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# Chromatography of $^{32}$P-Labelled Oligonucleotides on Thin Layers of DEAE-Cellulose

G. G. Brownlee and F. Sanger

Medical Research Council Laboratory of Molecular Biology, Cambridge

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A two-dimensional fractionation procedure has been developed for separating radioactively-labelled oligonucleotides of up to 50 residues long, using uniformly $^{32}$P-labelled 5S RNA of *Escherichia coli* as a model compound. The method uses ionophoresis on cellulose acetate at pH 3.5 in the first dimension; and ascending chromatography with a concentrated mixture of oligonucleotides on thin layers of mixed DEAE-cellulose and cellulose in the second dimension.

We have previously described two separate two-dimensional fractionation systems, using modified papers, suitable for studying enzymatic digests of $^{32}$P-labelled RNA. The first [1] is a two-dimensional ionophoretic procedure for studying the end-products of digestion with ribonuclease $T_1$ or pancreatic ribonuclease. The second procedure [2] uses both ionophoresis and chromatography and is suitable for studying the products of partial enzymatic digestion of purified low-molecular weight RNA, such as 5S RNA or transfer RNA. The second dimension is a type of displacement chromatography by which unlabelled oligonucleotides are used to displace labelled oligonucleotides fixed on DEAE-paper, and we have referred to this as "homochromatography". Homochromatography on DEAE-paper is limited to the fractionation of oligonucleotides of less than 25 residues in length, those larger than this remaining at, or near, the origin. It was of some importance to develop methods for extending this technique to larger fragments, especially for the determination of the sequence of molecules larger than tRNA or 5S RNA. A method suitable for fragments of up to 50 residues long was therefore developed using 5S RNA of known sequence [2] as a model compound, and this is described in this paper. It uses ionophoresis on cellulose acetate at pH 3.5 for the first dimension and homochromatography on thin layers of mixed DEAE-cellulose and cellulose in the second dimension. This method may also be used with advantage for separating smaller oligonucleotides (10–30 residues in length) as these are better resolved and show less streaking than on the DEAE-paper system.

*Unusual Abbreviations.* tRNA = transfer RNA; G>p = guanosine-2′,3′-cyclic phosphate; Cp, Ap, Gp and Up the 3′-phosphates of cytidine, adenosine, guanosine and uridine, respectively.

*Enzymes.* $T_1$-ribonuclease (EC 2.7.7.26); pancreatic ribonuclease (EC 2.7.7.16).

## MATERIALS AND METHODS

### *Preparation of Thin Layers*

The Desaga basic thin layer equipment and the adsorbants were purchased from Camlab (Glass) Ltd. (Milton Road, Cambridge). A slurry of DEAE-cellulose (MN 300 DEAE) and cellulose (MN 300 cellulose) was prepared sufficient for four long glass plates (20×40 cm) or eight short (20×20 cm) plates, as follows. For the "1:10" plates, 1.5 g DEAE-cellulose and 15 g cellulose were mixed and then added, with stirring, to 102 ml distilled water until the mixture was fairly evenly dispersed and wetted. The slurry was then homogenized thoroughly in a fast electric blender for about 3 min to break up aggregated particles. It was then de-aerated thoroughly and 50 ml poured into the spreader (giving a fixed 250 μ layer) which was then passed fairly rapidly across the plates positioned on the template. The other half of the slurry could be used when the spreader was reclosed and positioned to spread the remaining glass plates on a second template. The plates were separated slightly and allowed to dry at room temperature. On inspection they should have no streaks in the direction of spreading, which are usually caused by air bubbles trapped in the slurry and prevent even flow; nor should lumps be present, which are aggregated particles reflecting incomplete homogenization. Slight ridges perpendicular to the direction of spreading are less significant and indicate that the layer was not spread evenly (usually too slowly) or that the slurry was too dilute. Although perfect plates probably give the best separations, we have not observed markedly inferior fractionations with plates that were somewhat imperfect in any of the above respects. Plates containing a higher proportion of DEAE-cellulose to cellulose, in a ratio of 1 part to 7.5 parts instead of 1:10, were also prepared. Finally a razor blade was used to define a sharp edge to the thin layers.

*Fractionation Procedure*

$^{32}$P-labelled 5S RNA was extracted from *E. coli* (CA265) grown in the presence of [$^{32}$P]phosphate and was purified by acrylamide gel electrophoresis on 10% acrylamide slabs [3]. A different preparation purified by successive column chromatography on DEAE-Sephadex [4] and on a reversed phase column [5] was kindly provided by Dr. J. Abelson and was used for the fractionation shown in Fig. 3.

