ION-EXCHANGE THIN-LAYER CHROMATOGRAPHY

XVII. RESOLUTION OF OLIGONUCLEOTIDES FROM A PANCREATIC RIBONUCLEASE DIGEST OF RNA ON PEI-CELLULOSE*

K. RANDERATH AND E. RANDERATH

The Biochemical Research Laboratory and the John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, Boston, Mass. 02114 (U.S.A.)

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INTRODUCTION

In the preceding paper[1] we presented evidence that oligonucleotides obtained by enzymatic degradation of nucleic acids can be resolved by anion-exchange thin-layer chromatography on PEI-cellulose. The present communication will describe the chromatographic behavior of oligonucleotides in greater detail. A novel mapping procedure, which resolves all major mono-, di-, and trinucleotides from a pancreatic ribonuclease digest of RNA, will be described.

MATERIALS

For materials used in thin-layer chromatography, consult the preceding paper[1]. DEAE-cellulose, Whatman Chromedia DE 1 (Floc), lot No. 1349/57, 1 mequiv. N/g, was purchased from A. H. Thomas Co., Philadelphia, Pa., U.S.A. Ribonucleic acid purified according to CRESTFIELD *et al.*[2] and ribonuclease A, type 1A, 5 × crystallized, were supplied by Sigma Chem. Co., St. Louis, Mo., U.S.A. Thin-layer chromatography at elevated temperatures was carried out in a paper chromatography oven (No. 31492) and a separation chamber (No. 67319), obtained from Precision Scientific Co., Chicago, Ill., U.S.A.

METHODS

General

pH measurements were made with a Radiometer** pH-meter 22. All chromatographic systems were prepared from reagent grade chemicals.

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** Radiometer, Copenhagen NV, Denmark.

Pancreatic ribonuclease digestion of RNA

Four hundred mg RNA and 10 mg ribonuclease A were incubated in 30 ml 0.1 *M* Tris–HCl, pH 7.8, for 26 h at 25°.

Fractionation of oligonucleotides on DEAE-cellulose in the presence of urea[3–5]

Fifteen g DEAE-cellulose were washed successively with 3 l 1 *N* NaOH, 1 l 1 *N* HCl, 1 l 1 *N* NaOH, and water[6]. Fines were removed by sedimentation and the ion-exchanger was taken up in 300 ml starting buffer (0.05 *M* sodium acetate, 0.01 *M* Tris (base), 7.0 *M* urea, adjusted to pH 7.9 with acetic acid). After adjusting the pH to 7.9 the adsorbent was washed twice with 300 ml starting buffer. Finally, the material was suspended in 900 ml starting buffer and packed into a column (1.2 × 59 cm) under gradually increasing pressure (3–10 p.s.i.).

Fifteen ml of the digest (4200 O.D. units at 260 mμ) were diluted with 60 ml starting buffer and applied to the column. Elution was carried out with a linear gradient at a constant flow rate of about 35 ml/h for 50 h. The volume of the starting buffer was 1.5 l. The limit buffer (1.5 l) was 1.0 *M* sodium acetate, 0.01 *M* Tris, 7.0 *M* urea, pH 7.9 (acetic acid). Fractions were collected every 10 min. Ultraviolet transmittance at 254 mμ was recorded as a function of the tube number with an LKB* 4701 A ultraviolet absorptiometer and an LKB 6520 A chopper bar recorder, which were connected to a Beckman** model 132 fraction collector. Appropriate fractions of each peak (see below, Fig. 1) were pooled, and the O.D. at 260 mμ was determined.

Each pooled fraction was subsequently desalted by passing it through a fresh DEAE-cellulose column[7,8]. Fifteen g of washed (see above) DEAE-cellulose were taken up in 700 ml 0.01 *M* ammonium carbonate, pH 8.6, and the pH was adjusted to 8.6 with HCl. The DEAE-cellulose was washed with another portion (1 l) of ammonium carbonate at pH 8.6, subsequently suspended in 900 ml ammonium carbonate and poured into a column (4.5 × 20 cm) under pressure. Each oligonucleotide fraction was diluted 10–13 fold with 0.01 *M* ammonium carbonate and applied to the column. After the column had been washed with several hundred ml of 0.01 *M* ammonium carbonate, the oligonucleotides were eluted with 2.5 *M* ammonium carbonate, pH 8.6, at a flow rate of about 10 ml/min. 50-ml fractions were collected and their extinctions at 260 mμ determined. Fractions containing nucleotides were pooled and lyophilized. The residue of each pooled fraction was taken up in water (2–4 ml) and stored at —20°.

