Chromatography of Nucleic Acid Digests on Thin Layers of Cellulose Impregnated with Polyethyleneimine

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Methods are described for the separation of oligodeoxyribonucleotides and oligoribonucleotides by chromatography on thin layers of cellulose impregnated with polyethyleneimine.

Randerath & Randerath (1967) have given a thorough account of methods for separating mixtures of nucleotides and oligonucleotides on thin layers of cellulose impregnated with polyethyleneimine. We have adapted their procedures to the separation of the complex mixtures of oligonucleotides obtained during the sequence analysis of DNA and RNA. Our work has been mainly concerned with the sequence analysis of DNA and we have concentrated on developing methods for analysing pyrimidine tracts obtained by depurinating DNA (Burton & Petersen, 1960); preliminary experiments show that the thin layers are likely to be useful also for the analysis of enzymic digests of RNA.

MATERIALS AND METHODS

Escherichia coli M.R.E. 600 DNA uniformly labelled with ^{32}P was prepared as described by Grossman (1967). rRNA of *Xenopus laevis* uniformly labelled with ^{32}P was a gift from Dr Williamson. The '16S' rRNA of *Proteus mirabilis* N.C.T.C. 5887 uniformly labelled with ^{32}P was prepared from cells grown in the medium described by Grossman (1967), supplemented with 0.02% nicotinamide, and purified on 2.5% (w/v) polyacrylamide gels (Loening, 1970) from which it was eluted by shaking slices of the gel in 0.15M-NaCl overnight. Thin-layer plates (MN 300 polyethyleneimine) were obtained from Camlab (Cambridge) Ltd., Cambridge, U.K., and washed through the following cycle of solvents, as described by Randerath & Randerath (1967): 10 min in 10% (w/v) NaCl, dried at room temperature for a few hours; 10 min in water, dried at room temperature for a few hours; 10 min in 2M-formic acid adjusted to pH 2.2 with pyridine; 10 min in water. After being dried, the plates were stored at 4°C in the dark. This washing procedure improved the separation on two-dimensional chromatograms, but was not necessary for separations requiring development in one direction. For the latter separations it was enough to wash in 2M-pyridine-formate buffer, pH 2.2, and water. Certain batches of thin layers were dark yellow and gave streaky spots and irreversible adsorption of material at the

origin; these thin layers proved to be stock that had been stored for 3 years at room temperature. Thin layers made in the laboratory on either plastic or glass backing as described by Randerath & Randerath (1967) gave good results. Indeed the spots were generally more compact on these than on the commercial thin layers. Most of the work described in this paper was done on commercial thin layers.

Pyrimidine tracts were prepared by standard procedures (Burton & Petersen, 1960). Throughout the preparation, the sample volume was kept as small as possible because only small volumes can be applied to the thin layers. Before degradation the DNA was dialysed against several changes of water because salts interfere strongly with the chromatographic separation. The pyrimidine tracts used in the experiment described in Fig. 1 were prepared and fractionated by the procedure of Székely & Sanger (1969).

Complete digestion with ribonuclease A and ribonuclease T_1 and electrophoresis on cellulose acetate at pH 3.5 were carried out as described by Sanger, Brownlee & Barrell (1965), or in 5% (v/v) acetic acid, 7M-urea, pH 3.5. Samples for t.l.c. were applied, in 1-10 μl of solution containing as little salt as possible, at a point 1 cm from two edges of the layer, the thin layer being held horizontal and the capillary vertical. The nucleotides absorb into an area less than 1 mm in diameter. A small amount of marker dyes (1 μl of a 1:100 dilution of the mixture described by Sanger *et al.* 1965) was applied close to the point of application of the sample. The presence of salt, or of too high a concentration of nucleotides, is shown by a marked spreading of the dyes. Samples separated in the first direction on cellulose acetate were transferred to the thin layer by blotting with water as described by Sanger *et al.* (1965); considerable pressure can be applied during this process without risk of damaging the thin layer. The thin layers were transferred to the developing buffer, before the water dried.

