SEPARATION OF ^{32}P-LABELLED RIBONUCLEIC ACID COMPONENTS. THE USE OF POLYETHYLENIMINE-CELLULOSE (TLC) AS A SECOND DIMENSION IN SEPARATING OLIGORIBONUCLEOTIDES OF '4.5 S' AND 5 S FROM *E. COLI*

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1. Introduction

Preparations of RNA from several strains of *E. coli* have been found to contain a low molecular weight species which migrates on acrylamide gels between 5 S and 4 S RNA and contains no minor bases. This RNA has a base composition of 100 ± 2, and is here referred to as '4.5 S'. Molecules of a similar size, also without minor bases, have been found in Krebs 2 ascites tumour cells [1] and in HeLa cells [2], and it has been suggested that they serve as precursors for transfer RNA. However, the role of the 4.5 S RNA in *E. coli* is not yet clear.

In attempts to sequence a ^{32}P-labelled sample of this species several problems were encountered which could not be resolved using the method of Brownlee and Sanger [3]. The work of Randerath and Randerath [4] using thin layer chromatography on polyethylenimine (PEI)-cellulose as a method for separating small oligonucleotides, suggested that this system might be a useful one for sequencing studies. Moreover, recent work by Southern and Mitchell [5] illustrates the usefulness of PEI-cellulose for DNA sequencing, and, from preliminary experiments, these authors also suggest it might be used for the analysis of enzymatic digest of RNA. The work described in this letter, together with work in progress, fully substantiates the value of PEI-cellulose TLC as a useful method, alongside ionophoresis on DEAE-paper [3], for fractionating and sequencing oligoribonucleotides. In the case of products obtained from 4.5 S by ribonuclease T_1 digestion, some separations and purifications could be accomplished by the TLC route that were not accomplished on ionophoresis. For example, the 3'-end of the molecule streaked badly on ionophoresis but ran as a discrete spot on PEI-cellulose, and two of the octanucleotides which were not separated by ionophoresis (fig. 2) were separated on the TLC system (cf insert, fig. 1).

2. Methods

^{32}P-labelled RNA was obtained from *E. coli* (MRE600) grown in the presence of ^{32}P-phosphate, essentially as described by Brownlee and Sanger [3]. It was isolated by the phenol method, precipitated from dilute salt solution with ethanol, and purified by acrylamide gel electrophoresis using a 10% acrylamide slab gel [6]. The bands corresponding to 5 S and 4.5 S RNA were located by autoradiography, cut out, and eluted with M sodium chloride solution: the latter material ran ahead of 5 S RNA on the gel, with a mobility corresponding to a base composition of approximately 100 nucleotides. Both materials were precipitated by the addition of ethanol, carrier (unlabelled) RNA being added to aid recovery.

Ribonuclease T_1 digest of 5 S and 4.5 S RNA were carried out at 37° for 30 min using an enzyme to substrate ratio of 1:20. The products were partially separated in one dimension by high voltage

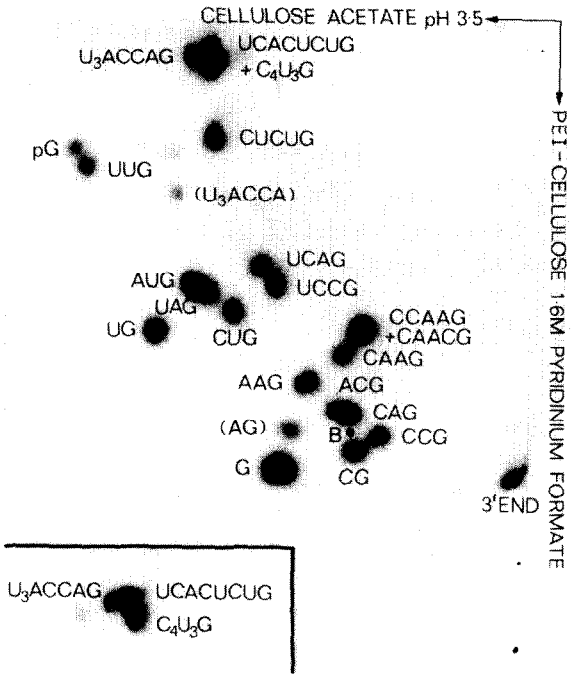

Fig. 1..A two-dimensional separation of the ribonuclease T_1 products of 4.5 S RNA from *E. coli* (MRE 600). Separation in the first dimension was by electrophoresis on cellulose acetate, pH 3.5 [3], and in the second by thin layer chromatography on PEI-cellulose, using 1.6 M pyridinium formate, as indicated. The insert shows the separation of all three octanucleotides, which was accomplished when 2.0 M pyridium formate was used to elute the TLC plate in a further experiment.

