The Behaviour of Oligodeoxynucleotides on Thin-Layer Chromatography on Polyethyleneimine-Cellulose and Ion-Exchange Paper Electrophoresis

APPLICATIONS IN FRACTIONATING AND SEQUENCING TERMINALLY LABELLED OLIGODEOXYNUCLEOTIDES

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The electrophoretic behaviour of oligodeoxynucleotides on ion-exchange papers was studied systematically with particular attention to applications in sequence analysis. Complex mixtures of larger oligonucleotides were fractionated by electrophoresis on cellulose acetate followed by t.l.c. on polyethyleneimine-cellulose. The behaviour of the oligonucleotides on polyethyleneimine-cellulose and on the two-dimensional system can provide a useful guide to their sequence. Detailed results from some of these experiments have been deposited as Supplementary Publication SUP 50031 (13 pages) at the British Library (Lending Division) (formerly the National Lending Library for Science and Technology), Boston Spa, Yorks. LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1973), 131, 5.

Terminal-labelling methods have been used to introduce radioactive atoms of very high specific radioactivity into precise regions of DNA to facilitate their isolation and sequence determination. Examples include sequences at the termini of bacteriophage DNA (Murray & Murray, 1973; Weigel *et al.*, 1973) and sites of cleavage of DNA by restriction enzymes (Kelly & Smith, 1970; Hedgpeth *et al.*, 1972; Bigger *et al.*, 1973). Progress in this area depended on the methods available for fractionating and sequencing oligodeoxynucleotides.

Electrophoresis on DEAE-cellulose paper was used in separation and sequencing of micro-quantities of uniformly ^{32}P -labelled oligoribonucleotides (Sanger *et al.*, 1965). Small oligoribonucleotides were partially digested with exonuclease and the resulting series of products was fractionated by DEAE-cellulose paper electrophoresis. The composition of these partial digestion products could be found by digesting them completely with exonuclease or alkali, and hence the sequence of the parent oligonucleotide could be deduced. But it was also found that the sequence of an oligonucleotide could be deduced from the relative positions of its partial digestion products on DEAE-cellulose paper, since the relative change in mobility of an oligonucleotide on addition (or subtraction) of a mononucleotide (i.e. M value) was characteristic of that mononucleotide.

For a terminally labelled oligonucleotide, deduction of its sequence from M values is the only method possible, because the composition of its partial digestion products cannot be ascertained by complete

digestion with exonuclease as with a uniformly labelled oligonucleotide.

The behaviour of oligodeoxynucleotides on DEAE-cellulose paper is similar to that of equivalent oligoribonucleotides (Murray & Offord, 1966; Murray, 1970), but the unambiguous deduction of the sequence of a terminally labelled oligonucleotide from M values is not possible, because G and T residues cannot be distinguished in this system. However, electrophoresis on AE (aminoethyl)-cellulose paper at pH 3.5 (Murray, 1973) distinguishes the M -value of G from that of T, and the two systems together provide the information to deduce the sequence of a terminally labelled oligonucleotide without ambiguity.

[Electrophoresis of partial digests on DEAE-cellulose paper alone is sufficient to determine the sequence of oligoribonucleotides derived from digestion of RNA with RNAase (ribonuclease) T_1 or pancreatic RNAase, because the base specificities of these endonucleases ensure that the oligonucleotides contain only one G residue or one pyrimidine respectively, which is 3'-terminal in each case].

These methods, however, are limited to relatively small oligonucleotides (five to ten residues long, depending on composition). Larger oligoribonucleotides have been fractionated by t.l.c. on polyethyleneimine-cellulose (Southern & Mitchell, 1971; Griffin, 1971) and by 'homochromatography' on thin layers of DEAE-cellulose (Brownlee & Sanger, 1969; Ling, 1972). We have explored other fractionation conditions with ion-exchange papers by varying the pH

and molarity of the electrophoresis buffers, to see whether the size limits of nucleotide which these systems can fractionate could be improved, or if one single system could replace the two systems, while still retaining unambiguous *M* values for each mononucleotide. Also, following the work of Southern & Mitchell (1971), we have examined the use of t.l.c. on polyethyleneimine-cellulose for fractionating oligodeoxynucleotides and deducing their sequences from *M* values.