Thin-layer chromatography

Thin-layer procedures were generally as described in the preceding paper[1]. For separations at elevated temperatures, the oven mentioned in Section "Materials" was employed. Descending runs were always carried out in a water-saturated atmosphere. Oligonucleotides were characterized according to the procedures described in the preceding paper[1]. A great number of solvent systems was examined for their capacity to resolve oligonucleotides in the pancreatic ribonuclease digest.

A mixture of mono-, di-, and trinucleotides was mapped on a PEI-cellulose layer in the following way. The solution, which contained 2–10 mμmoles of each compound

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* LKB Instruments, Rockville, Md., U.S.A.
** Beckman Instruments, Spinco Division, Palo Alto, Calif., U.S.A.

in a volume of 150 μl, was applied in portions of 10 μl, without intermediate drying, close to one corner of a PEI-cellulose sheet (24 × 19.5 cm).

First dimension. After the starting area had been dried in a current of cool air, the layer was developed at right angles to the 24-cm edge by descending irrigation (see Section "Descending continuous-flow chromatography" of the preceding paper[1]) at 24–26° with water up to the folding line of the transfer flap followed by 0.09 *M* $MgCl_2$, 0.18 *M* Tris–HCl at pH 8.45 (24°). After a development time of 7 h and 20 min the wicks were cut off and the sheet was dried in a stream of cool air. The sheet was then treated for 10 min with 1 l anhydrous methanol, dried and treated for 15 min with a solution of 5 ml acetic acid in 1 l methanol. The chromatogram was dried and stored overnight at —20°.

Second dimension. A strip at right angles to the first dimension containing the nucleoside monophosphates, which were well separated from the oligonucleotides, was cut off and subjected to ascending chromatography with an acetic acid/LiCl system[9]. This procedure separated 3'-CMP and 3'-UMP. The remainder of the sheet was subjected to descending chromatography at 37° with water up to the folding line of the transfer flap followed by 0.5 *M* magnesium acetate (adjusted to pH 5.7 (25°) with acetic acid). After a development time of 7 h and 20 min the wicks were cut off and the sheet was dried and photographed in short-wave ultraviolet light.

Application of Gp-rich oligonucleotides in the presence of urea and subsequent development were carried out as follows. Thirty μl 8.5 *M* urea were added to a 5-μl sample containing about 5 mμmoles of GpGpCp and the mixture was heated at 100° for 2 min. The solution was then applied slowly in one portion without drying. For a subsequent descending run, the wet zone was covered with a piece of Parafilm until the solvent front reached the starting line. Development was carried out at room temperature with 0.15 *M* Tris acetate, pH 8.5, to the starting line followed, without intermediate drying, by 0.6 *M* magnesium acetate, pH 5.7, for several hours (continuous-flow chromatography).

RESULTS

Fractionation of the pancreatic ribonuclease digest in the presence of 7 M urea

Fig. 1 shows that the digest obtained under the conditions described above is resolved into ten major fractions, the first three of which (numbered 1, 2, and 3) have been characterized as containing mainly mono-, di-, and trinucleotides, respectively.