Partial $T_1$-ribonuclease (Sankyo, Japan) digests were prepared in 2—5 µl in 0.02 M Tris-chloride, 0.02 M magnesium chloride, pH 7.5, incubated for 30 min at 0° and fractionated on cellulose acetate strips (Oxoid or Schleicher & Schüll) at pH 3.5 in the first dimension, as before [1]. The solution used to wet the strips (5% acetic acid—7 M urea, adjusted to pH 3.5 with pyridine) has a very weak buffering capacity and the pH was found to increase at room temperature. It was therefore stored at 4° and rejected when the pH rose higher than 3.7. Nucleotides were blotted from the cellulose acetate to the plates as follows. The strip was removed from the tank used for the first dimensional run and monitored with a portable Geiger counter to find the position of the oligonucleotides, which in partial digests was rather fast, usually in the region just slower than the major red, acid fuchsin, marker. This part of the strip was then placed on the thin layer plate, 3 cm from one of the short ends, perpendicular to the direction of spreading of the plate. Three moist, but not too wet, strips of Whatman 3 MM paper were carefully placed on top followed by a glass plate to maintain efficient contact. Water thus flowed into the layer, transferring the oligonucleotides onto the DEAE-cellulose layer. Care is needed in this procedure to obtain efficient transfer and we prefer to use strips that still have some "white spirit" on their surface, rather than let the buffer in the strip dry out. Excess water or pressure applied to the glass plate does not help the transfer but rather tends to dislodge the layer. Transfer is not entirely quantitative and is probably poorer for the larger fragments. However over 80% of the total material is usually transferred. Because only 20 cm was available for the second dimension we preferred to use short runs in the first dimension on approx. 50 cm cellulose acetate strips for about 1 h, applying 6 kV. This spread out the partial products less well than the longer runs on 85 cm strips, but allowed them to be included in a single second-dimensional run.

Fractionation in the second dimension was carried out at 50—60° in an oven using one or other of the homomixtures [2] described below. Which particular mixture was used depended on whether the largest fragments, 15 to 40—50 residues long, were to be isolated (homomixture *a*); an intermediate size, in the range of 10 to approx. 25 residues (homomixture *b*); or even smaller oligonucleotides, 1 to approx. 15 (homomixture *c*). The thin-layer tank, used for the short plate, or the tall tank (formed by inverting one thin-layer tank on top of another) used for long plates, are equilibrated with approx. 100 ml of the homomixture at 60° in the oven. The plate is also equilibrated at 60°, but outside the tank. Before starting the ascending homochromatography, the plate is briefly chromatographed with distilled water until the front is about 5 cm up the plate. This is designed to wash excess urea, transferred from the first dimension, away from the origin which otherwise interferes in the fractionation, especially of faster moving oligonucleotides (as can be seen in Fig. 3, which was not washed). An alternative procedure, which is quicker and therefore results in less cooling of the plate, is to spray the region of the origin with distilled water. After this the plate is transferred into the equilibrated tank and ascending homochromatography continued until the front reaches the top of the plate. This usually takes about 2 h for short plates and 5 h for long ones, although we have noticed considerable variation in running time, which we cannot account for.

The plate is then dried, marked with red ink containing [$^{35}$S]sulphate, and a radioautograph prepared by exposing to an X-ray film in a lead-lined folder. It was convenient to prepare two separate exposures (one for an elution template and the other for the records) by exposing two films one on top of the other.

Homomixtures were prepared as follows:

*Homomixture a* was essentially as before [2] and was prepared by dissolving 10 g of yeast RNA (British Drug Houses Ltd.) in 200 ml of 7 M urea and adjusting to pH 7.5 with 10 N KOH. The mixture was kept frozen and was used in the separation shown in Fig. 3. It has also been used to isolate a $T_1$-oligonucleotide (21 residues long) from R 17 bacteriophage RNA [6].

*Homomixture b* was a preparation of homomixture *a* dialysed against 7 M urea for 2—3 h at 4°. In this time there was approximately 10% increase in volume. It was used for the fractionation shown in Fig. 2.

