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Use of Reverse Phase Ion Pair Chromatography to Fractionate and Purify DNA Fragments and Monomeric Components of RNA

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A study of the separation of oligonucleotides using reverse phase ion pair chromatography is reported. The work includes a comparison of several countercations (triethylammonium, tetraethylammonium, tetrabutylammonium and tetrapentylammonium) as possible candidates for the formation of ion pairs with oligonucleotides in order to obtain an optimum separation on a C_{18} reverse phase support, using high performance liquid chromatography. The effect of the countercation concentration has also been explored. It is shown that 0.0075 *M* tetrabutylammonium sulfate (pH 6.9) brings about a satisfactory separation up to a hexadecanucleotide DNA sequence. It was also found that the following mixtures are clearly resolvable into two groups using tetrapentylammonium phosphate (6.25×10^{-4} *M*) and a linear gradient of acetonitrile:

(1) Four ribonucleosides and their 2′,3′-cyclic phosphates;

(2) four ribonucleosides and their 5′-monophosphates;

(3) four ribonucleosides and their 2′- and 3′-monophosphates.

During the last few years the chemical synthesis of oligodeoxyribonucleotides by the solid phase methodology has almost become routine work especially with the introduction of automated machines.[1a] The introduction of a number of new, complementary protecting groups, more active condensing agents and improved methods for separation of intermediates has also led to spectacular development of solution phase syntheses of nucleic acids in general. At present most of the synthetic work on oligodeoxyribonucleotides is based on solid phase methods.[1] This gas greatly reduced the time necessary for the synthesis, but yielded a product which is highly contaminated by various truncated sequences resulting from inefficient coupling reactions. This has created an urgent need for a sensitive and efficient technique that could be used both for analytical and preparative separation of the desired oligonucleotide from the crude mixture.

It is a frequent observation that the anion exchange chromatography commonly used for the separation of oligonucleotides generally suffers from some of the following disadvantages:

(1) The relatively short life-time of ion-exchange columns, together with their high costs, makes their routine use very expensive.

(2) Low efficiency of separation, especially for higher oligomers.

(3) A high risk of depurination of chromatographed oligodeoxynucleotides due to the use of an acidic eluent for efficient separations.

(4) The high concentration of inorganic salts in the eluent causes a gradual corrosion of the stainless steel valves and the pump heads of the expensive HPLC equipment.

Reverse phase chromatography, particularly the approach which utilizes partially deprotected oligonucleotides, can be an alternative to ion-exchange chromatography.[2] The hydrophobic properties of a desired oligodeoxynucleotide, containing the highly lipophilic 4,4′-dimethoxytrityl- (DMTr) group at the 5′-position, is sufficiently different from all other compounds in the crude mixture to allow its separation. However,

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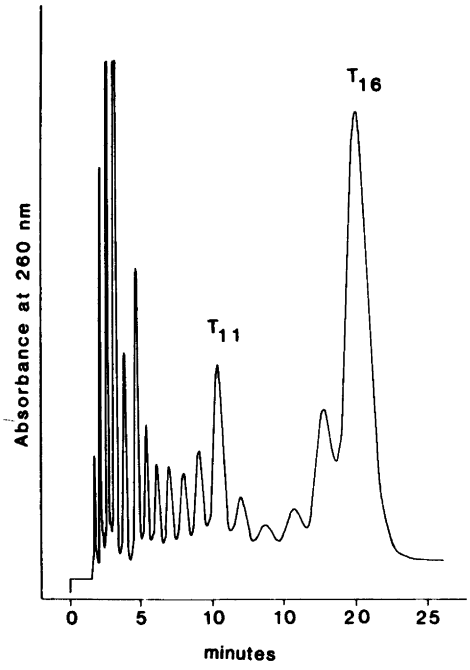


Figure 1a

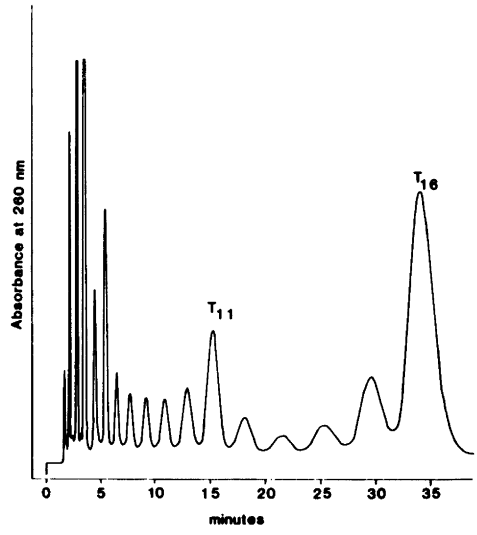


Figure 1b

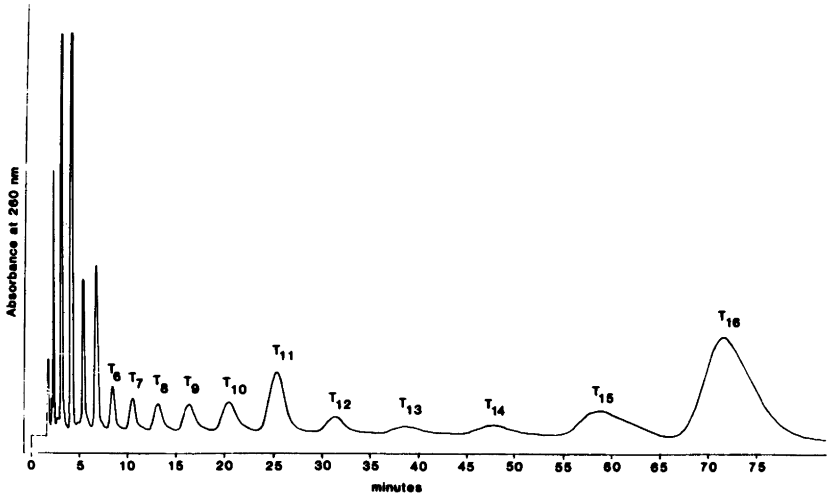


Figure 1c

Fig. 1. a. Linear gradient elution (30 min) of the T_{16} mixture with 0.1 M triethylammonium acetate (pH 7±0.1) containing 13 % acetonitrile in solvent A and 14 % acetonitrile in solvent B. *b.* Linear gradient elution (30 min) of the T_{16} mixture with 0.1 *M* triethylammonium acetate (pH 7±0.1) containing 12.5 % acetonitrile in solvent: A and 13.5 % acetonitrile in solvent: B. *c.* Linear gradient elution (30 min) of the T_{16} mixture with 0.1 *M* triethylammonium acetate (pH 7±0.1) containing 12 % acetonitrile in solvent: A and 13 % acetonitrile in solvent: B.

