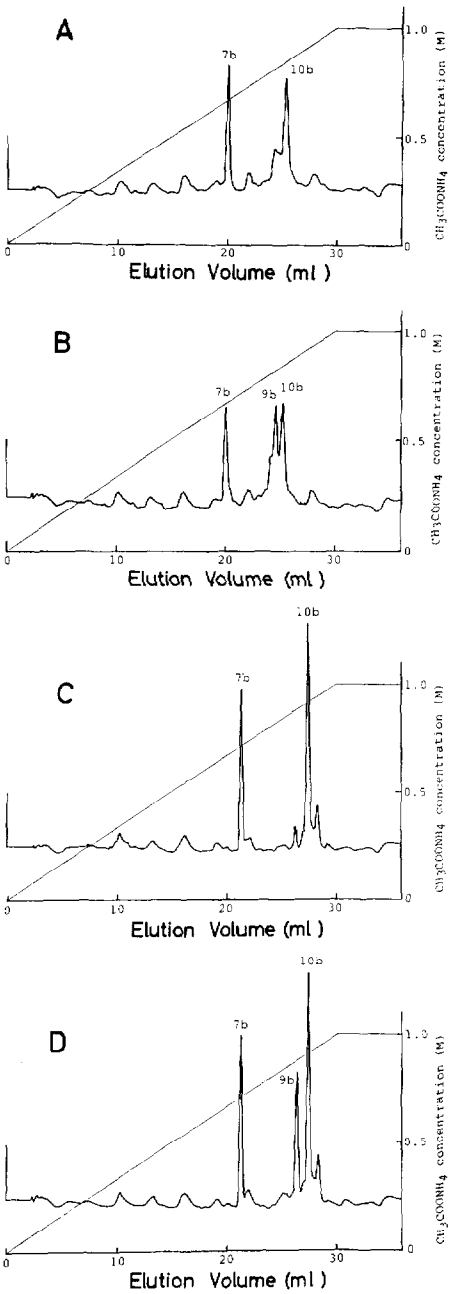
TABLE III

PEAK WIDTH AT HALF-HEIGHT OBTAINED FOR THE OLIGODEOXYRIBONUCLEOTIDES AT DIFFERENT TEMPERATURES

Conditions as in Fig. 6.

Temperature (°C)	*Peak width at half-height (ml)*							
	6b	*7b*	*9b*	*10b*	*12b*	*16b*	*23b*	*26b*
35	0.32	0.30	0.34	0.38	0.50	0.36	0.42	0.34
40	0.30	0.28	0.32	0.32	0.44	0.34	0.40	0.44
50	0.30	0.28	0.34	0.28	0.40	0.36	0.36	0.50
60	0.26	0.24	0.30	0.32	0.40	0.32	0.44	0.68
70	0.26	0.24	0.28	0.32	0.38	0.34	0.50	0.72
80	0.22	0.22	0.24	0.30	0.36	0.34	0.54	0.80

changed to 0.93, as shown in Fig. 7B. This implies that 7b and 9b are composed of a double-stranded fragment and, therefore, the height of 7b decreased. This result indicates that at low temperatures, effective purification of such molecules is difficult. The peak of the double-stranded fragment could not be observed in this experiment. On increasing the temperature to 50°C, the change in height of 7b disappeared. As shown in Fig. 7C and D, the relative ratio was 0.73 for both the peak-height measurements. This strongly suggests that with single-stranded DNA fragments containing approximately ten bases, an elevated temperature is required to prevent the formation of double strands, which interferes with accurate measurements. Also, as there is a possibility that oligodeoxyribonucleotides may be hydrolysed at high temperatures, separation at about 50°C is reasonable.