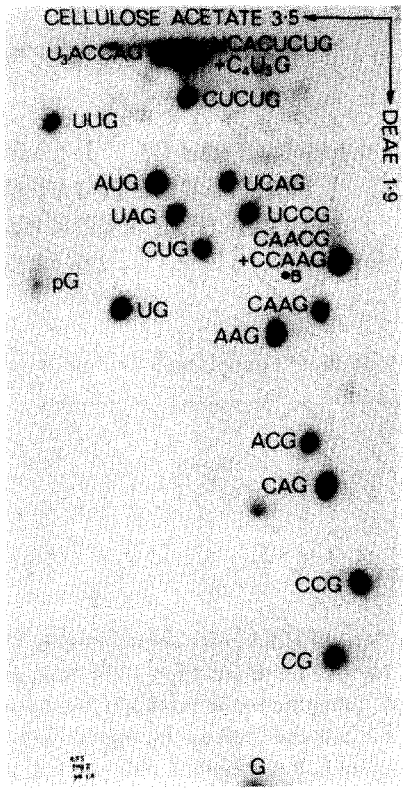

Fig. 2. Same as in fig. 1, except for separation in the second dimension, which was carried out by ionophoresis on DEAE-paper (43 × 84 cm) at pH 1.9 [3]. Missing from this photograph is the 3'-terminal fragment which runs near the origin in the first dimension and streaks in the second dimension.

electrophoresis on cellulose acetate paper at pH 3.5 in 7 M urea as described in [3]. For separation in the second dimension, thin layer chromatography on PEI-cellulose plates was carried out. The plates (20 × 40 cm) were made from a 1:1 mixture of MN 300 PEI-cellulose (1.0 meq/g) and MN 300 HR cellulose powder (both from Macherey, Nagel and Co., West Germany, purchased from Camlab (Glass) Ltd, Cambridge). In a typical procedure 8.5 g of MN 300 PEI-cellulose and 8.5 g MN 300 HR cellulose were stirred together in 110 ml water to a thick slurry, which was then homogenised in a fast electric blender for about 5 min, or until the mixture was uniformly dispersed. After de-aeration, the material was spread onto the glass plates in a manner similar to that described by Randerath and Randerath [4]. The plates were allowed to dry overnight at room temperature and, before use, the layer edges were made smooth and sharp. The ribonuclease T_1 products were blotted from the cellulose acetate strip directly onto the PEI-cellulose plate, as described for DEAE-cellulose plates by Brownlee and Sanger [7], and chromatographic separation was carried out in an ascending manner at room temperature until the solvent front was approximately one-quarter of the way from the top of the plate. In the examples shown (see figs. 1 and 3) the eluting solvent was made by dissolving 15 ml formic acid and 7.5 ml pyridine (both Analar, from BDH Chemicals Ltd, Poole, Dorset) in 7 M urea, to a volume of 250 ml. This is referred to as a 1.6 M solution in the following discussion. For other experiments, not herein described, where PEI-cellulose was used to fractionate partial enzymatic digests of RNA, solutions of higher concentration (2.0–3.0 M) were used to develop the plates [8]; in every case

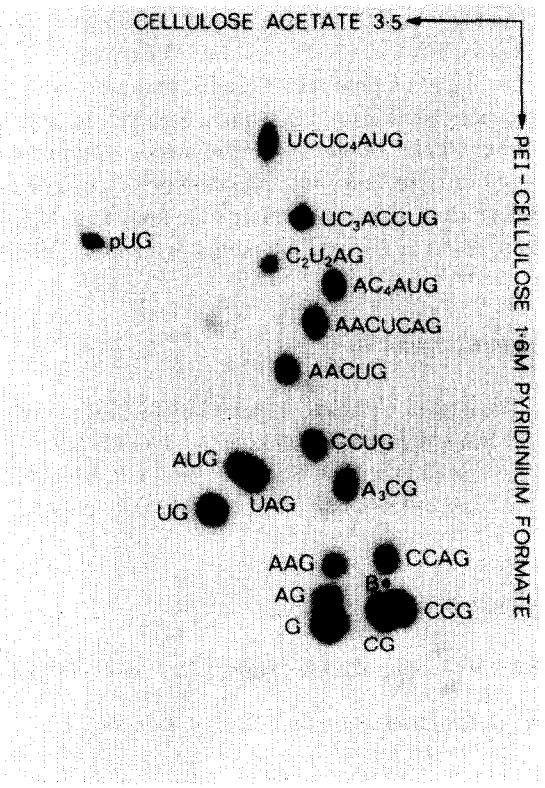

Fig. 3. A two-dimensional separation of the ribonuclease T_1 products of 5 S RNA from *E. coli* (MRE 600). Separation in the first dimension was by electrophoresis on cellulose acetate, pH 3.5 [3], and in the second by thin layer chromatography on PEI-cellulose, using 1.6 M pyridinium formate, as indicated. Missing from this photograph is the 3′-terminal product which runs slowest in the first dimension and fastest in the second. The product identifications are those made by Brownlee and Sanger [3].