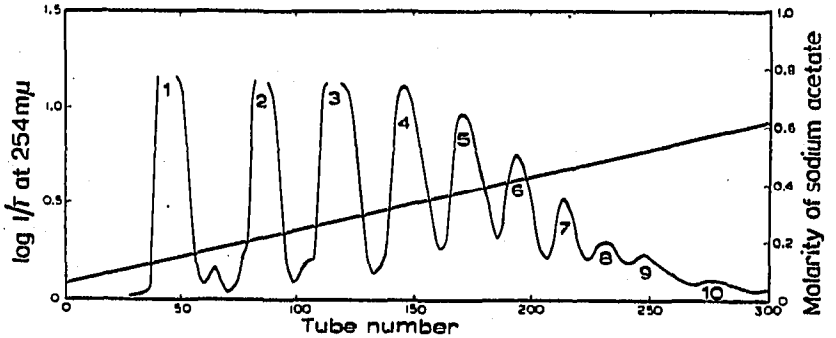

Fig. 1. Separation of a pancreatic ribonuclease digest of RNA on a DEAE-cellulose column with a linear gradient of sodium acetate, at pH 7.9, in the presence of 7 *M* urea.

The peaks numbered 4–10 presumably represent groups of oligonucleotides whose net negative charge is given by their respective numbers in the diagram[3,8].

The chromatographic behavior of the compounds obtained after desalting of the first three peaks was investigated in some detail, in the hope that it might be possible to infer the behavior of higher oligonucleotides from these results. Particular consideration was given to the problem of separating positional (sequential) isomers, *i.e.*, isomeric oligonucleotides differing only with regard to the sequence of the mononucleotide constituents.

Characterization of compounds in fractions 1–3 (Fig. 1)

Fraction 1 contained a mixture of 3′-CMP and 3′-UMP as the main components. These compounds were characterized by their chromatographic behavior on PEI-cellulose in an acetic acid/LiCl system[9] and by spectrophotometric analysis after elution.

Fraction 2 contained a mixture of four main components, which were degraded by alkaline hydrolysis to a mixture of nucleoside-2′- and -3′-monophosphates. From each of the four compounds two nucleoside monophosphates were obtained. Quantitative analysis of these compounds revealed a purine : pyrimidine ratio of 1:1. The ultraviolet spectra of the four compounds were in agreement with those reported by STANLEY AND BOCK[10] for the dinucleotides in pancreatic ribonuclease digests of RNA. From these data and from the behavior of the compounds in the DEAE-cellulose/urea system it could be concluded that peak 2 consisted of the four dinucleotides ApCp, ApUp, GpCp and GpUp.

Fraction 3 contained eight main components, which were degraded with LiOH to a mixture of nucleoside-2′- and -3′-monophosphates. For each compound, a purine: pyrimidine ratio of 2:1 was found. These results have been presented in Table II of the preceding paper[1]. The ultraviolet spectra of the compounds were in agreement with those reported by STANLEY AND BOCK[10] for the trinucleotides in pancreatic ribonuclease digests of RNA. From these data and from the column chromatographic behavior of the compounds it could be inferred that peak 3 consisted mainly of the eight trinucleotides ApApCp, ApApUp, ApGpCp, GpApCp, ApGpUp, GpApUp, GpGpCp, and GpGpUp.

Behavior of oligonucleotides under different conditions of elution

Among the solvents examined, weakly alkaline and weakly acidic buffer solutions proved to be most useful. A difficult problem was the resolution of positional isomers. The tendency of oligonucleotides rich in Gp to aggregate[11] on ion-exchange columns[12,13] was also noticed on PEI-cellulose layers and presented additional problems. Because we anticipated such difficulties to increase with increasing chain-length of the oligonucleotides, we used the trinucleotides as model substances to study the separation of positional isomers and to find conditions, which would reduce or eliminate the aggregation tendency of Gp-rich oligonucleotides.

(*a*) *Alkaline systems.* Tris–HCl systems at pH 8.5 were found to fractionate the individual members of oligonucleotide groups chiefly on the basis of their base composition. The four dinucleotides are completely separated by 1.0–1.3 *M* Tris–HCl at pH 8.5, the mobilities decreasing in the order ApCp > ApUp > GpCp > GpUp. GpCp and GpUp overlap with some of the trinucleotides. As pointed out in the

preceding paper[1], the eight trinucleotides in the RNA digest are resolved into 6–7 zones under the same conditions. The sixteen tetranucleotides are resolved into ten distinct zones by developing at 25° with water followed by 1.6 *M* Tris–HCl at pH 8.5 (ref. 14). Base analysis revealed the following order of decreasing mobility: ApApApCp > ApApApUp > (ApApGp)Cp > (ApApGp)Up > (ApGpGp)Cp > (ApGpGp)Up > GpGpGpCp > GpGpGpUp. The isomeric tetranucleotides terminating in Up are resolved into two fractions. (Complete resolution would yield three positional isomers). Several tetranucleotide fractions overlap with trinucleotides under these conditions.

