ION-EXCHANGE THIN-LAYER CHROMATOGRAPHY

XVI. TECHNIQUES FOR PREPARATION AND ANALYSIS OF OLIGONUCLEOTIDES*

E. RANDERATH AND K. 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.)

(Received July 24th, 1967)

INTRODUCTION

Effective and sensitive techniques for the fractionation of oligonucleotides are essential for the study of nucleotide sequences in nucleic acids. The methods used at present are anion-exchange column chromatography on modified cellulose[1-3], a two-dimensional combination of electrophoresis and chromatography on paper[4], and two-dimensional electrophoresis[5,6]. The recent elucidation of the complete primary structures of s-RNA species[7-11] depended largely on the application of column chromatographic methods for separation of oligonucleotide mixtures obtained by enzymatic digestion of purified s-RNA samples. With the exception of the procedure published recently by SANGER *et al.*[5,6], the resolution power of the conventional two-dimensional "mapping" procedures for oligonucleotides appears to be too low to be applied to complex oligonucleotide mixtures obtained by digestion of nucleic acids.

The procedure by SANGER *et al.*[5,6] affords a considerably better resolution. This system is, however, not well suited for the preparative isolation of larger quantities of the compounds, and it requires *in vivo* labeling of the nucleic acids with radioactive isotopes.

The high resolution power of PEI-cellulose** thin-layer chromatography for mononucleotides has been demonstrated in a series of papers from these laboratories[13-16]. More recently, we have systematically explored the suitability of this material for separating and preparing oligonucleotides. We found that the experimental procedures previously described for mononucleotide separations on PEI-cellulose had to be modified considerably in order to achieve some more difficult oligonucleotide separations.

This paper describes the general techniques we have developed for preparation and analysis of oligonucleotides. The accompanying paper[17] will report the resolution

* This work has been supported by grants-in-aid from the U.S. Public Health Service (AM 05507), the Wellcome Trust, the U.S. Atomic Energy Commission (AT(30-1)-2643) and the U.S. Public Health Service (CA 5018).

This is publication No. 1310 of the Cancer Commission of Harvard University.

** A cellulose anion-exchange material obtained by treating chromatography cellulose with poly(ethyleneimine)[12].

of all major mono-, di-, and trinucleotides obtained by digesting RNA with pancreatic ribonuclease.

MATERIALS

MN 300 Cellulose powder, manufactured by Macherey und Nagel, Düren, West Germany, and a Desaga applicator with adjustable slit width were supplied from Brinkmann Instruments, Westbury, N.Y., U.S.A. Poly(ethyleneimine) (Polymin P "BASF") was obtained from BASF Colors and Chemicals, Inc., 845 Third Avenue, New York, N.Y. 10022. Bakelite ® Rigid Vinyl Sheets (21 × 50 in.), type VSA 3310 clear 31 Matte 06, 0.010 in., manufactured by Union Carbide Corporation, Cincinnati, Ohio, U.S.A., were obtained from Commercial Plastics and Supply Corporation, 630 Broadway, New York, N.Y. 10012.

Intramedic ® polyethylene tubing PE 10 (I.D. = 0.011 in., O.D. = 0.024 in.) and PE 50 (I.D. = 0.023 in., O.D. = 0.038 in.) manufactured by Clay-Adams, Inc., New York, was obtained from E. F. Mahady Co., Cambridge, Mass., U.S.A. Tape with adhesive on both sides was Scotch ® brand double-coated tape No. 665. Syringes, Luer-Slip, B-D Yale, with glass tip (1 ml, 2 ml), and syringe needles, Luer-Lok, B-D Yale, stubs gauge 18, length 2 in., were purchased from A . H. Thomas Co., Philadelphia 5, Pa., U.S.A. Chromatography paper, phosphorylated cellulose, Whatman P 81, was obtained from the same company.

Trinucleotides from a pancreatic ribonuclease digest of yeast ribonucleic acid were prepared as described in the following communication[17]. The mixture contained about 0.2–0.5 mμmole/μl each of ApApCp*, ApApUp, GpApCp, ApGpCp, GpApUp, ApGpUp, GpGpCp, and GpGpUp.