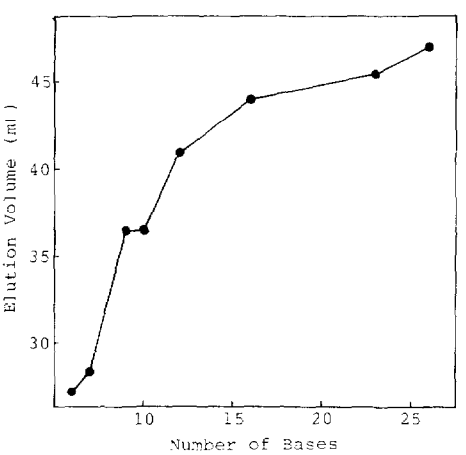
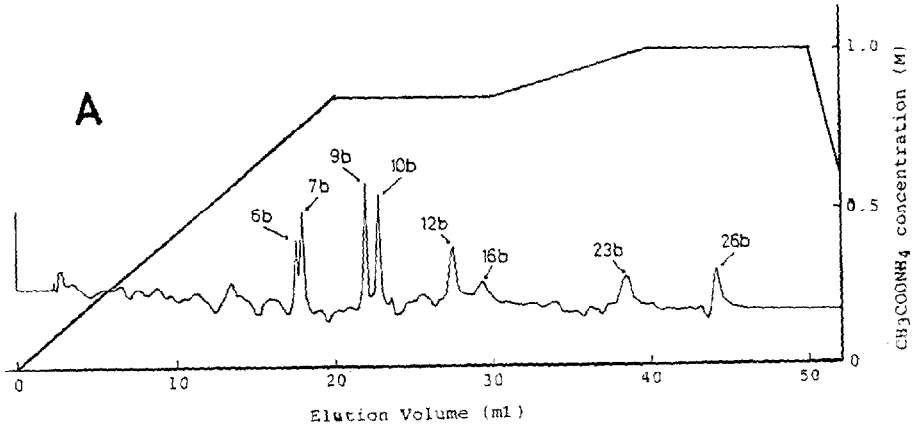


Fig. 7. Elution profiles of samples containing 7b (dTCAAATC) and 10b (dCGACCCGGGT). (A) and (C), obtained in the absence of 9b (dCGGATTTGA), which is complementary to 7b; (B) and (D), obtained in the presence of 9b. (A) and (B), measured at 16°C: (C) and (D), measured at 50°C. Other conditions as in Fig. 2.

Fig. 8. Relationship between the number of bases and the elution volumes of single-stranded oligodeoxyribonucleotides obtained at pH 5 by use of a linear gradient from 0.01 to 2.5 *M* ammonium acetate–acetic acid buffer (pH 5) in 75 min. Other conditions as in Fig. 1.

The effect of the pH of the eluent on retention was also studied. The chromatographic behaviour of the samples was found to vary when the pH of the eluent was changed. At pH 5, the retention became much higher than that observed at the pH of the eluent that contained only ammonium acetate (6.4–7.0). Therefore, a much higher concentration of the salt was required in order to achieve a similar retention of the solutes. In order to obtain the relationship represented in Fig. 8, a high final



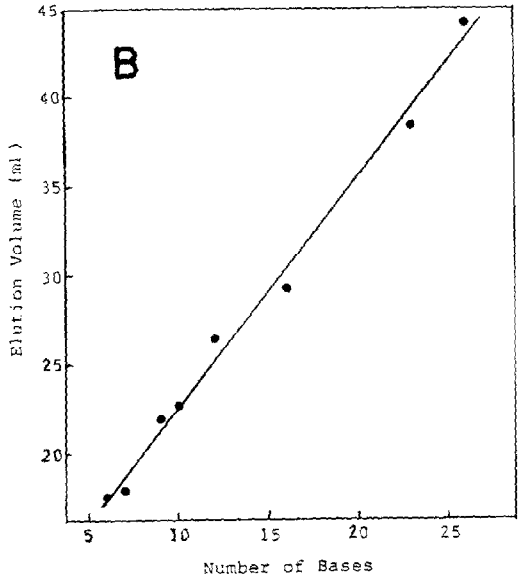


Fig. 9. (A) Separation of single-stranded oligodeoxyribonucleotides under the optimum gradient conditions shown. (B) Relationship between the number of bases and elution volumes of single-stranded oligodeoxyribonucleotides with the gradient system shown in (A). Other conditions as in Fig. 1.

concentration (*ca.* 2.0 *M*) was required for gradient elution, whereas at pH 7, 1 *M* was sufficient. This result implies that it is disadvantageous to lower the pH for the separation of such substrates. Also, a higher pH may lead to hydrolytic degradation of the samples. Consequently, a neutral pH was considered to be best for the separation of the substrates on DEAE-5PW.