this method of final separation is clearly not feasible in combination with a strategy for the synthesis of oligoribonucleotides, which employs acid-labile groups for the protection of 2'- and 5'-hydroxyl, respectively, due to the possible degradation and migration of the 3'→5' phosphate linkages during the final acid treatment.

This report describes the use of reverse phase ion pair chromatography (RPIPC) as a method for the separation of mononucleotides and oligonucleotides both for analytical and preparative use. It should be pointed out that this method has been sometimes called just "reverse phase chromatography", although it is really *reverse phase ion-pair chromatography*. Ion pair chromatography (RPIPC)[4] is a very powerful tool for the separation of ionic solutes, but most work has dealt with monofunctional compounds. RPIPC has been used for separations of mono[7] and oligonucleotides[6] also. However, in only a few cases have crude synthetic, as opposed to artificial, mixtures of oligomers been studied. Earlier data[6a,6b] throws more light on the mechanism of ion pair chromatography of oligonucleotides. However, these data were considered insufficient for the preparative isolation of oligonucleotides. This report presents studies on the main variables that control the retention of oligonucleotides by ion pair chromatography on a C_{18} reverse phase support and also on the application of these systems to the solution of practical separation problems in nucleic acid chemistry. A new chromatographic system has also been investigated for the separation of a mixture of ribonucleosides and their monophosphates, formed during enzymatic and chemical digestions of oligoribonucleotides. Such analyses are the essential step in confirming the structure of synthetic RNA fragments. This system can also be used for determination of oligonucleotide chain length[5a] and for many other biochemical purposes.

RESULTS AND DISCUSSION

Effect of the countercation concentration on the separation. In the initial studies, triethylammonium acetate was chosen, firstly, because the earlier work[5f,6d,6e] did not specify optimum condition for separations and secondly, it is a volatile salt.

A crude synthetic mixture of oligothymidilic acids was first examined using 0.1 M triethylammonium acetate. The use of an additional buffer was avoided for the following reasons: (1) any extra salt would invalidate the idea of a volatile buffer in preparative runs; (2) a small change in the pH of the eluent, (pH 7±0.1), would have a very small effect when the difference in the first pK_a of the phosphoric acid (pK_a *ca.* 1) with that of the mobile phase is considered. The pH of the solution remained unchanged in a stoppered flask over a period of three weeks at room temperature.

The advantages of using the crude mixture of oligothymidylic acid (from mono to hexadecanucleotides) generated by the solid phase DNA synthesis methodology,[1] were firstly, that it contained a well characterized mixture of substrates to be separated and secondly, that the problem of secondary effects in the elution properties due to base-pairing was not possible. Fig. 1 demonstrates the importance of the nature of the gradients that have been used for separations. It has been observed that a small change in acetonitrile concentration in the mobile phase

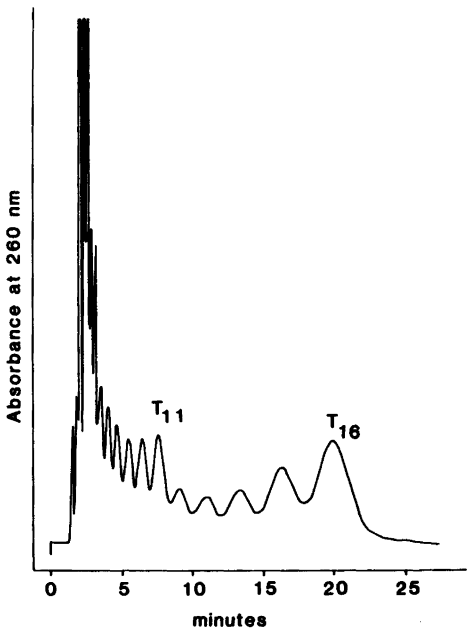


Fig. 2. Isocratic elution of the T_{16} mixture with 0.1 *M* triethylammonium acetate (pH 7±0.1) containing 13.5 % acetonitrile.

$Et_3\overset{+}{N}H\ OAc^-$ (mol/l).

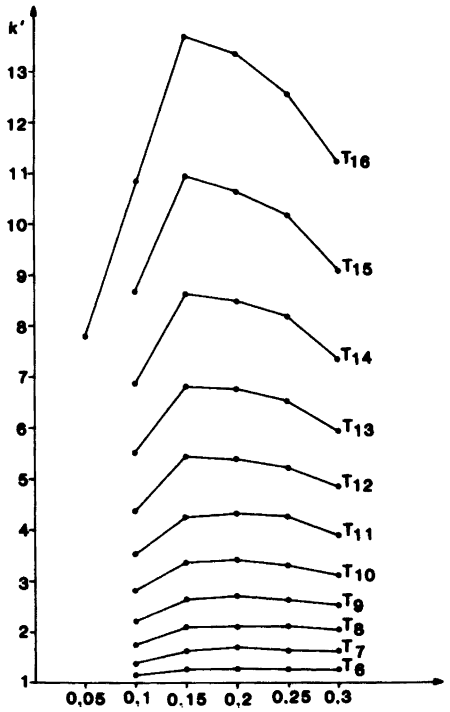


Fig. 3. A plot of the capacity factor (k') of oligothymidylic acids (T_6 to T_{16}) against different concentrations of triethylammonium acetate (pH 7±0.1) with a constant acetonitrile concentration (13.5 % v/v) showing the optimum retention at 0.15 *M* salt concentration.