Ribonuclease T1 (activity 330,000 U/mg, protein content 1.0 mg/ml) was obtained in 70 % saturated ammonium sulfate solution from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Dialyzer tubing, flat width 3/8 in., was purchased from Visking Company, Division of Union Carbide Corp., Chicago, Ill., U.S.A.

METHODS

Preparation of PEI-cellulose sheets

Rigid vinyl sheets (21 × 125 cm) were coated on one smooth side with a homogenized suspension of 22 g Cellulose MN 300 in 145 ml 1 % PEI hydrochloride solution (pH 6) using the Desaga applicator (slit width 0.5 mm). Details of the procedure were given previously[18]. Some cellulose batches required the addition of about 10 ml water to the PEI-cellulose suspension before homogenizing in order to obtain a uniform layer.

After the sheets had been allowed to dry in the air, they were cut into smaller pieces (usually 25 cm wide) and subjected to either one of the following washing procedures:

---

* For abbreviations of nucleotides, see "Abbreviated Instructions to Authors" as published in the issues of the *Journal of Biological Chemistry*. Oligonucleotides are written with the 5'-OH to the left and the 3'-OH to the right. The letter "p" to the left of the nucleoside symbol indicates a 5'-phosphate, the letter "p" to the right a 3'-phosphate. Phosphate groups are omitted in some cases; thus AAC indicates ApApCp.

1. The sheet was soaked for 1 min in 1 l 10% NaCl solution. Immersion was started from one end of the sheet and continued in slow and steady motion. After excess solution had been allowed to drain and the rear had been dried with a paper towel, the sheet was suspended using a clip attached to a cord and dried at room temperature for several hours. It was then soaked for about 5 min in 1 l distilled water and was again dried. Subsequently, the layer was developed at right angles to the coating direction by ascending irrigation with distilled water in a closed jar. When the front reached the top of the sheet, the jar was partially opened for 2–3 h so that the band of impurities at the top became very narrow.

2. The sheet was soaked for 1 min in 1 l 10% NaCl solution and dried as in procedure 1. The sheet was then soaked in 1 l distilled water for 5 min, transferred, without intermediate drying, to fresh distilled water (1 l), soaked for 10 min, and dried at room temperature. Large trays allowed several sheets to be washed simultaneously.

The dry sheets were kept at —20°.

Chromatography of trinucleotides

Ascending chromatography was always carried out in the direction of the ascending development with water. Descending chromatography was usually done in the coating direction. For continuous-flow chromatography, the impurity zone (washing procedure 1) was cut from the sheet.

(*a*) *Ascending continuous-flow chromatography*[18]. A sheet, 18 × 4 cm, with its long edge at right angles to the coating direction, was used. The starting line was marked at 2 cm from the bottom edge. A wick of Whatman No. 3 MM paper (16 × 4 cm) was attached to the top by stapling and folded back behind the uncoated side. Five μl of a trinucleotide mixture (see Section "Materials") was applied in one portion and the start spot was dried with cool air. The chromatogram was developed with water up to the starting line followed, without intermediate drying, by 1.3 *M* Tris–HCl (pH 8.5 at 25°) for 4 h. After development the wick was cut off and the sheet was dried in a stream of cool air and examined under short-wave U.V.-light. (To make the background appear uniform under U.V.-light, the sheet may be soaked in 300 ml anhydrous methanol for 5–10 min.)

(*b*) *Descending continuous-flow chromatography*. A sheet, 25 × 4 cm, with its short edge at right angles to the coating direction, was used. The starting line was marked at 2 cm from one narrow edge and 5 μl of the trinucleotide mixture was applied. A 5 cm long wick of Whatman No. 1 paper was sewed onto the sheet so that layer and paper overlapped 0.8 cm. The paper was folded against the layer at a distance of 4 cm from its end to serve as a solvent transfer flap. A glass bar (28 × 2 × 1 cm) was attached with double-coated tape to the rear of the sheet between 1.2 and 3.2 cm from the edge of the sheet. The glass bar was not allowed to overlap with the seam. A thick absorbent wick consisting of three 20 cm long Whatman No. 3 MM paper sheets laid on top of each other was stapled to the bottom edge of the PEI-cellulose sheet. The length of the wick was chosen so that during development the solvent front did not reach its lower edge.