Before development with solvent a piece of filter paper was fixed to the top of the thin layer with 'bulldog' clips. The edge of the thin layer was dipped into water to a depth of 0.5 cm until the water had risen 1 cm past the origin. This step was included before the development of chromatograms to prevent the concentration of salt by evaporation at the solvent front.

The solvents used were: (1) 1.0–3M-formic acid adjusted to pH 3.2–3.6 with pyridine; (2) LiCl at concentrations 0.5–1.0M; the pH of this solution is usually about 5; (3) 0.6M-LiCl in 60mM-acetic acid.

If development in formate was to be followed by development in another solvent, the pyridine-formate was removed by leaving the thin layer at room temperature overnight. LiCl can be removed by washing in anhydrous methanol (Randerath & Randerath, 1967). Spots were detected by radioautography. Before exposure of the thin layer to X-ray film the pyridine-formate was removed as described above to avoid chemical fogging of the film. LiCl is hygroscopic and sticks to the film; to prevent this a thin sheet of paper or plastic was placed between them. Alternatively the film can be held against the plastic backing material of the thin layer.

It is sometimes useful to use two films, one above and one below the thin layer, with widely different exposures so that spots due to the more abundant nucleotides are not overexposed on the first film and the rarer nucleotides are detected on the second. For radioactive counting, the areas of the radioactive spots were marked on the plastic back of the thin layer and cut out with scissors. The nucleotides can readily be eluted from the thin layer. The area containing the nucleotide was cut to a pennant shape and if the chromatogram had been developed with LiCl this was removed by washing in methanol. The square end of the pennant was laid, layer side down, against a wad of filter paper wetted with 2M-triethylammonium hydrogen carbonate [aq. 30% (v/v) triethylamine saturated with CO₂, pH 8–9]. The pointed end was laid against the pointed end of a pennant of phosphocellulose paper. About 10–20 μ l of triethylammonium hydrogen carbonate solution was collected on the phosphocellulose, which was dried and then washed in ether. Nucleotides were washed from the phosphocellulose with water as described by Sanger *et al.* (1965).

RESULTS AND DISCUSSION

Separation of pyrimidine tracts. Other systems have been described for separating pyrimidine tracts on paper by two-dimensional chromatography (Burton & Petersen, 1960; Shapiro & Chargaff, 1963) or by electrophoresis followed by chromatography (Petersen, 1961) or by two-dimensional ionophoresis (Székely & Sanger, 1969; Murray, 1970; Southern, 1970). The most effective method, which separates pyrimidine tracts of up to 20 nucleotides long, requires a series of analyses on columns of DEAE-cellulose (Petersen & Reeves, 1966). The method described here has advantages over earlier methods: it is rapid, requires little material, does not require dephosphorylation of the nucleotides and separates tracts up to the octamers. It does not replace column separation for a complete quantitative analysis, because the longer tracts are not separated.

For qualitative analysis of pyrimidine tracts, which have the general formula Y_nP_{n+1}, two-dimensional chromatography was used. The thin layers (10cm × 10cm) were developed in the first

direction with 2M-pyridine-formate buffer, pH 3.4, and in the second with 1M-lithium chloride (Plate 1). Certain batches of thin layers gave a poorer separation in the first dimension than that shown in Plate 1: the slower-moving nucleotides were closer to each other and to the origin, which would be expected of thin layers with a higher ion-exchange capacity. Some samples gave streaky spots in the first dimension, probably because of the presence of traces of salts. In these cases the thin layers were developed first in 2M-pyridine-formate buffer, pH 3.4, dried, sprayed with dilute formic acid, pH 3.2, and then developed in the same direction with 2M-pyridine-formate buffer, pH 3.2. This procedure increased the separation of the slower-moving nucleotides, compressed the faster-moving spots and decreased tailing (see Plate 4a). For quantitative analysis, where clear resolution between spots is required, larger plates (20cm × 20cm) were used.