may lead to a loss of efficiency of separation of oligonucleotides (Fig. 1). It was also found that an isocratic elution with 13.5 % of acetonitrile gave a satisfactory separation of different compounds in the crude T_{16} mixture (Fig. 2). The effect of the concentration of the triethylammonium acetate, under isocratic conditions with 13.5 % acetonitrile was then investigated to optimize the separation of each compound in the T_{16} mixture. The variations of the ionic strength were not corrected due to the reasons already mentioned. Fig. 3 shows that the optimum retention can be achieved with 0.15 M, although, a satisfactory separation is obtained within the concentration range 0.1 to 0.3 M triethylammonium acetate. It should be added that the separation of the T_{16} mixture was not possible using 0.01 *M* triethylammonium acetate irrespective of the acetonitrile concentration in the mobile phase or the range and type of gradient used. This is clearly in contrast to the results reported earlier by MacLaughlin.[6e]

Comparison of various countercations in the mobile phase. A concentration of 0.05 M was chosen for these studies, because this was the lowest concentration that was effective with triethylammonium acetate. The retention time of hexadecathymidilic acid was maintained by varying the amount of acetonitrile in the mobile phase under an isocratic condition. Due to the large differences in hydrophobicity of the various countercations it was not possible to maintain a constant acetonitrile concentration, along with a constant concentration of various countercations (0.05 M) even in the case of two homologues, *e.g.* triethylammonium- and tripropylammonium acetate. The data are, therefore, presented in the form of a plot (Fig. 4) denoting the type of the

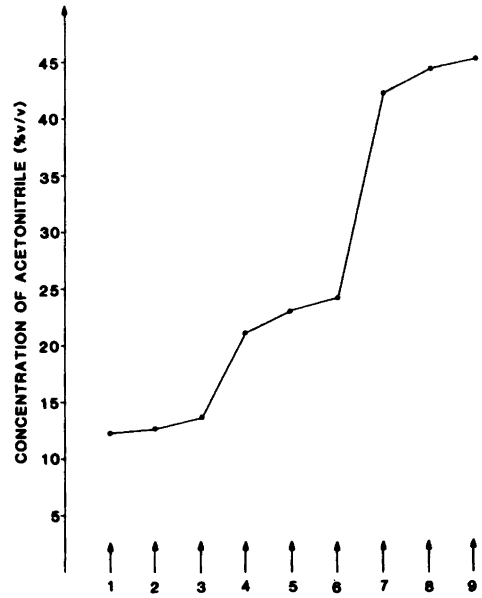


Fig. 4. Concentration of acetonitrile used to obtain a capacity factor (*k'*) of 19 for hexathymidylic acid with different countercation in the mobile phase showing the effect of the hydrophobicities of various ion-pairs: 1 (triethylammonium acetate); 2 (tetraethylammonium phosphate); 3 (tetraethylammonium acetate); 4 tripropylammonium acetate); 5 tetrapropylammonium phosphate); 6 (tetrapropylammonium acetate); 7 (tributylammonium acetate); 8 (tetrabutylammonium phosphate); 9 (tetrabutylammonium acetate).