Considering all the results, a gradient condition was designed so that a linear relationship between the number of bases and the elution position of the samples could be obtained. A typical separation obtained under optimum conditions is shown in Fig. 9A. The relationship between the elution volume and the number of bases in the samples obtained under the conditions used in this analysis is shown in Fig. 9B.

The separation of sequential isomers, the number of bases in which is the same, was performed in order to establish the extent of the deviation in retention between the isomers. The substrates were separated from each other but were eluted closely, as shown in Fig. 10. The observed separation was small in comparison with those for the samples containing a different number of bases. This indicates that the method used here is sufficient for the purification of single-stranded DNA fragments with different chain lengths and that it can even be used for the isolation of some sequential isomers.

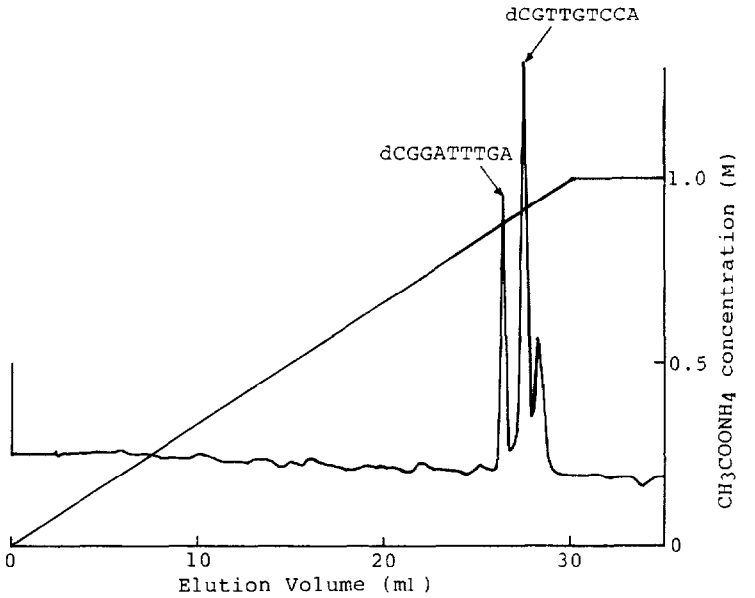


Fig. 10. Separation of sequential isomers containing nine bases. Conditions as in Fig. 2.

The results obtained suggest that anion-exchange chromatography with a DEAE-5PW column and eluents containing only volatile salts is highly efficient for the separation of single-stranded oligodeoxyribonucleotides with chain lengths up to *ca.* 26 base numbers.

REFERENCES

1 M. D. Matteucci and M. H. Caruthers, *J. Amer. Chem. Soc.*, 103 (1981) 3185.
2 E. Ohtsuka, M. Ikehara and D. Söll, *Nucleic Acids Res.*, 10 (1982) 6553.
3 L. J. McBride and M. H. Caruthers, *Tetrahedron Lett.*, 24 (1983) 254.
4 S. D. Putney, S. J. Benkovic and P. R. Schimmel, *Proc. Nat. Acad. Sci. U.S.*, 78 (1981) 7350.
5 D. F. Smith, P. F. Searle and J. G. Williams, *Nucleic Acids Res.*, 6 (1979) 487.
6 M. J. Gait and R. C. Sheppard, *Nucleic Acids Res.*, 4 (1977) 1135.
7 C. F. Crampton, F. R. Rankel, A. M. Benson and A. Wade, *Anal. Biochem.*, 1 (1960) 249.
8 A. M. Maxam and W. Gilbert, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 560.
9 H. Domdey and H. J. Gross, *Anal. Biochem.*, 98 (1979) 346.
10 E. Y. Chen and B. A. Roe, *Anal. Biochem.*, 89 (1978) 45.
11 J. D. Pearson and F. E. Regnier, *J. Chromatogr.*, 255 (1983) 137.
12 W. Haupt and A. Pingoud, *J. Chromatogr.*, 260 (1983) 419.
13 K. Itakura, N. Katagiri and S. A. Narang, *Can. J. Chem.*, 52 (1974) 3689.
14 E. Ohtsuka, Y. Taniyama, R. Marumoto, H. Sato, H. Hirosaki and M. Ikehara, *Nucleic Acids Res.*, 10 (1982) 2597.