Similar fractionations are obtained with Tris–HCl systems containing $MgCl_2$ (see below, Fig. 3) and with ammonium carbonate systems at pH 8.4–8.6. The elution pattern of the di- and trinucleotides closely resembles the order of elution obtained for these compounds on DEAE-cellulose columns at pH 8.6 (*cf.* ref. 10).

The overall resolution appears to be best at pH values around 8.5. At pH values between 7.5 and 8.0 only three dinucleotide and four trinucleotide zones are obtained. Increasing the pH to values around or above 9, on the other hand, results in enlarged zones because of the decrease in net positive charge of the exchanger.

The positional isomers ApGpCp and GpApCp could not be resolved at pH values between 8 and 9, whereas, in the pH range from 8.2 to 9, most systems separate the corresponding isomers terminating in Up. These results are in agreement with those reported by STANLEY AND BOCK[10] and by SYMONS *et al.*[15] for DEAE-cellulose columns at pH 8.6 and pH 8.5, respectively. It is interesting to note that neither pair of isomers could be resolved under similar conditions on DEAE-Sephadex columns[16].

(*b*) *Acidic systems.* The difficult separation of the positional isomers ApGpCp and GpApCp could be achieved best at pH values between 5.5 and 6.0. In this pH range the differences in mobilities of the corresponding isomers terminating in Up are also most pronounced. The trinucleotide isomers are well resolved with ammonium acetate buffers containing EDTA and with magnesium acetate solvents. In our experience, the presence of high magnesium concentrations in the solvent definitely improved the separation of the isomers.

At pH 6.5–7.0 and below 5.5 mobilities of the isomers were found to be less different. Buffer systems of pH 6.5 (ref. 10) and 7.0 (ref. 17) have been used to separate compounds not resolved at pH 8.6 on DEAE-cellulose columns. As far as we can see, more acidic eluants (pH < 6.5) have not been investigated so far for their capacity to resolve positional isomers by chromatography on modified celluloses. The possibility of separating ApGpUp from GpApUp (not the corresponding compounds terminating in Cp) on Dowex-1 columns with HCl/NaCl mixtures has been reported in the literature[13,18].

Fig. 2 depicts a separation of the trinucleotide isomers by a magnesium acetate system at pH 5.7. It will be noted that the amounts of the two isomers terminating in Cp in the digest are clearly different[17].

The Gp-rich trinucleotides GpGpCp and GpGpUp exhibit a strong tendency to aggregate under acidic and neutral conditions. We found this phenomenon to be influenced by the ambient temperature and also by the cation of the separating buffer. Ammonium, triethylammonium, lithium, and sodium acetate solvents at pH 5.5–6.0, with or without the addition of EDTA, resolve the isomeric trinucleotides, but GpGpCp and GpGpUp streak and remain partially at the origin. This tendency is much less pronounced with Tris, triethanolammonium or magnesium acetate systems.