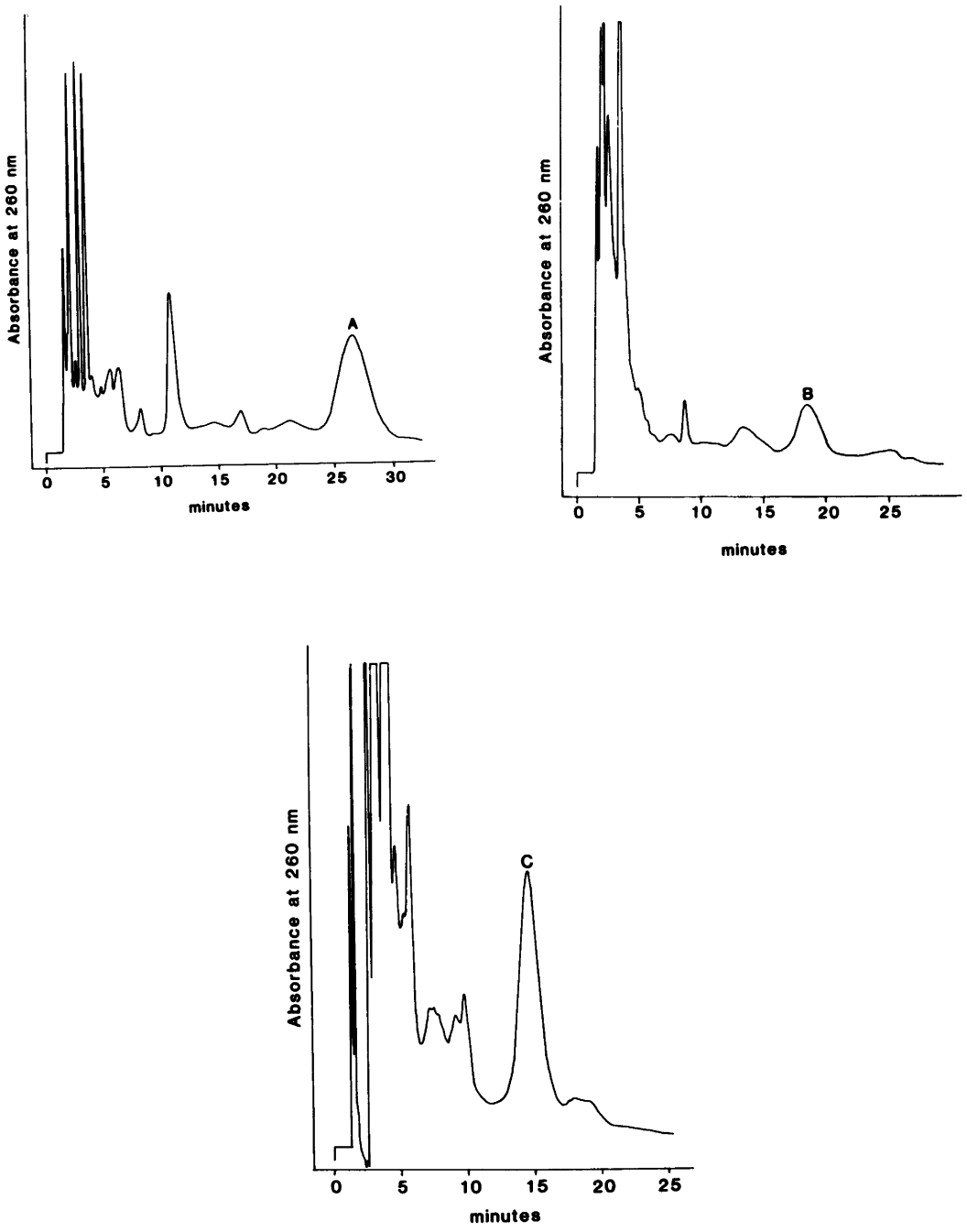


Fig. 5. a. The purification of $^{5'}$d(TCAATCAATCA)$^{32'}$ (peak: A) from a crude mixture using 0.1 *M* triethylammonium acetate (pH 7±0.1) containing 11.5 % acetonitrile. b. The purification of $^{5'}$d(AATTCCTGGTCCA)$^{3'}$ (peak: B) from a crude mixture using 0.1 *M* triethylammonium acetate (pH 7±0.1) containing 12 % acetonitrile. c. The purification of $^{5'}$d(ACACAAAGAC)$^{3'}$ (peak: C) from a crude mixture using 0.3 *M* triethylammonium acetate (pH 7±0.1) containing 12 % acetonitrile.

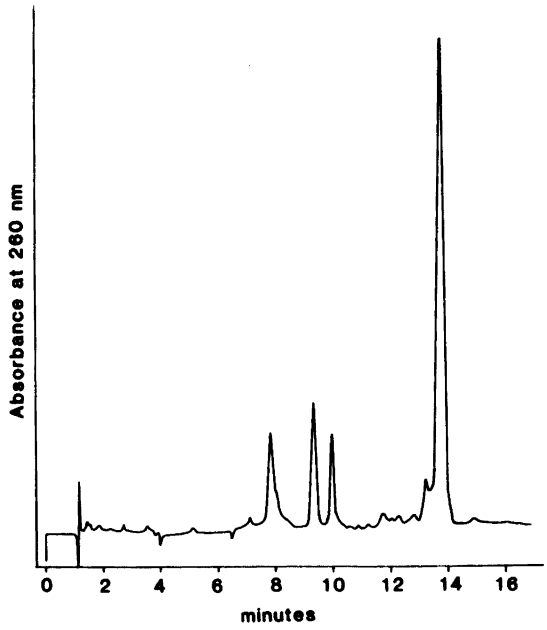


Fig. 6. The linear gradient elution (20 min) of the crude octathymidylic acid with 0.005 M tetrabutylammonium sulfate (pH 7±0.1) containing 0 % acetonitrile in solvent: A and 75 % acetonitrile in solvent: B showing coelutions of most of the components of the T_8 mixture under the main peak (as was clearly evident by ^{32}P-labelling, gel electrophoresis followed by autoradiography).

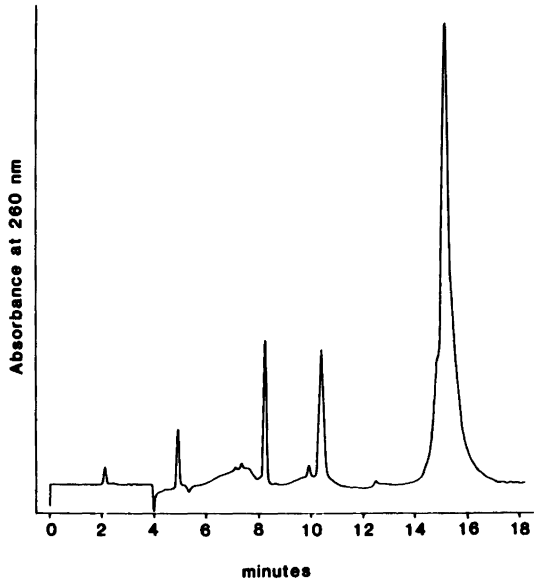


Fig. 7. The linear gradient elution (20 min) of the T_{16} mixture with 0.005 M tetrabutylammonium sulfate (pH 7±0.1) containing 0 % acetonitrile in solvent: A and 75 % acetonitrile in solvent B showing the coelutions of most of the components of the T_{16} mixture (compare with Fig. 9).

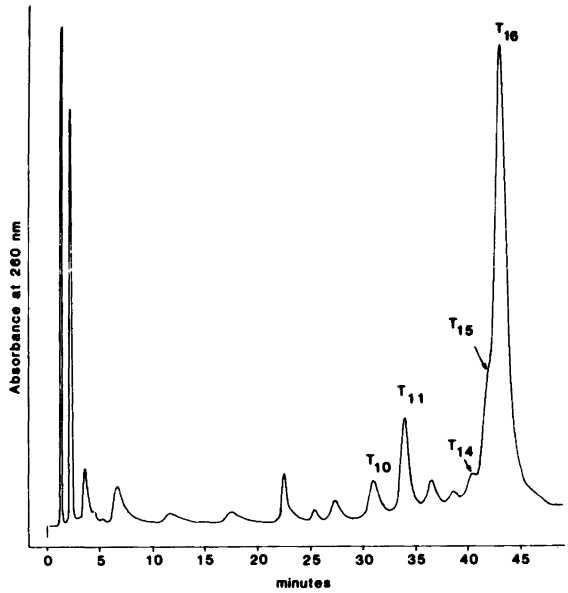


Fig. 8. The linear gradient elution (40 min) of the T_{16} mixture with 0.0075 M tetrabutyl ammonium sulfate (pH 7±0.1) containing 30 % acetonitrile in solvent: A and 42 % acetonitrile in solvent: B showing the coelutions of the final and penultimate peaks despite a lower concentration of acetonitrile in the final eluting media (solvent: B) than the condition which is shown in Fig. 9 for an optimum separation of the components of the T_{16} mixture.