In evaluating fractionation systems, it is necessary to have nucleotides of suitable size and known sequence. Nuclease digests of DNA have usually been used previously, but their complexity causes difficulties. We have used the homologous oligodeoxynucleotide series oligo(dT), oligo(dA), oligo(dC), and oligo(dG) as model nucleotides.

Abbreviations

Since only deoxynucleotides are discussed in this paper, A is used for deoxyadenylic acid, G for deoxyguanylic acid etc. All the nucleotides referred to in the text, Plates, Figures and Tables have a 5'-phosphate, unless stated otherwise, which has been omitted throughout.

Materials and Methods

Materials

Cellulose acetate strips were from Oxo Ltd., London E.C.4, U.K.; ion-exchange papers (AE-81 and DE-81) were Whatman Chromedia, Whatman Biochemicals, Maidstone, Kent, U.K.; polyethyleneimine-cellulose layers (Polygram CEL 300 PEI), manufactured by Macherey Nagel and Co., Düren, Germany, were obtained through Camlab (Cambridge) Ltd., Cambridge, U.K., and were washed by ascending chromatography in the following series of solvents: 1.5M-formic acid adjusted to pH3.7 with pyridine (two washes if necessary), methanol (to improve flow rates) and water. The dye markers Xylene Cyanol FF (blue), Acid Fuchsin (red), Orange G (yellow) were from G. R. Gurr Ltd., London S.W.6, U.K.

Pancreatic deoxyribonuclease I, snake venom phosphodiesterase and bacterial alkaline phosphatase were from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; polynucleotide kinase was prepared from *Escherichia coli* infected with bacteriophage T₄ by the procedure of Richardson (1965), with the modification of Wu & Kaiser (1968), as described by Murray (1973).

[γ -³²P]ATP was from The Radiochemical Centre, Amersham, Bucks., U.K. The copolymers poly(dA)·poly(dT) and poly(dG)·poly(dC) were from Miles Seravac (Pty) Ltd., Maidenhead, Berks., U.K.

Dinucleoside monophosphates were from Sigma Ltd., Norbiton Station Yard, Kingston-upon-Thames, Surrey, U.K.

Methods

Details of electrophoresis, radioautography and micro techniques for nucleotide analysis are as described by Sanger *et al.* (1965) and Murray (1970). Mobilities of compounds are given with respect to the blue dye (xylene cyanol ff) unless otherwise stated. Polyethyleneimine-cellulose chromatography was largely as described by Randerath & Randerath (1967) and Southern & Mitchell (1971).

Preparation of terminally labelled homo-oligodeoxynucleotides. Poly(dA)·poly(dT) and poly(dG)·poly(dC) were dissolved in 20mM-Tris-HCl (pH7.5)-1mM-MgCl₂, and dialysed against the same buffer.

The copolymers were partially digested with deoxyribonuclease I (Bollum, 1965). Reaction mixtures containing 0.1-0.4mg of copolymer/ml, 0.1-10 μ g of deoxyribonuclease I/ml, 5mM-MgCl₂ and 20mM-Tris-HCl, pH7.5, in a total volume of 20 μ l were incubated for 10-30min at 37°C in a capillary tube. The terminal 5'-phosphates of the digestion products were removed with phosphatase: 5 μ l of a solution containing 50mM-EDTA and 0.2mg of bacterial alkaline phosphatase/ml, was added and the mixture incubated for 1h at 37°C. The phosphatase was removed by absorption on to phosphocellulose paper (Szekely & Sanger, 1969). The phosphocellulose paper was pre-washed in dilute NH₃ solution and rinsed in water. The copolymer digests were applied to phosphocellulose paper pennants, which were allowed to dry and eluted with water into a capillary tube. The eluates were evaporated to dryness on polythene in a vacuum desiccator.