For descending chromatography, ordinary paper chromatography equipment (rectangular jar, 24 × 12 × 12 in., with corresponding solvent trough assembly) was used. The atmosphere of the tank was saturated with water vapor by lining the walls with wet filter paper in the usual way. The glass bar attached to the rear of the sheet

served as a support of the chromatogram; it was laid on the rack holding the solvent trough so that the transfer wick was immersed into the solvent. For transferring the chromatogram from one solvent to another, it was taken out of the solvent vertically and the edge of the transfer flap was blotted briefly with a paper towel.

The chromatogram was developed with water up to the folding line of the transfer flap followed, without intermediate drying, by 1.2 *M* Tris–HCl (pH 8.5 at 25°) for 16 h. The solvent front migrated about 15 cm on the wick. After chromatography the wick was cut off and the sheet was dried in a stream of cool air and examined under U.V.-light.

(*c*) *Pre-equilibration*. Sheets were pre-equilibrated with the buffer to be used for subsequent descending or ascending development and compounds were applied to the wet sheets. Descending chromatography was carried out in the following way.

A sheet, 25 × 4 cm, the short edge at right angles to the coating direction, was used. The starting line was marked at 4.5 cm from one narrow edge. As described previously[18], a small part of the sheet itself served as a solvent transfer flap. The sheet was laid carefully, layer side down, on the surface of the solvent (300 ml), 1 *M* Tris–HCl (pH 8.5 at 25°), starting slowly from one end and continuing in slow and steady motion. After it had been soaked for 10 min, it was blotted with Whatman No. 1 paper. Five μl of the trinucleotide solution was applied immediately in one portion. During application of the sample the layer (except the starting area) was kept covered with a plastic sheet to prevent evaporation. The sheet was then folded along a line drawn on the uncoated side at 3.5 cm so that the layer formed an angle of about 45°. The antisiphon rod of commercial paper chromatography equipment was attached with adhesive tape to the uncoated side of the solvent transfer flap between 3.0 and 3.4 cm from the end of the sheet. A wick consisting of two 32 cm long Whatman No. 3 MM paper sheets was attached to the bottom part of the chromatogram by stapling. All operations were done quickly to minimize evaporation.

The sheet was then developed with the same buffer in a water-saturated atmosphere at 25° for 16 h using commercial paper chromatography equipment. The solvent front migrated about 27 cm on the wicks.

A paper wick could also serve as a transfer flap (see Section "Descending continuous-flow chromatography") instead of folding the sheet. In this case the wick was attached prior to the equilibration.

Preparative chromatography

Dry start and continuous-flow development were used for preparative chromatography. The sample was applied either by hand or a sample streaker.

(*a*) *Application by hand*. The sample was applied with a capillary pipette extended with flexible capillary tubing. A 1 μl-Carlsberg pipette, the upper part of which served as a calibrated (50 μl, 100 μl) reservoir, was well suited for this purpose. Two connected pieces (each about 1.2 cm long) of polyethylene tubings PE 10 and PE 50, the narrow tubing PE 10 pushed about 2 mm into the PE 50 tubing, were attached to the tip of the pipette. The narrow polyethylene tubing prevented damage of the layer during application and ensured that the sample was applied slowly. Application of the sample in a straight line was accomplished with the aid of a ruler (which was not allowed to touch the layer at the line of application).

(*b*) *Application by a sample streaker*. The sample streaker was built as described

by BACON[19]. The solution was applied with a syringe fastened to the streaker. A piece of polyethylene tubing PE 10 (7 cm) was pushed into a piece of PE 50 tubing (0.5–0.7 cm), which was then pushed about 2 mm into the glass tip of the syringe. The syringe needle, which was bent in the middle at an angle of about 100°, was attached to the syringe so that the tubing protruded about 1 cm from inside the needle. The needle was turned arround until the polyethylene tubing touched the layer. While the streaker was moved slowly and steadily along a ruler parallel to the starting line, the solution was delivered continuously from the syringe.