*Homomixture c* was a dialysed and hydrolysed homomixture prepared by dissolving 10 g of yeast RNA in 100 ml of 1 N KOH and hydrolysing it for 15 min at room temperature. The solution was then neutralized to pH 7.5 with concentrated HCl and dialysed against distilled water for 2—4 h. 84 g of urea was then added and the volume made up to 200 ml with distilled water to give a 5% homomixture in 7 M urea. Homomixture *c* may be diluted with 7 M urea to give a 3% mixture, which gives somewhat sharper spots than the 5% mixture, although the $R_F$ values are lower.

Homomixture *c* was not used in the experiments quoted in this paper but is described here for com-

pleteness. It is useful for the fractionation of the oligonucleotides encountered in complete $T_1$-ribonuclease digests of RNA, when it is usually used as a 3% mixture on 1:7.5 plates. Fragments from 1 to about 15 residues long are separated from one another approximately according to chain length. The resolution of the smaller oligonucleotides is generally poorer than on the standard two-dimensional ionophoretic fractionation system [1], although fractionation of those in the range 10—15 residues is usually rather better. Longer ones are better separated using homomixture *b* or homomixture *a*.

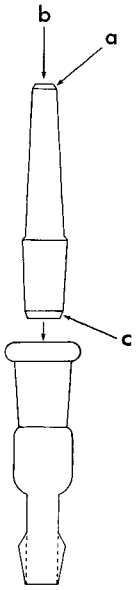


Fig. 1. *Diagram of a thin layer elution device.* This has a B10/19 ground glass joint which can be connected to a vacuum line. The upper part has an orifice "b" of approx. 2 mm internal diameter and is ground on its circumference to give a sharp edge, "a", suitable for scraping and dislodging the thin layer. A sintered disc of porosity 1 or 2 is welded in at "c". Device available from T. W. Wingent Ltd. (Milton, Cambridge, England)

### *Elution and Analysis*

Those spots to be eluted were marked and numbered on the radioautograph that showed the [$^{35}$S]sulphate ink marks, and holes were cut out with a razor blade corresponding to these spots. This radioautograph then functioned as a "template" and was stuck with sellotape onto the thin layer, aligning it by means of the ink marks. The thin layer spots thus exposed through the holes of this template could then be scraped off, using the elution device shown in Fig. 1 which is connected to a vacuum line. The cellulose is thus sucked up through the narrow orifice (b) and is trapped in the sinter (c). The porosity of the sinter should be number 1 or 2. If it is too course the sinter lets some of the smaller particles through in the succeeding washing and elution procedure, whilst if it is too fine particles do not easily suck up and subsequent elution is very tedious. Ten to twenty samples were usually eluted, using separate top sections of the elution device and the same bottom section. These were then inverted and held in position in a stand with clips. Urea was first removed from the samples by washing with 95% ethanol before eluting oligonucleotides with triethylamine carbonate, pH 10.0 [1]. Two or three drops (0.2 ml) were collected into small silicone-treated [1] test tubes, to which a further two or three drops of water was added, and the triethylamine carbonate removed *in vacuo* using three successive washes with water.

The analysis of partial digestion products by further treatment with $T_1$ and pancreatic ribonuclease was as before [2] except that lower enzyme concentrations and shorter digestion times could be used as less carrier nucleotide was present on the DEAE-thin layers than on the DEAE-paper. Suitable conditions for both $T_1$ and pancreatic ribonuclease were 10 µl of 0.1 mg/ml of enzyme and digestion was for 30 min at 37°. Conditions for other sequence procedures of spots from thin layers are presented by Adams *et al.* [6].

## RESULTS

Fig. 2 shows in its upper half a radioautograph of a partial $T_1$-ribonuclease digest (1:4000 for 30 min at 0°) of 5S RNA using a long 1:10 plate and homomixture *b* for the second dimension. Although there is a large number of products they are resolved from one other as discrete spots, except for the faster moving and smaller products which move as bands, and the slower products where the mixture is too complex to resolve. The sequence of some of the fragments was determined by further $T_1$-ribonuclease digestion and by reference to the known sequence of 5S RNA [2] reproduced in Fig. 4. In all cases identical partial digestion fragments had been previously isolated by homochromatography on DEAE-paper, so that there was no difficulty in identification. These sequences are recorded by the residue number (see Fig. 4) of the two ends of the fragment in the lower half of Fig. 2. Oligonucleotides from 13 to 28 residues long were isolated and this suggests that the method is suitable for fractionation of oligonucleotides of this size range, assuming the mixture to be fractionated is no more complex than in Fig. 2. If it is simpler the size range of fractionation may well be extended to fragments over 30 residues long.