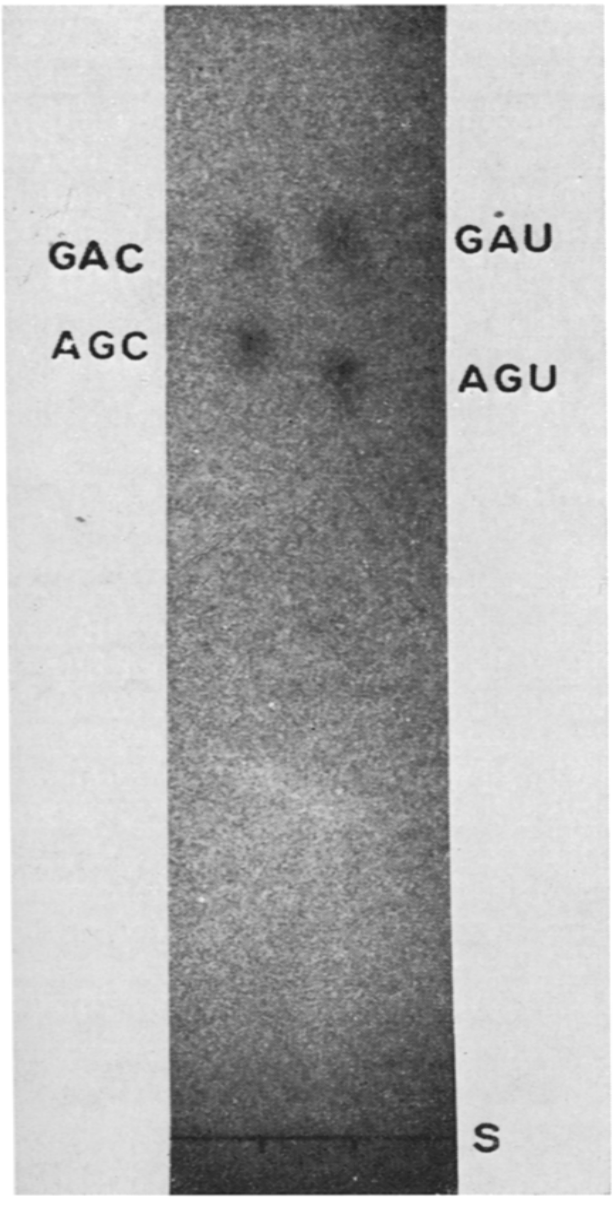

Fig. 2. Separation of positional trinucleotide isomers in a magnesium acetate system on a PEI-cellulose layer. Development (continuous-flow, descending): water to the folding line of the solvent transfer wick followed by 0.65 *M* magnesium acetate, pH 5.7, for 18 h at room temperature. GAC = GpApCp, etc. S = Starting line. Photographed by short-wave ultraviolet light.

The aggregation appears to be somewhat less dependent on the anion, although in buffers containing multivalent anions like citrate or EDTA relatively more of the Gp-rich compounds remains at the origin than in buffers containing only univalent anions. If the ambient temperature was kept at 35–40°, sharp zones were obtained with magnesium acetate solvents, and no U.V.-absorbing material remained at the origin. In the case of ammonium acetate solvents, streaking could not be eliminated by raising the temperature. Resolution of positional isomers is somewhat less distinct at 35–40° than at 25°. Trailing of Gp-rich compounds was found to be increased at low temperature (4°).

Aggregation of Gp-rich oligonucleotides was also observed in more acidic solvent systems. High concentrations of urea ($> 7\ M$) in the solvent appear to prevent aggregation, at least of shorter oligonucleotides, although it was, in our experience, not possible to resolve sequential isomers under such conditions.

Our results indicate that aggregation observed on the layer may be due to two processes. (1) Nucleotide material not moving from the origin during the run is probably caused by aggregation of nucleotides in the solution applied. That Gp-rich oligonucleotides form complexes of high molecular weight in aqueous solution has been demonstrated previously[11]. (2) Streaking of compounds is probably caused by reversible aggregation and dissociation during chromatography.

Initial aggregation of GpGpCp could be prevented by adding urea to the nucleotide solution and heating (see Section "Methods"). The pre-development with dilute alkaline buffer was required to remove the urea completely before the concentrated magnesium acetate buffer reached the area of application. (If this step was omitted, part of the nucleotide migrated within the urea zone.) No nucleotide material remained at the origin. GpGpCp migrated as a single, non-trailing spot.

Aggregation phenomena observed in the magnesium acetate system thus seem to be mainly due to initial aggregation in the solution applied. In ammonium acetate systems, on the other hand, where extensive trailing and smearing are observed, aggregation–dissociation phenomena appear to occur during the run in addition to the initial aggregation.