countercation versus the acetonitrile concentration under an isoeluting condition. The plot shows that the retention time of the hexadecathymidylic acid is dependent on the alkyl chain length of the countercation and not on the total number of carbon atoms. The isoeluting concentration of acetonitrile is approximately the same for both tributylammonium acetate and tetra-

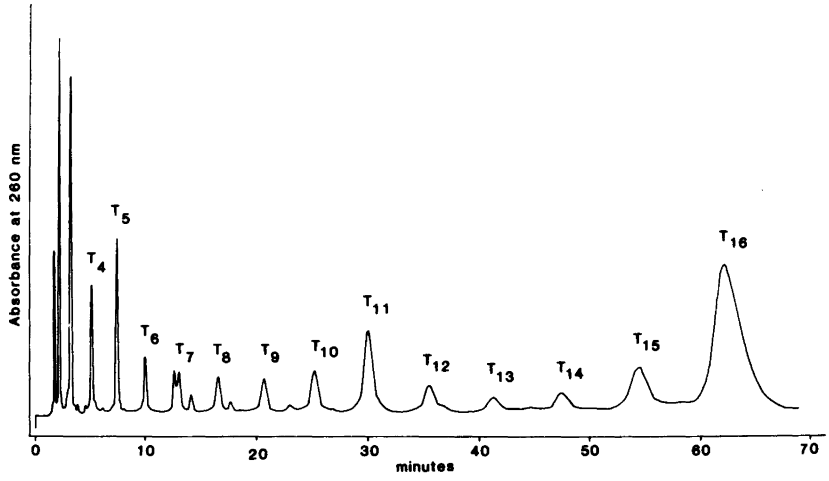
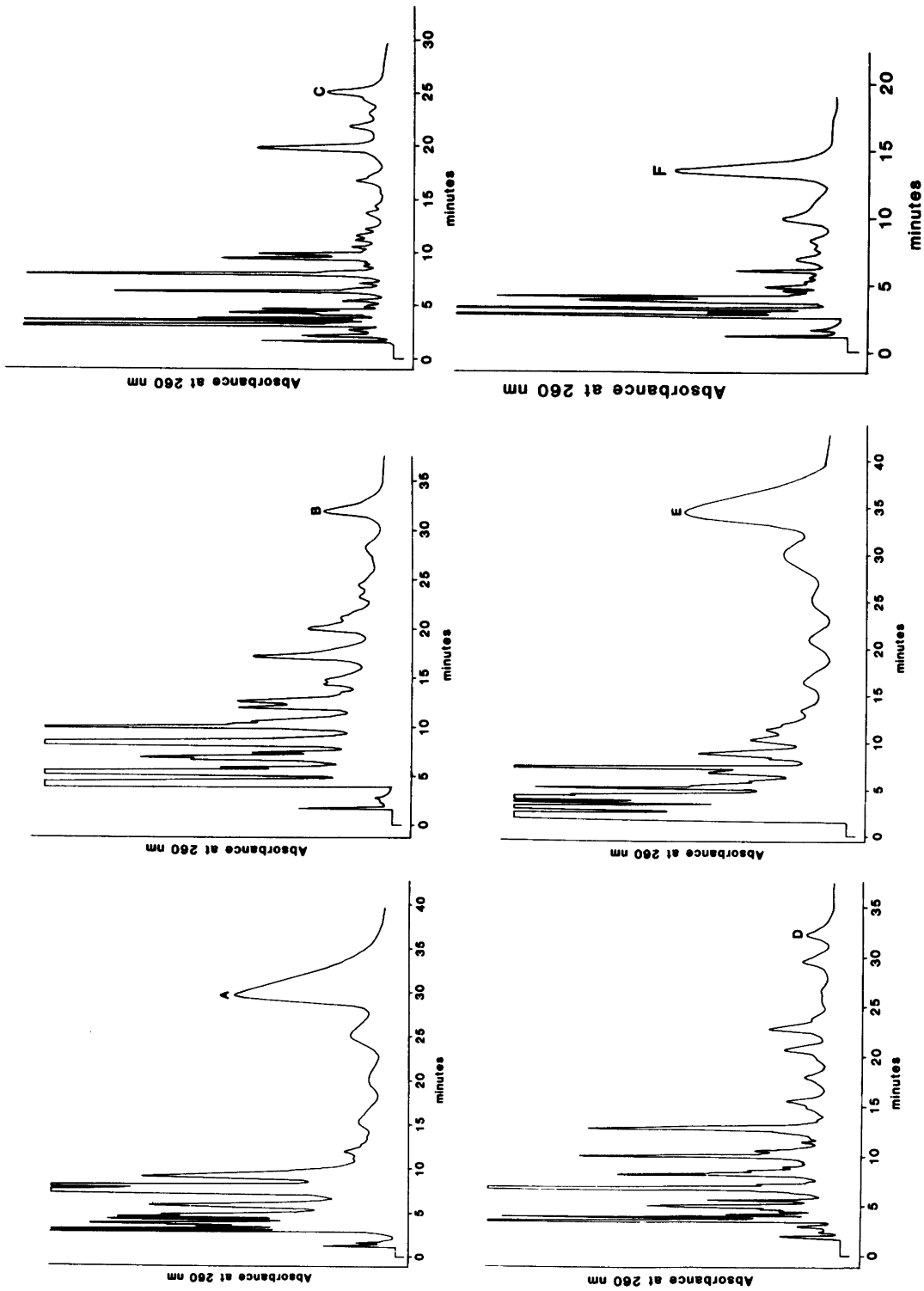


Fig. 9. A parabolic gradient (M=0.2; for exact instrument condition, see LDC Gradient Master model 1601) elution (40 min) of the T_{16} mixture with 0.0075 *M* tetrabutylammonium sulfate (pH 7±0.1) containing 35 % acetonitrile in solvent: A and 45 % in solvent: B.