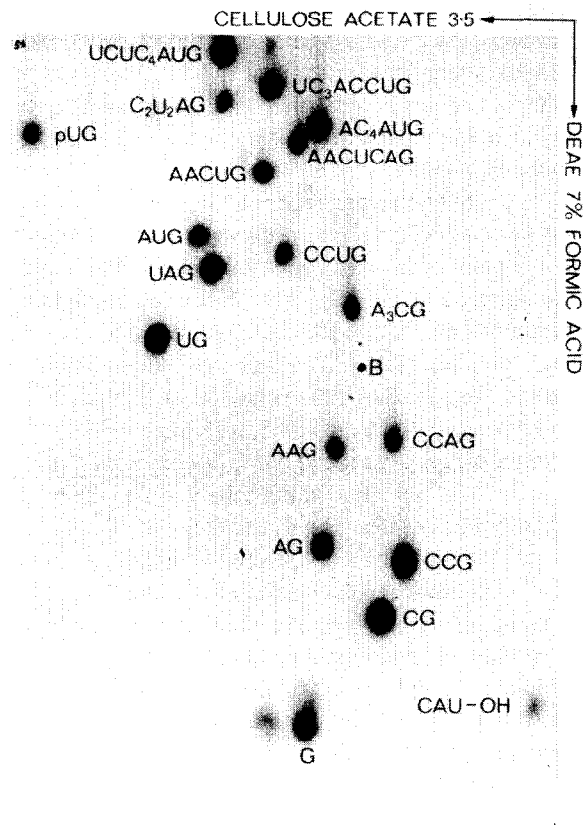

Fig. 4. Same as fig. 3, except for separation in the second dimension, which was carried out by ionophoresis on DEAE-paper (43 × 84 cm) in 7% formic acid.

good results were obtained when the volume ratio of formic acid to pyridine was kept at 2:1 and solutions made up in 7 M urea. The plates were allowed to dry at room temperature for a few hours before being autoradiographed.

For sequence determination, where relevant, the positions of the ^{32}P-labelled oligoribonucleotides were determined by autoradiography, and the spots removed from the plates and eluted as described for DEAE-cellulose plates [7]. Sequences were determined by a combination of chemical and enzymatic methods [3, 9].

Figs. 1 and 3 show the results obtained when the products from ribonuclease T_1 digests of 4.5 S and 5 S, respectively, were separated on PEI-cellulose, as described above. For comparison, separations of the same materials (albeit from different preparations) using ionophoresis on DEAE-paper are shown in figs. 2 and 4. Product sequences are as shown.

3. Results and discussion

4.5 S RNA appears to be a specific molecular species normally present in the several strains of *E. coli* examined to date. It contains no minor bases, and the sequence of its components obtained by ribonuclease T_1 or A digests does not immediately suggest a relationship with any of the *E. coli* RNA

molecules already sequenced. An RNA described by Hindley [10] may have contained this material, but Hindley's sample would appear to have contained other molecules as well, since the fingerprint he gave for a ribonuclease A digest is too complex to belong to the molecule described here as 4.5 S.

In studying this species a fractionating method has been worked out which involves a first dimension separation by electrophoresis on cellulose acetate paper at pH 3.5 [3], and a second dimension separation by TLC on PEI-cellulose, using formic acid–pyridine (2:1) in 7 M urea as eluting solvent. This is illustrated with the ribonuclease T_1 digests of 4.5 S and 5 S (figs. 1 and 3, respectively). The chromatography is carried out at room temperature in order to effect a maximum separation of the larger oligoribonucleotides. Chromatography at 60° increases the separation of the smaller oligoribonucleotides, somewhat at the expense of the larger ones. In some cases a 60° separation has proved a useful adjunct to the separation at room temperature.

In the example given (fig. 1) a 1.6 M buffer concentration was used for chromatography of 4.5 S products. This failed to separate two of the octanucleotides. However, in a separate experiment (cf. insert, fig. 1) when a 2.0 M buffer solution was used, complete separation of these two components was effected.

To date, the use of PEI-cellulose TLC as a second dimensional separation procedure for oligoribonucleotide mixtures, using conditions similar to those herein described, has found wide general application. For example, in addition to experiments discussed here and elsewhere [4, 5], it has been used for the fractionation of enzymatic digests of tRNA (1.5–2.0 M buffer), ribonuclease T_1 digests of whole R17 (2.5 M buffer), ribonuclease A digests of 5 S and 4.5 S after blocking the uracil (and guanine) residues with *N*-cyclohexyl-*N'*-(β-morpholinyl-4-ethyl) carbodiimide-methyl-*p*-toluene sulphonate (0.05 M buffer) [8], and for studies on the ribosomal 16 S precursor RNA [11]. The method is fast, easy to use, and gives reproducible results. It should find particular application for sequencing work in laboratories where extensive electrophoresis equipment is not available, and for sequencing non-radioactive nucleic acids by the polynucleotide kinase route [12], where some of the existing methods are not applicable.

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