Polynucleotide kinase was used to transfer [³²P]-phosphate groups to the 5'-termini of the oligonucleotides (Richardson, 1965). The dephosphorylated oligonucleotides were dissolved in 20 μ l of a solution containing 0.1M-Tris-HCl, pH7.5, 10mM-MgCl₂, 20mM-2-mercaptoethanol, 0.5 unit of polynucleotide kinase (Richardson, 1965) and 0.1mM-[γ -³²P]ATP (specific radioactivity approx. 14Ci/mmol) and incubated for 30min at 37°C.

The labelled copolymer digests, prepared as described above, were used in most experiments. Electrophoresis on cellulose acetate (pH3.5, 7M-urea) in the first dimension, followed by paper electrophoresis or t.l.c. on polyethyleneimine-cellulose, separated the homo-oligodeoxynucleotide series derived from either copolymer.

Sometimes it was necessary to purify the homo-oligomers from undigested polynucleotides and low-molecular-weight contaminants such as ATP and P_i. The digests were first desalted by applying them to

DEAE-cellulose paper pennants [which had been washed previously in 30% (w/v) triethylamine carbonate] washing with water, and eluting with 30% triethylamine carbonate. The triethylamine carbonate was removed by evaporation in a vacuum desiccator, and the nucleotides were applied in a volume of about 3 μ l, as a 0.5cm streak, to cellulose-acetate strips and electrophoresed (pH3.5, 7M-urea; 100V/cm, 10min). The nucleotides were transferred to polyethyleneimine-cellulose layers which were developed in pyridine-formate buffers, pH3.7, the thin layer being transferred during chromatography to solvents of progressively increasing concentrations of formic acid as follows: for poly(dG)·poly(dC) digests, 0.75, 1.5, 2.5 and 3.0M; for poly(dA)·poly(dT) digests, 1.2, 1.5, 2.5, and 3.0M. Each homo-oligodeoxynucleotide series was eluted.

Preparation of di-deoxyribonucleoside monophosphates. A solution (10 μ l) containing 0.2 μ g of uniformly 32 P-labelled bacteriophage lambda DNA (7.5×10^5 c.p.m.), 8mM-MnCl₂ and 1mg of deoxyribonuclease I/ml was incubated at 37°C for 1h and fractionated on cellulose acetate (pH3.5) and then polyethyleneimine-cellulose (in pyridine-formate, 1.5M, pH3.7). The main products of this digestion were 5'-phospho-dinucleotides. The spots were located by radioautography, eluted, electrophoresed in some cases on AE-cellulose paper to separate sequence isomers, and treated with phosphatase to remove the terminal phosphates (10 μ l of solution containing 0.1mg of bacterial alkaline phosphatase/ml and 0.1M-Tris-HCl, pH7.5 was incubated at 37°C for 1h). Their identities were deduced from (i) the positions of the spots on the cellulose acetate/polyethyleneimine-cellulose 'map', (ii) the mobilities of some of the parent dinucleotides on AE-cellulose paper (pH3.5), and (iii) digestion with venom exonuclease and electrophoresis of the products on AE-cellulose paper, pH3.5.

Elution from polyethyleneimine-cellulose layers. The following method (A. Mirzaberkov, unpublished work) was used. The spot, having been located by radioautography, was cut out and held, layer side down, to the pointed end of a 3MM paper pennant, by using flat-tipped forceps. The other end of the paper pennant was placed between two glass slides in an elution tray containing 30% triethylamine carbonate, so that liquid flowed along it and through the polyethyleneimine-cellulose spot. The forceps were then removed and a glass capillary was used to collect the liquid from the tip of the paper pennant.

Results and Discussion

Use of homo-oligodeoxynucleotides in sequencing investigations

Nuclease digests of RNA and DNA, and Burton digests (Burton & Peterson, 1960) of DNA (pyrimi-

dine tracts) have been used previously to investigate systems for fractionating oligonucleotides (Sanger *et al.*, 1965; Murray, 1970; Southern & Mitchell, 1971). Endonuclease digests of DNA are unsuitable for evaluating methods of sequencing larger oligodeoxynucleotides, because they comprise very complex mixtures whose larger constituents (particularly sequence isomers) usually cannot be resolved. Burton digests can obviously only give information about pyrimidines.