Absorbance at 260 nm
minutes
A
B
C
D
E
F

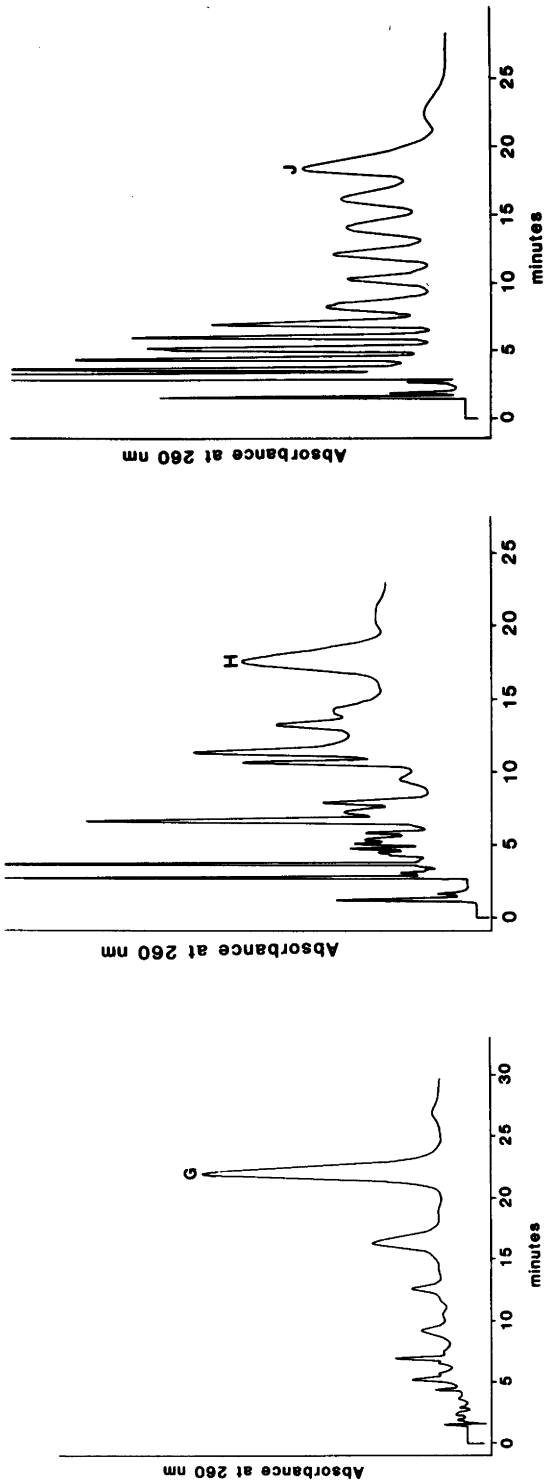


Fig. 10. The purification of DNA molecules from crude mixtures using 0.0075 M tetrabutylammonium sulfate (pH 7±0.1) using a parabolic gradient (M=0.2, LDC Gradient Master, model 1601) for 40 min for separations except for the elution profile (Fig. 10 J) which was for 30 min. The specific purified sequences of DNA and the range of acetonitrile gradients that was used for separations are as follows:
a. (Peak A): $^{5'}$d(AATTCCTGGTCCA)$^{3'}$; gradient 30–36 %. b. (Peak B): $^{5'}$d(TGCGACCCAGACTC)$^{3'}$; gradient: 35–45 %.
c. (Peak C): $^{5'}$d(GCTTTCCAAGCTC)$^{3'}$; gradient: 35–45 %. d. (Peak D): $^{5'}$d(CTCGATGTGCATGCA)$^{3'}$; gradient: 35–45 %.
e. (Peak E): $^{5'}$d(GTAGTCCATCCACCA)$^{3'}$; gradient: 30–37 %. f. (Peak F): $^{5'}$d(TCAATCAATCA)$^{3'}$; gradient: 30–40 %.
g. (Peak G): $^{5'}$d(AAAAAAAA)$^{3'}$; gradient: 26–36 %. h. (Peak H): $^{5'}$d(ATGGGTTTCTTCGC)$^{3'}$; gradient 30–40 %.
j. (Peak J): $^{5'}$(TGGATGCACGATGC)$^{3'}$; gradient: 35–45 %.

butylammonium acetate, but is different from that required for tetrapropylammonium acetate. The nature of the anion in the ion pairing reagent has no real effect on the concentration of acetonitrile required, as can be seen from a comparison of the results for acetates and phosphates (Fig. 4).

Application of 0.1 M triethylammonium acetate to the separation of oligonucleotide mixtures. The above system was then employed for the separation of the pure DNA sequence from the crude synthetic mixtures[5f] containing other truncated chains as shown in Fig. 5. During these studies it became clear that hydrophobic interactions of the ion paired oligonucleotide with the support would be dependent both on the hydrophobicity of the countercation and that of the bases. Thus, a mobile phase comprised of a smaller countercation like the triethylammonium ion, would be very sensitive to the actual base composition of the DNA sequence to be purified. The effect is particularly significant in cases where the sequence contains a large number of guanine bases, when the oligomer was eluted almost in the void.

Application of tetrabutylammonium (TBA) sulfate for the separation of oligodeoxyribonucleotides. It was anticipated that the contribution of the hydrophobicity from the bases would become negligible when compared to that from an appropriate countercation of a certain chain length, allowing the separation by charge only. This was observed when 0.0075 *M* TBA sulfate was used in the mobile phase.