Fig. 3 shows a less extensive but simpler $T_1$-ribonuclease digestion of 5S RNA which had been purified by column chromatography. Although the conditions of digestion are more violent than those of the previous experiment, the digestion is less complete, which presumably reflects differences in the con-

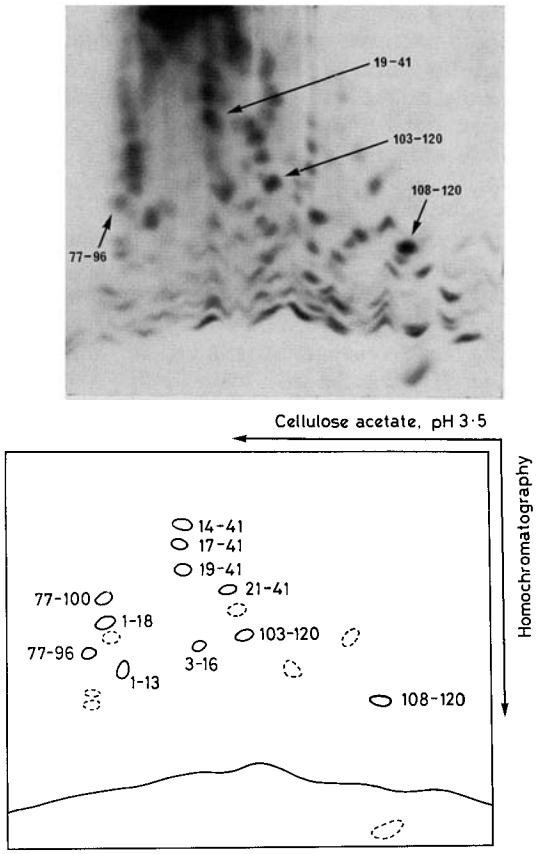


Fig. 2. *Radioautograph and diagram of a two-dimensional fractionation of a partial $T_1$-ribonuclease digest (1:4000, 30 min at 0°) of 5S RNA using a 50 cm cellulose acetate strip (Schleicher & Schüll) in the first dimension at pH 3.5 in 7 M urea; and using homomixture* b *for homochromatography on a long, 1:10, thin layer plate in the second dimension.* The numbers on the diagram are an abbreviation for the sequence which can be read off by referring to Fig. 4. The diagram and radioautograph are aligned vertically to allow the reader to recognize which spots on the radioautograph the numbers on the diagram refer. To aid identification a few are marked directly on the radioautograph. Dotted circles are included in the diagram to show some fairly strong spots that were not eluted. The major nucleotide front has an $R_F$ of approx. 0.5 relative to the solvent front. The single nucleotide ahead of this is G>p

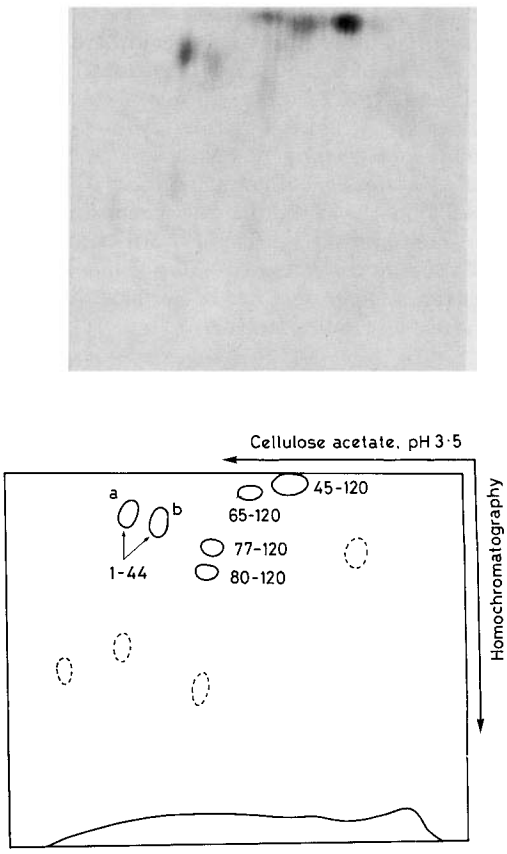


Fig. 3. *Radioautograph and diagram of a two-dimensional fractionation of a partial $T_1$-ribonuclease digest (1:500, 30 min at 0°) of 5S RNA (purified by column chromatography).* The sample was fractionated as in Fig. 2 except for the following: "Oxoid" cellulose acetate (57 cm) was used in the first dimension, and homomixture *a* and a short plate in the second dimension. The meaning of symbols is as in the legend to Fig. 2 and the text. The wavy line at the bottom of the diagram marks the nucleotide front which in this case is close to the solvent front