Fig. 1. Electrophoresis of homo-oligodeoxynucleotides on AE-cellulose paper at pH3.5 (a) and DEAE-cellulose paper at pH2.0 (b)

Electrophoresis buffers were: 10% (v/v) acetic acid-1% (v/v) pyridine (pH3.5) for AE-cellulose paper, and 8.75% (v/v) acetic acid-2.5% (v/v) formic acid (pH2.0) for DEAE-cellulose paper. The fastest moving spot is in each case the mononucleotide. b, blue marker dye.

Copolymer digests give relatively long homo-oligodeoxynucleotides whose sequences are, of course, known. These can be used to distinguish effects due to composition from effects due to size. However, their behaviour when fractionated on a system might not vary to the same extent as that found with oligodeoxynucleotides of random sequence.

The behaviour of homo-oligomers was examined in established systems used in sequencing, i.e. electrophoresis on AE-cellulose paper and DEAE-cellulose paper (Fig. 1). An oligonucleotide can be sequenced by fractionating its partial nuclease digest with these

two systems (Murray, 1973), because M values [the ratio of the distance between two oligonucleotides that differ by the addition (or loss) of one mononucleotide, to the distance from the origin of the larger oligonucleotide], are specific for the mononucleotide by which the two oligonucleotides differ.

M values for most of the homo-oligomers (Table 1) lie within the limits found for values from partial digests of oligodeoxynucleotides from a digest of DNA (Table 2). Table 1 also reveals that M values usually increase as nucleotides get longer and shows the necessity of using the two systems together to confirm the sequence of a nucleotide.

Fig. 1 shows the limitations in sizes of nucleotides that can be fractionated on these paper electrophoresis systems. G residues are particularly retarding and (dG)₄ is the largest member of the oligo(dG) series which can be conveniently fractionated on AE-cellulose paper.

The mobilities of dinucleoside monophosphates on the two paper electrophoresis systems are given in Table 3.

Table 1. M values for homo-oligodeoxynucleotides on AE-cellulose and DEAE-cellulose papers

Homo-oligonucleotides	M value	
	AE-cellulose (pH 3.5)	DEAE-cellulose (pH 2.0)
A ₂ -A ₃	1.13	0.28
A ₃ -A ₄	1.68	0.47
A ₄ -A ₅	1.57	0.54
A ₅ -A ₆	1.90	0.60
A ₆ -A ₇		0.80
G ₂ -G ₃	2.74	1.93
G ₃ -G ₄	3.04	1.81
G ₄ -G ₅		2.20
C ₂ -C ₃	0.40	0.03
C ₃ -C ₄	0.54	0.03
C ₄ -C ₅	0.92	0.04
C ₅ -C ₆	1.30	
T ₂ -T ₃	1.17	2.26
T ₃ -T ₄	1.47	2.38
T ₄ -T ₅	1.71	2.30
T ₅ -T ₆	1.50	

Table 2. M values calculated from partial nuclease digests of nucleotides from DNA digests (Murray, 1970, 1973)

Mononucleotide added	M value	
	AE-cellulose (pH 3.5)	DEAE-cellulose (pH 2.0)
A	0.9-1.5	0.4-1.0
G	2.0-3.0	1.4-3.0
C	0.5-0.7	0-0.3
T	1.0-1.5	1.4-3.0

Table 3. Mobilities of dinucleoside monophosphates on DEAE-cellulose paper (pH 2.0) and AE-cellulose paper (pH 3.5)

Mobilities are expressed with respect to blue dye (Xylene Cyanol FF). Electrophoresis buffers were as described in the legend to Fig. 1.