Apart from the variations between plates, mentioned above, the general pattern of spots is quite reproducible, though there are small variations in the relative distances of movement, especially in the second dimension. The relative movements are not always those predicted from the composition: for example, d(pCpCp) moves ahead of d(pCp) in the first dimension and d(pTpTp) moves ahead of d(pTp) and d(pTpTpTp) in the second. The small effect of deoxyribosylcytidine residues on mobility in the first dimension is expected, but it is surprising to find such a small effect of thymidine residues as compared with deoxyribosylcytidine residues in the second. Together these observations suggest that there might be a non-ionic interaction between the amino group of cytosine and the matrix. A comparable separation of pyrimidine tracts by ionophoresis on the cellulose acetate/DEAE-cellulose paper or cellulose acetate/aminoethyl-cellulose paper systems (Murray, 1970; E. M. Southern, unpublished work) would require two runs, because in these systems the small nucleotides move very much faster relative to the long ones. A further advantage in the use of thin layers for quantitative analysis is the avoidance of a transfer step between the two dimensions, which in the cellulose acetate/DEAE-cellulose paper system might lead to unequal losses of nucleotides.

The thin layers are also useful for separation of pyrimidine tracts of general formula Y_nP_n produced by the action of alkaline phosphatase followed by polynucleotide kinase and ATP (Richardson, 1965), as described by Murray & Wu (1968) and by Székely & Sanger (1969). In this case the thin layers are developed in the first direction with 1.5M-pyridine-formate buffer, pH 3.5, and in the second with 0.5M-lithium chloride. Alternatively the oligonucleotides may be separated first by ionophoresis

on cellulose acetate at pH3.5, after which they are transferred to polyethyleneimine layers, which are developed in 1.5M-pyridine-formate buffer, pH3.5. The separation of these oligonucleotides obtained by developing the thin layers only in pyridine-formate buffer (Fig. 1) suggests that this system may be useful for separating the products of partial venom phosphodiesterase digests of pyrimidine tracts. Again, as compared with ionophoresis on DEAE-cellulose paper or aminoethyl-cellulose paper the pattern is more compact because of the greater relative mobilities of oligonucleotides rich in thymi-

dine. The relative mobilities of oligonucleotides, however, are probably more variable, so that these cannot be used diagnostically for sequence determination until further evaluation of the method is complete.

Digests of RNA. The most widely used methods for separating the products of nuclease digests of RNA are ionophoresis on cellulose acetate followed by ionophoresis on DEAE-cellulose paper or 'homochromatography' (Brownlee & Sanger, 1969). Although these give excellent separations, the former method has the disadvantage that the relative

Fig. 1. Separation of pyrimidine tracts, labelled with polynucleotide kinase, on polyethyleneimine-cellulose. The nucleotides were prepared as described by Székely & Sanger (1969) and applied to the thin layers in groups according to their cytosine content. The layers were then developed with 1.5M-pyridine-formate buffer, pH3.2 (a), pH3.4 (b) and pH3.6 (c).

mobilities of long and short nucleotides are such that they do not usually allow effective separation of all the nucleotides on one ionophoretogram. Homochromatography is useful for separating long sequences, but, because the eluent contains a high concentration of nucleotides, further analysis of the radioactive spots is more difficult and subsequent labelling with polynucleotide kinase is impossible. Preliminary experiments suggest that the polyethyleneimine-cellulose layers will be useful for the analysis of digests of RNA. The results shown in Plate 2 were obtained with the two-dimensional system 1.5M-pyridine-formate buffer, pH 3.5, followed by 0.8M-lithium chloride. Clearly this system did not give good resolution of ribonuclease A digests, and this is probably because of the low mobility of guanosine residues in lithium chloride (Randerath & Randerath, 1967). Resolution of ribonuclease T₁ digests was somewhat better, probably because each nucleotide contains only one guanosine residue.