It has also emerged that the acetonitrile concentration in the eluting mixture and the gradient time should be carefully chosen as is evident from comparison of the elution profiles of the octathymidylic and the hexadecathymidylic acid mixtures (Figs. 6 and 7 respectively). The difference in acetonitrile concentrations in the initial and the final mobile phase should not exceed 10 % and the gradient should be run preferably in a non-linear fashion as shown by an optimum separation of the T_{16} mixture (Fig. 9). A poor separation of the same T_{16} mixture by a linear gradient of acetonitrile (30–42 %) (Fig. 8) further clarifies the need of a non-linear gradient. The type of the gradient is important especially for a complete separation between the final and the penultimate peaks. Figs. 10A–J are elution profiles which demonstrate the separations of desired DNA sequences (8–16 units long) from the respective crude synthetic mixtures, using 0.0075 *M* TBA sulfate in the mobile phase. The products were homogeneous as shown by gel electrophoresis of the ^{32}P-labelled material followed by autoradiography, after the removal of the TBA sulfate by dialysis through acetylated dialysis bags. The use of tetrapentylammonium (TPeA) phosphate gave no improvement over TBA sulfate.

Separation of ribonucleosides and monoribonucleotides monophosphates by RPIPC. Anion exchange chromatography[10] is the most commonly used method for the separation of mononucleotides; however, the separation has also been achieved by RPIPC.[5] Crowther *et al.*[5a] have separated nucleosides and mononucleotides (both in ribo- and deoxyriboseries) using an isocratic elution system containing 2 mM TBA phosphate, buffered to pH 4.9. However, the order of the elution of these monomeric components did not follow any regular pattern. In addition good resolution could only be achieved by using carefully controlled conditions. We anticipated that a more hydrophobic countercation might be able to perform a group separation in such a way that the nucleosides, mononucleotides and cyclic monophosphates eluate as well separated groups. It was desired that the above group separation was well resolved and relatively insensitive to the small changes of countercation concentration, pH, temperature and the flow rate, enabling us to adopt the technique as a routine analytical method.

A solution of tetrapentylammonium phosphate (6.25×10^{-4} M) in 0.06 M NaH_2PO_4 (pH 7±0.1) was thus employed for a linear gradient elution of the mixture which made the system adaptable for the simultaneous separation of higher charged mononucleotide diphosphates and the triphosphates.

The results of our experiments are summarised in Figs. 11, 12 and 13. It should be added that during these efforts, no complete group separation was possible for 2′,3′-cyclic monophosphates and 5′-monophosphates, since 2′,3′-cyclic AMP co-eluates with the 5′-CMP. It is presumed that an additional increase of the lipophilicity of the countercation (*e.g.* tetrahexylammonium (THA) phosphate) may resolve this problem.

We have subsequently used[3b] the present chromatographic systems successfully for the analyses of the purities of two synthetic RNA

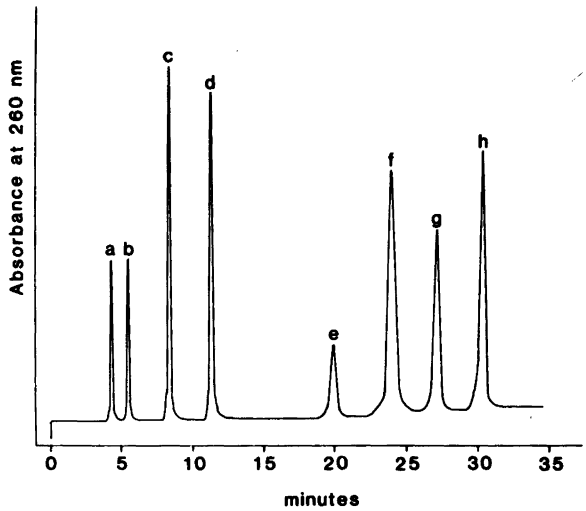


Fig. 11. Analytical separation of four common ribonucleosides and their 5′-phosphates using 6.25 m*M* tetrapentylammonium (TPeA) phosphate (pH 7±0.1) using a linear gradient of acetonitrile: 0–20 % (30 min) a: cytidine; b: uridine; c: guanosine; d: adenosine; e. 5′-CMP; f: 5′-UMP; g: 5′-GMP; h: 5′-AMP.

fragments: CCC and AUAA and their spleen phosphodiesterase and 0.1 *M* NaOH digestion products to establish the general applicability of our procedures.

EXPERIMENTAL

Instrumentation. The high performance liquid chromatograph (HPLC) was an LDC type consisting of two LDC pumps (model III), spectromonitor III with variable wavelength detector, a

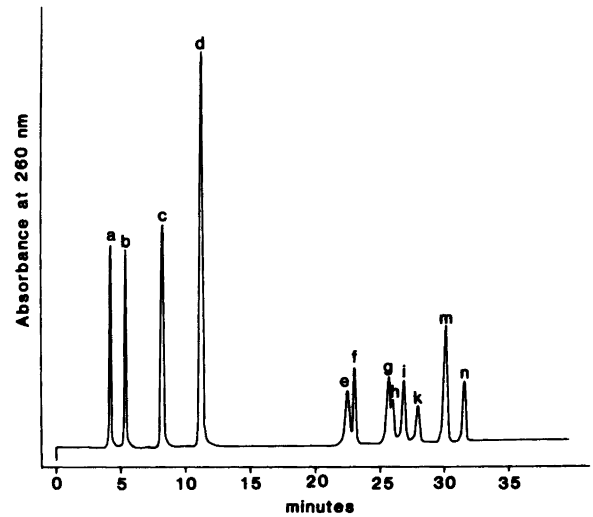


Fig. 12. Analytical separation of four common ribonucleosides and their 2′- and 3′- phosphates with 6.25 m*M* TPeA phosphate (pH 7±0.1) using a linear gradient of acetonitrile: 0–20 % (30 min). a: cytidine; b: uridine: c: guanosine; d: adenosine; e: 3′-CMP; f: 2′-CMP; g: 3′-UMP; h: 2′-UMP; i: 3′-GMP; k: 2′-GMP; m: 3′-AMP; n: 2′-AMP.