figuration of the RNA prepared by two different methods. Fractionation in this case was on a short 1:10 plate using homomixture *a* in the second dimension. The faster moving products are very streaky as in this experiment no attempt was made to chromatograph urea away from the origin before homochromatography (see Methods section). Nevertheless some of the slower moving spots were eluted and their sequences determined by further digestion with $T_1$ and pancreatic ribonuclease.

As is shown in the lower half of Fig. 3, oligonucleotides of from 41 to 76 residues long were isolated pure. These fragments had not been isolated in our previous work and this provided confirmation that the sequence shown in Fig. 4 was correct. Two alternative forms of the sequence of residues 1 to 44 were isolated, marked "a" and "b" on Fig. 3, which on analysis were found to differ only in the residue at position 12. This was adenine in spot "a" and cytosine in spot "b". These separate fragments derive from the two major species of 5S RNA and are presumed to separate in the first dimension on the basis of differences in shape, as the single base change would probably not be sufficient to alter mobility on the basis of a charge difference. The unequal yields of the two fragments presumably reflect a different susceptibility of the two forms of 5S RNA to cleavage with $T_1$ ribonuclease under the conditions used, perhaps because of slight differences in shape. Thus, using this system, fragments of up to 45 residues long are well separated, and even larger

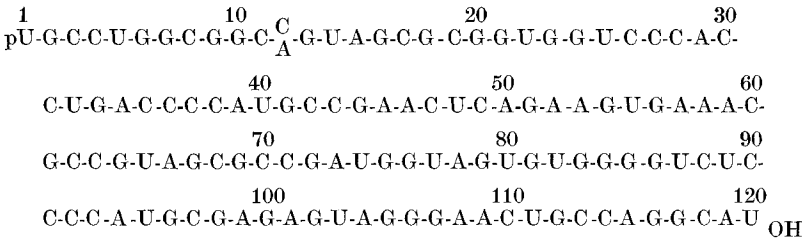


Fig. 4. *The nucleotide sequence of 5S RNA of* E. coli *(strain CA265).* Both cytosine and adenine occur in position 12, in approximately equal yields, as deduced from the yields of the $T_1$-ribonuclease end-product, CpApGp, which is 0.5 relative to other end-products present in 1.0 relative molar yields

fragments may be isolated if the mixture is not too complex. Fragments smaller than 15 residues long move at, or near, the nucleotide front and are not well fractionated.

## DISCUSSION

Thin layer techniques are well known to offer advantages over paper fractionation systems [7]. The operator has strict control over the composition, the thickness and uniformity of the system, and usually considerably better separations are observed in shorter times than with paper. In the work reported here the technique allowed us to reduce the "effective" capacity of the DEAE-cellulose by mixing it with an excess of cellulose. We were thus able to isolate larger fragments by homochromatography on DEAE-thin layer than was possible on DEAE-paper. Moreover, the resolution obtained was better as the spots were less diffuse. In comparing the homomixtures described, mixtures *b* and *c*, which are both dialysed mixtures, give better resolution than mixture *a*, which usually gives slightly more streaky, though acceptable, results. The first two mixtures are thus preferable for separating mixtures of fragments of less than 30 residues long, while for larger fragments mixture *a* is best. The reason for the improved resolution with the dialysed mixtures may be due to removal of salt, as the conditions of dialysis do not result in the loss of a significant amount of nucleotide material from the mixtures. This would explain why the mobility of a given oligonucleotide is in fact slower with the dialysed than with the undialysed mixture. Thus it is possible that with the homomixtures *b* and *c* a true displacement chromatography is occurring.

The fractionation system described in this paper should be useful for separating fragments of ribonucleic acids of unknown sequence, especially for the larger RNA molecules where rather complex mixtures of products may be obtained.

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G. G. Brownlee and F. Sanger
M.R.C. Laboratory of Molecular Biology
Hills Road, Cambridge, CB2 2QH, Great Britain