Both kinds of ribonuclease digest were well resolved when ionophoresis on cellulose acetate at pH 3.5 was followed by chromatography on polyethyleneimine cellulose. The choice of buffer for development depends on the composition of the nucleotides. The separation of a pancreatic ribonuclease digest of total rRNA *Xenopus laevis* (Plate 3a) was obtained with 1.5M-pyridine-formate buffer, pH 3.5. The relative mobilities of the oligonucleotides in the second dimension appear to be similar to their relative mobilities during ionophoresis on DEAE-cellulose at acid pH values, giving a similar overall appearance to the 'maps'. The important advantage of the thin layers is in the higher mobilities of the slower relative to the faster-moving nucleotides (i.e. oligonucleotides that remain at or near the origin on ionophoresis on DEAE-cellulose paper or AE-cellulose paper are separated).

The separation of a ribonuclease T₁ digest of the 16S component of rRNA from *Proteus mirabilis* (Plate 3bi, ii and iii) shows first that quite complex mixtures of oligonucleotides including moderately long sequences can be separated on polyethyleneimine layers [compare, for example, Fellner, Ehresmann & Ebel (1970), Fig. 1]; second, that the relative mobilities of the nucleotides are similar to those on DEAE-cellulose paper at acid pH values; and finally, how a system may be evolved for separating an unknown mixture of nucleotides. In this case the thin layer was developed first with low concentrations of buffer. An exposure of the thin layer at this stage (Plate 3bi) showed that the fast but not the slow-moving nucleotides were separated. The thin layer was then developed in a more concentrated buffer which separated the slower nucleotides, but brought together some of the faster ones (Plate 3bii).

A third development using a buffer at low concentration rescued the separation of the faster nucleotides, leaving the slow-moving nucleotides still separated. Clearly, any area in the chromatogram could be 'stretched' in this way, with the right choice of buffer, giving the technique a greater flexibility than the corresponding ionophoretic methods. In the example shown here, it is clear that we chose too high a buffer concentration for the second development, which moved out even the very slowest nucleotide a few cm from the origin but decreased the separation of nucleotides containing 2 uridine residues.

General comments. Our main reason for choosing to work with polyethyleneimine layers was the possibility they offer for varying the ion-exchange capacity of the thin layer. This appeared to be necessary because the capacities of commercially available ion-exchange materials are generally rather high, with the consequence that molecules with a high charge, e.g. long oligonucleotides, have very low mobilities. So far we have not exploited this potential of polyethyleneimine thin layers because the commercial thin layers gave satisfactory separations with quite long oligonucleotides, but trials on thin layers prepared with 0.2% polyethyleneimine showed that on these layers the pyrimidine tracts moved much faster than on 1% layers (Plate 5) and gave more compact spots. This does not show, however, that it is better to decrease the ion-exchange capacity of the layer than to increase the concentration of the developing buffer in order to resolve the longer oligonucleotides and further work is needed to clarify this point. We have concentrated, so far, on developing systems that allow the separation on one chromatogram of all the short nucleotides; under the conditions

Fig. 2. Titration of polyethyleneimine with HCl. A solution (0.21% approx. 50 mM) of polyethyleneimine in water (O) or 0.1M-NaCl (●) was titrated with 0.2M-HCl. The pH was read with a glass electrode.

EXPLANATION OF PLATE I

Separation of pyrimidine tracts from *E. coli* ^{32}P -labelled DNA on thin layers of polyethylencimine-cellulose. The mixture of nucleotides (about 10^5 c.p.m.) in $2\mu\text{l}$ of water was applied to a thin layer ($10\text{cm} \times 10\text{cm}$) and the chromatogram developed as described in the text, with 2M-pyridine-formate buffer, pH 3.4, in the first direction and 1M-lithium chloride in the second. The photograph is of a radioautograph obtained after 18h exposure.