Di-ribonucleoside monophosphate	Mobility on DEAE-paper	Mobility on AE-paper	Di-deoxynucleoside monophosphate	Mobility on AE-paper
ApA	0.93	0.76	ApA	0.84
ApG	1.66	0.77	ApG	0.90
ApC	0.79	0.65	—	—
ApU	2.35	1.04	ApT	1.12
GpA	—	0.85	GpA	0.96
—	—	—	GpG	0.85
GpC	1.75	0.93	—	—
GpU	2.00	0.99	GpT	1.12
CpA	0.79	0.63	(CpA)	0.53
CpG	1.67	0.88	(CpG)	0.93
CpC	0.81	0.89	—	—
CpU	2.46	1.15	(CpT)	1.18
UpA	2.40	1.12	TpA	1.19
UpG	2.05	0.99	TpG	1.14
UpC	2.46	1.16	—	—
—	—	—	TpT	1.41

Fig. 2. Effect of concentration of the electrophoresis buffer on M values of homo-oligodeoxynucleotides on AE-cellulose paper at pH 3.5

Homo-oligodeoxynucleotides were electrophoresed on AE-cellulose paper with buffers of varying molarities, but all adjusted to pH 3.5 with pyridine. The buffers contained: 10% acetic acid (0.09M); 9.55% acetic acid, 0.9% formic acid (0.17M); 9.1% acetic acid, 1.75% formic acid (0.25M); and 8.75% acetic acid, 2.5% formic acid (0.32M). The molarity refers to total anion concentration calculated by using the Henderson-Hasselbach equation. (a) shows M values for A (▲) and C (□); (b) shows M values for G (■) and T (○). Symbols joined by lines refer to M values for a given pair of homo-oligodeoxynucleotides. Lines of increasing M values refer to homo-oligodeoxynucleotides of progressively increasing size listed in Table 1.

Fig. 3. Effect of pH of the electrophoresis buffer (10% acetic acid) on M values of oligodeoxynucleotides on AE-cellulose paper

Terminally labelled oligonucleotides were prepared by degrading DNA with deoxyribonuclease I, treating with phosphatase, labelling with polynucleotide kinase and [γ - 32 P]ATP, and fractionating the products by two-dimensional electrophoresis on DEAE-cellulose paper (Murray, 1973). The larger oligonucleotides were eluted, partially digested with venom exonuclease, and electrophoresed on AE-cellulose paper, with buffers containing 10% acetic acid, adjusted to various pH values with pyridine. (a) shows M values for A (▲) and C (□); (b) shows M values for T (○); 1 = TGC $\rightarrow$ TGCA; 2 = TG $\rightarrow$ TGA; 3 = GG $\rightarrow$ GGA; 4 = AG $\rightarrow$ AGA; 5 = TG $\rightarrow$ TGC; 6 = TGT $\rightarrow$ TGTT; 7 = TG $\rightarrow$ TGT; 8 = GC $\rightarrow$ GCT; 9 = TC $\rightarrow$ TCT; 10 = AG $\rightarrow$ AGT.

EXPLANATION OF PLATE I

Two-dimensional fractionation of terminally labelled 5'-oligodeoxynucleotides by electrophoresis on cellulose acetate and chromatography on polyethyleneimine-cellulose layers

Labelled oligonucleotides were prepared by digesting DNA with deoxyribonuclease I, treating with phosphatase, and then labelling with polynucleotide kinase and [γ - 32 P]ATP. The digest was electrophoresed on cellulose acetate [in 5% (v/v) acetic acid–7M-urea, pH3.5], at 100 V/cm. The yellow dye was run about 40 cm from the origin in this case to increase separation, and the nucleotides were transferred to two polyethylenimine-cellulose layers. After developing with 1.5M-pyridine-formate, pH3.7, the spots were located by radioautography, eluted, and their major components purified by electrophoresis on AE-cellulose paper (pH3.5). Nucleotides were sequenced by electrophoresis of their partial venom digests on AE-cellulose paper, pH3.5, and on DEAE-cellulose paper, pH2.0. PEI-cellulose is polyethyleneimine-cellulose. The line drawing (*ii*) is a key to (*i*).