(*c*) *Mapping of mono-, di-, and trinucleotides.* Fig. 3 depicts a map obtained by a two-dimensional combination of a $MgCl_2$/Tris–HCl system with a magnesium acetate system (see "Methods", Section "Thin-layer chromatography"). It was necessary to adjust the pH of the layer with 0.5 % acetic acid in methanol prior to the development in the second dimension. Omission of this step caused a considerable distortion of the spot pattern.

Cytidylic and uridylic acids were developed separately in the second dimension because they migrated too fast in the magnesium acetate solvent under the conditions used.

Characteristics of descending anion-exchange thin-layer chromatography

As shown in Fig. 4, the distance from the origin travelled by individual mono-, di-, and trinucleotides is a linear function of time in descending continuous-flow chromatography on PEI-cellulose layers. In ascending chromatography, on the other hand, the rate of migration decreases during the run, a fact responsible for the lower degree of resolution characteristic for long-time ascending runs.

DISCUSSION

A critical evaluation of the methods currently available for the fractionation of oligonucleotides reveals a remarkable difference between ion-exchange column procedures, which afford a high degree of resolution, and conventional chromatographic–electrophoretic "sheet" techniques, which are considerably less effective, unless ion-exchange mechanisms are superimposed on the electrophoretic fractionation (as is the case in the mapping procedure of SANGER *et al.*[19]). This difference is not due to an intrinsic superiority of column procedures over sheet procedures, but

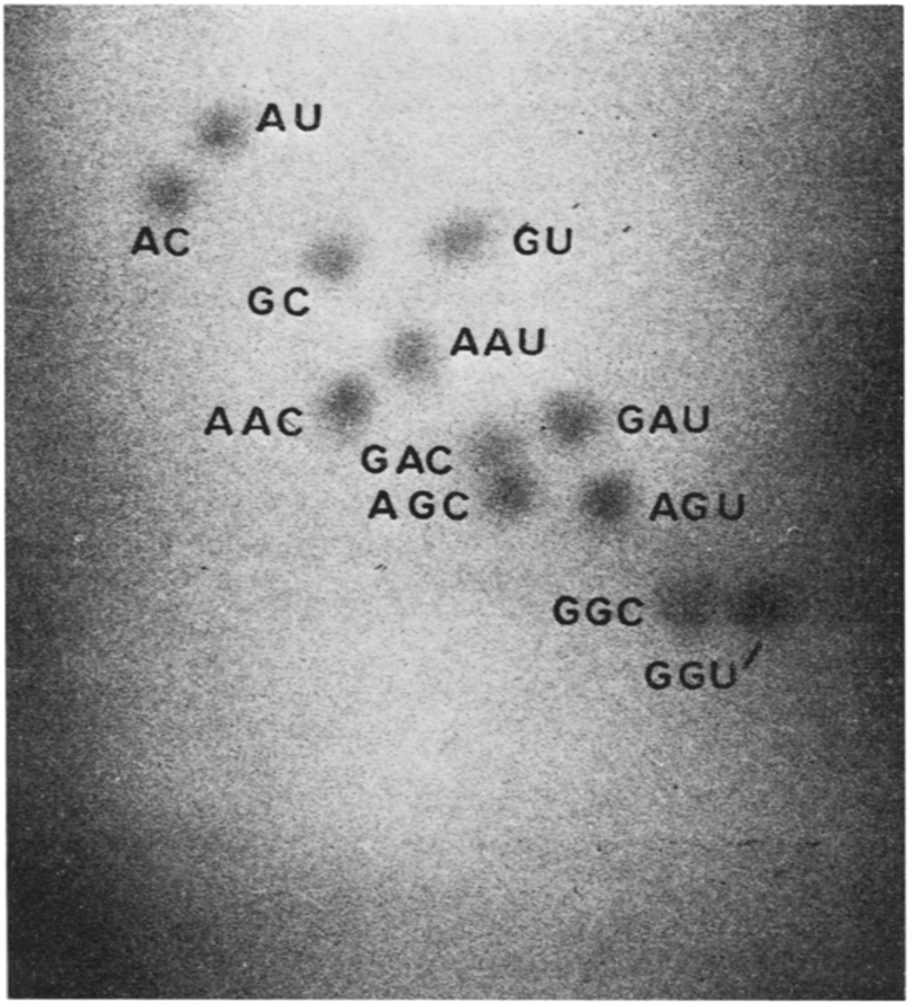

Fig. 3. Two-dimensional separation of di- and trinucleotides on a PEI-cellulose layer. First dimension from right to left, second dimension from bottom to top. Starting point is in the right-hand lower corner. AU = ApUp, etc. Photographed by short-wave ultraviolet light.