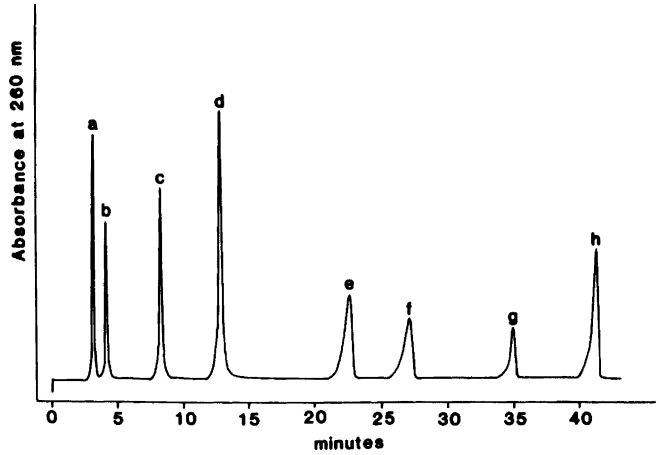


Fig. 13. Analytical separation of four common ribonucleosides and their 2',3'-cyclic phosphates using 3.125 m*M* TPeA phosphate (pH 7±0.1) using a linear gradient of acetonitrile: 0–20 % (30 min) a: cytidine; b: uridine; c: guanosine; d: adenosine; e: 2',3'-cCMP; f: 2',3'-cUMP; g: 2',3'-cGMP; h: 2'3'-cAMP.

gradient controller (Gradient Master 1601) and Rheodyne 7125 injection valve. In all experiments a fixed 260 nm wavelength was used for detection. The data were electronically integrated using a Hewlett-Packard 3390A electronic integrator.

Columns. The separation of oligonucleotides was performed on 250×4.6 mm columns packed with Spherisorb ODS (10 μ). A column of the same size, but packed with Nucleosil C_{18} (5 μ) was used for the separation of ribonucleosides and ribomononucleotide monophosphates. In each case a guard column 50×4.6 mm, packed with the same material was employed to protect the analytical column.

Chemicals. Triethylamine (*purum*, Fluka), Tripropylamine (*purum*, Fluka) and Tributylamine (synthesis grade, Merck) were distilled and immediately neutralized in a water suspension with analytical grade acetic acid to obtain stock solutions of 1.0, 0.25 and 0.25 M concentrations, respectively. Tetraethylammonium iodide (*puriss.*, Fluka), Tetrapropylammonium bromide (Kodak), Tetrabutylammonium chloride (*purum*, Fluka), Tetrabutylammonium hydrogen sulfate (*puriss.*, Fluka) were used for the preparation of the different quaternary ion-pair reagents:

(i) the aqueous solution of Tetrabutylammonium hydrogen sulfate (TBA HSO_4) were neutralized with 0.1 M NaOH to pH 7±0.1

(ii) all other solutions of the quaternary ammonium acetates or phosphates were prepared by converting quaternary halides to their hydroxides with silver oxide[11] followed by neutralization with acetic or phosphoric acid to pH 7±0.1.

The concentration of the quaternary salts was measured by the picrate method. All solutions were made with double distilled water, the acetonitrile used for solvent preparation was of HPLC grade (Merck).

Samples. The various oligodeoxyribonucleotides were prepared in our laboratory *via* the phosphite-triester methodology.[1] 5'-*O*-dimethoxytrityl (DMTr)-3'-phosphoromorpholidites[1] were used, and were activated by tetrazole during the actual condensation reactions. As a prevention against failure sequences a capping step was also included as has been described in the literature.[1b] Finally, the DNA sequences were deprotected in the following manner: (1) thiophenolate treatment for 1 h; (2) treatment with aq.NH_3 (d 0.9) at 50° overnight; (3) removal of the 5'-DMTr group with 80 % aqueous acetic acid. The crude mixtures were then used for the chromatographic studies without any further purifications.

The nucleosides, and all mononucleotides used in the second part of this work were purchased from Sigma Chemical Company.

Mobile phases and chromatographic conditions. The various mobile phases were prepared on a volume percent basis, and were well degassed before use. The aqueous solvents were filtered through 0.45 μm filters. Both gradient and isocratic elutions have been used for the separation of oligonucleotides. In each case the amounts of acetonitrile in the mobile phase, and the range of gradient were dependent on the type of the countercation used for the separation. In

the comparative studies with all tertiary ammonium acetates and quaternary ammonium acetates and phosphates, we used a uniform concentration of 0.05 M of the solute. In the concentration dependent studies, using triethylammonium acetate a range from 0.05 to 0.3 M at pH 7±0.1, together with a fixed concentration of acetonitrile, 13.5 % v/v, was used.

TBA sulfate 0.0075 M pH 7±0.1 has also been used as the mobile phase for oligonucleotide separations. No attempts have been made to correct the changes of ionic strength during the concentration studies. No additional buffering salts were added into any of the chromatographic solvents used for the oligonucleotide separation.

The system designed for the separation of the mononucleotides contained 6.25 mM tetrapentylammonium (TPeA) phosphate, buffered with NaH_2PO_4, 0.06 M, pH 7±0.1, and the separations have been made using a linear gradient elution from 0 to 20 % acetonitrile in 30 min.

The flow rate in all experiments was 2.0 ml/min, and all separations were carried out at ambient temperature (20 °C±1°).

Acetylated dialysis bag. Dialysis bag (Sigma, Inc.) was placed in 100 % acetic anhydride in a stoppered flask at 20 °C for 16 h. The bags were then extensively washed with distilled water. They were then soaked overnight in 30 % aq. glycerol at 20 °C. The bags, thus treated, were transferred in a chamber containing 10 % glycerol and stored at +4 °C. Such peracetylated bags can retain from −3 charged phosphate residue selectively over the salts.[9]

Characterization of the peaks. The structure of eluted oligodeoxyribonucleotides have been confirmed by ^{32}P-labelling at the 5'-hydroxyl terminal of the oligodeoxyribonucleotide with ^{32}P-γ-ATP and kinase,[8] followed by the electrophoresis on a 20 % polyacrylamide gel along with the authentic markers and then visualized by the autoradiography.

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