Fig. 6. Relationship between M -value and R_p for oligodeoxynucleotides separated on polyethyleneimine-cellulose layers

The ratio of distance by which an oligonucleotide is retarded on polyethyleneimine-cellulose when another mononucleotide is added to it to the distance of the resulting oligonucleotide from the origin, i.e. M value, is plotted against the R_p of the resulting oligonucleotide. Measurements were taken from fractionations on polyethyleneimine-cellulose (with 1.5M-pyridine-formate, pH3.7) of homo-oligomers, pyrimidine tracts and nucleotides from the ends of bacteriophage DNA (Murray & Murray, 1973), which had been labelled in their 5'-phosphates by using polynucleotide kinase, and separated in the first dimension on cellulose acetate. ●, G; △, T; ■, A; ○, C.

effect, as is seen with the addition of C residues to the oligo(dC) series (see Fig. 5)]. These effects decrease as nucleotides get larger (Smith, 1967).

On polyethyleneimine-cellulose, the retarding effect of adding mononucleotides to a given oligonucleotide is in the order $G > T > A > C$, although the actual and relative sizes of these effects vary with the position of the oligonucleotide on the chromatogram.

These considerations enabled the sequences of terminally labelled nucleotides from the ends of bacteriophage DNA to be extended as far as 12 bases from the 5' end (Murray & Murray, 1973). However, this was a special case, since each chromatogram contained two families of complementary sequences, providing internal comparisons of the effects of adding nucleotides.

In general, ambiguities might arise between the effects of T and G residues and perhaps between A and C residues, in both dimensions of the fractionation. A similar discussion of the two-dimensional

system consisting of electrophoresis on cellulose acetate and 'homochromatography' on thin layers of DEAE-cellulose was given by Rensing & Schoenmakers (1973).

Sequencing on polyethyleneimine-cellulose

Examination of Plate 1 suggested that oligonucleotides might be sequenced by a one-dimensional separation of their partial exonuclease digests on polyethyleneimine-cellulose, if unique M values* could be obtained for each mononucleotide, taking into account the variation of M values with the position of the nucleotide on the chromatogram.

M values (defined as the ratio of the distance by which an oligonucleotide is retarded when a mononucleotide is added to it to the distance of the resulting oligonucleotide from the origin) were calculated from the results of several fractionations on polyethyleneimine-cellulose with 1.5M-pyridine-formate, pH3.7, of homo-oligomers, pyrimidine tracts and nucleotides derived from ends of bacteriophage DNA (all of which had been sequenced by electrophoresis of their partial digests on ion-exchange papers). These were plotted against the R_p (the rate of movement with respect to P_i) of the larger of the two oligonucleotides from which the M value had been derived (Fig. 6).

The results for the homo-oligomer series are usually consistent with those for the ends of bacteriophage DNA and pyrimidine tracts. But Fig. 6 shows that the M values for the four mononucleotides are not distinctive enough to determine a sequence unambiguously. There is doubt particularly in distinguishing between M values for G and T residues and between M values for A and C residues.

Effect of varying the developing solvent

The effects of concentration and pH of the pyridine-formate solvent on the R_F values of homo-oligomers are shown in Figs. 7(a)-(d) and 8(a)-(c). Conditions may be varied to suit the mixture to be fractionated; for example, one can separate quite long nucleotides and resolve small ones on the same chromatogram by progressively increasing the concentration of the developing buffer.

Conclusion

Electrophoresis of partial exonuclease digests of terminally labelled oligodeoxynucleotides on AE-cellulose paper (pH3.5) and DEAE-cellulose paper

* M value is used here in chromatography in an analogous way to its use in electrophoresis.

Fig. 7. Effect of concentration of formate buffer (made pH 3.7 with pyridine) on R_F values of homo-oligomers on polyethyleneimine-cellulose

(a) Oligo(dA); (b) oligo(dC); (c) oligo(dG); (d) oligo(dT).

(pH 2.0) provides the most reliable method for sequencing smaller oligodeoxynucleotides. No equally good method applicable to larger terminally labelled oligodeoxynucleotides is yet available. The two-dimensional method using cellulose acetate and

polyethyleneimine-cellulose, though not itself always dependable, has already proved valuable, and, in conjunction with other techniques, may provide enough information for the unequivocal determination of sequences.

Fig. 8. Effect of pH of 1.5M-pyridine-formate buffer on R_F values of homo-oligomers on polyethyleneimine-cellulose
(a) Oligo(dC) and oligo(dG); (b) oligo(dA); (c) oligo(dT).

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