EXPLANATION OF PLATE 2

Separation of ribonuclease A (a) and ribonuclease T₁ (b) digests of *X. laevis* rRNA by two-dimensional chromatography on polyethyleneimine-cellulose (20 cm × 20 cm). Solvents were 1.5 M-pyridine-formate buffer, pH 3.5, and 0.8 M-LiCl.

EXPLANATION OF PLATE 3

(a) Separation of a ribonuclease A digest of *Xenopus laevis* rRNA by ionophoresis on cellulose acetate in pyridine-acetate buffer, pH 3.5, and chromatography on polyethyleneimine-cellulose (20 cm × 20 cm) developed with 1.5M-pyridine-formate buffer, pH 3.5.

(b) Separation of a ribonuclease T₁ digest of *Proteus mirabilis* 16S rRNA by ionophoresis (anode on left) on cellulose acetate in 5% acetic acid, 7M-urea, pH 3.5, and t.l.c. on polyethyleneimine-cellulose (20 cm × 40 cm) developed with 1.5M-pyridine-formate buffer, pH 3.5 (4h) followed by 1.5M-pyridine-formate buffer, pH 3.7 (2h). The layer was dried and exposed to X-ray film (bi). It was then sprayed with dilute formic acid, pH 3.2, and developed (4h) in 3M-pyridine-formate buffer, pH 3.6, dried and exposed (bii). It was again sprayed with dilute formic acid, pH 3.2, and developed (20h) in 1.6M-pyridine-formate buffer, pH 3.6 (biii).

EXPLANATION OF PLATE 4

(a) Separation of pyrimidine tracts of DNA by chromatography on polyethyleneimine-cellulose layers in 2M-pyridine-formate buffer. The chromatogram on the right was developed twice at pH 3.3, with intermediate wetting with formic acid, as described in the text.

(b) Separation of a ribonuclease A digest of RNA by two-dimensional chromatography on polyethyleneimine-cellulose. The solvents were as for Plate 2a, except that the LiCl contained 7M-urea.

EXPLANATION OF PLATE 5

Pyrimidine tracts from *E. coli* [^{32}P]DNA chromatographed on polyethyleneimine layers, 1% (a) and (c), and 0.2% (b) and (d), in pyridine-formate buffer, pH 3.5, 0.5M (a) and (b), 1.0M (c) and (d).

described here, nucleotides with net negative charges ranging from 3 to 10 can be separated at a single salt concentration.

We have found pyridine-formate buffer to be a very useful solvent at acid pH values, its main advantage being its volatility. The mobilities of the nucleotides in this solvent are readily altered by varying the pH (Plate 4a and Fig. 1); mobilities increase with pH. Part of this effect is due to increased formate ion concentration and part to decreased ionization of polyethyleneimine (Fig. 2). The useful pH range of polyethyleneimine is below pH 9. Long nucleotides need rather high salt concentrations to move them at low pH values; for example, to move the mixed tRNA species of *E. coli* on polyethyleneimine-cellulose needs 3M-lithium chloride at pH 3.5, 1.2M-lithium chloride at pH 5 and 0.9M-lithium chloride at pH 8.5.

Addition of urea to either the first or second solvent, or increasing the temperature to 70°C, or addition of formamide to the first solvent of the system used for separating the pyrimidine tracts did not increase resolution. The addition of urea to lithium chloride used to elute ribonuclease A digest on a two-dimensional chromatogram gave a curious result (compare Plates 2a and 4b): the nucleotides were separated into three distinct lines and the mobilities of the slower-moving nucleotides were increased markedly. This suggests that the addition of urea may be useful for separating sequences rich in guanosine, as is found in column chromatography on DEAE-cellulose (Bartos, Rushizki & Sober, 1963). From the fractionation of the ribonuclease T₁ digest (Plate 3b) it appears that a few sequence isomers such as C-A-G and A-C-G are separated to about the same extent as on DEAE-cellulose at

acid pH values. A much better separation of sequence isomers of short oligonucleotides is given by the system developed by Murray (1970).

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