rather to the underlying physical principles of separation. We were led to assume, therefore, that ion-exchange sheet chromatography, under the appropriate conditions, would afford an equal or even greater degree of resolution than ion-exchange column techniques. This expectation appeared justified to us, because (1) there is ample evidence for the equal or superior resolution capacity of sheet chromatography, especially on thin layers, over column techniques, (2) two-dimensional chromatography is feasible on sheets, and (3) anion-exchange thin-layer chromatography had previously been shown to afford high resolution of mononucleotides.

That more difficult separations of oligonucleotides (for example, the resolution of positional isomers) can be achieved by ion-exchange sheet chromatography, has, to our knowledge, been demonstrated here for the first time. The resolution capacity of conventional mapping techniques by partition chromatography and electrophoresis on paper[20–24] or cellulose thin layers[25], in their present form, is considerably lower than that of column and thin-layer chromatography on cellulose ion-exchangers.

TYNDALL *et al.*[26] have presented preliminary data on the possibility of mapping enzymatic digests of RNA by two-dimensional ion-exchange chromatography

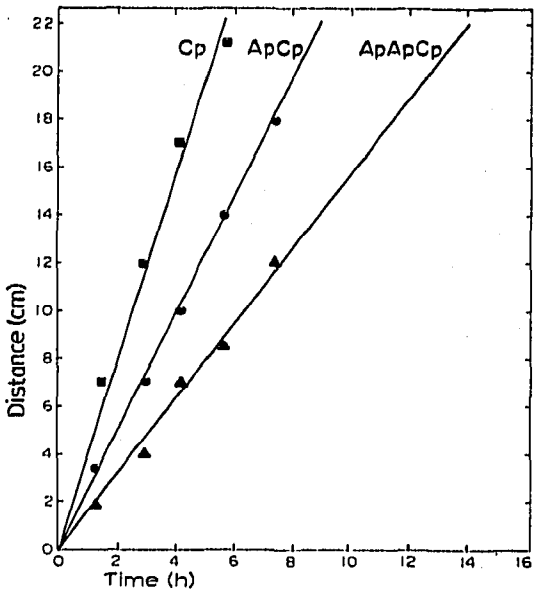

Fig. 4. Relationship between migration distance and development time in descending continuous-flow chromatography on a PEI-cellulose thin layer. Development: water to the folding line of the solvent transfer wick followed by 0.5 *M* magnesium acetate, pH 5.7, in a warm room (37°).

on DEAE-paper. According to SANGER *et al.*[19] considerably better separations are obtained by high voltage electrophoresis on DEAE-paper. Preliminary work in our laboratory[27] had indicated that thin-layer chromatography on DEAE-cellulose or PEI-cellulose also affords better resolution of oligonucleotides than DEAE-paper chromatography. Of the three materials, PEI-cellulose layers gave the most distinct chromatographic separations[27].

As the results presented in this and the preceding paper[1] indicate, anion-exchange thin-layer chromatography on PEI-cellulose is capable of resolving complex oligonucleotide mixtures. This novel method may be regarded as complementary to and may be used in conjunction with column chromatography and two-dimensional high-voltage electrophoresis[19] in studies on the primary structure of nucleic acids.

SUMMARY

The present paper gives detailed descriptions of oligonucleotide separations on thin layers of PEI-cellulose. The factors which influence the chromatographic behavior of these compounds are analyzed. A mapping procedure is presented which separates all common mono-, di- and trinucleotides in a pancreatic ribonuclease digest of RNA.

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