For the preparation of trinucleotides, 500 μl of the mixture were applied to a sheet (25 × 16 cm, the short edge at right angles to the coating direction, washed according to procedure 1) at a starting line 2 cm from one narrow edge. One cm on either side was left free. The applicator was moved several times along the starting line without intermediate drying. After application of the sample the starting zone was dried in a stream of cool air. Further preparation of the sheet and development were carried out as described in Section "Descending continuous-flow chromatography".

Preparative isolation of trinucleotides

The preparative chromatogram of the trinucleotides described in the previous paragraph was desalted[13] with two 1-l portions of anhydrous methanol (10 min each). The six major substance zones (see below, Fig. 1) were marked with a pencil under U.V.-light. The partially resolved band (No. 4 in Fig. 1) was treated as a single zone. The bands were then cut from the sheet.

Elution was carried out in paper chromatography solvent troughs: 10 ml 0.5 *M* ammonium carbonate (pH 8.6) was poured into the trough and the cut-out was placed slowly, layer side down, on the solvent surface starting from one end, while the other end was held with a pair of tweezers. Compounds were eluted for 15 min at room temperature; the trough was agitated occasionally.

After the cut-out had been removed with a pair of tweezers, the eluate was filtered through Whatman No. 1 paper (which had been pre-washed with ammonium carbonate and water) on a small Büchner funnel to remove a few PEI-cellulose particles. The trough was rinsed with 2 ml 0.1 *M* ammonium carbonate and the washing liquid was filtered also. The combined filtrate was diluted with water to give a 0.1 *M* ammonium carbonate solution. In order to remove small amounts of poly(ethyleneimine) from the sample, the diluted eluate was then filtered slowly through 6 layers of wet phosphate paper (4.2 cm diameter) on a Büchner funnel. The phosphate paper had been thoroughly pre-washed with 0.5 *M* ammonium carbonate and water by soaking and rinsing on the funnel.

The filtrate was lyophilized and the residue, which contained some cellulose components soluble in ammonium carbonate beside nucleotide, was taken up in 1 ml of hot water (60°). The solution was transferred to a small tube and lyophilization was repeated. The residue was taken up in 400 μl hot water. Drying in two steps rather than in one improved the recoveries.

Determination of recoveries

Ten μl each of compound 3 and compound 6 (Fig. 1), and three 5-μl portions of the original trinucleotide mixture were chromatographed in the system described in Section "Descending continuous-flow chromatography". After the chromatogram

had been desalted with 1 l methanol for 15 min, compounds 3 and 6, the corresponding spots from the original mixture, and appropriate blank areas were marked with a pencil and cut out. The cut-outs were placed, layer side down, onto the bottom of 5-ml beakers, and 500 μl 1.5 *M* LiCl, 0.02 *M* Tris–HCl, pH 7.4, were added slowly with a Carlsberg pipette so that the cut-out floated on the surface. Elution was carried out at room temperature for 1 h with occasional agitation.

The eluates were centrifuged at 2000–3000 r.p.m. for 5 min. Extinctions at 260 mμ of compounds were measured against the corresponding blank solutions in cells of 2 mm inside width and 1 cm light path in a Zeiss PMQ II spectrophotometer. Recoveries of compounds 3 and 6 were calculated from the extinction values at 260 mμ on the basis of the extinction values of the reference eluates from the original trinucleotide mixture.

Base analysis of trinucleotides

(*a*) *Hydrolysis.* Trinucleotides prepared as described above were hydrolyzed with alkali to give 2'- and 3'-ribonucleoside monophosphates. Fifteen-μl portions of 1 *N* LiOH were added to 60-μl samples of compounds 1, 2, 5, and 6 (containing about 0.4 mμmole trinucleotide/μl), and 10-μl portions of 1 *N* LiOH were added to 40-μl samples of compounds 3 (containing about 0.8 mμmole trinucleotide/μl) and 4 (containing about 1 mμmole trinucleotide/μl). All samples were hydrolyzed at 37° for 19 h.

After hydrolysis about 15.5 μl 1 *N* HCl were added to samples 1, 2, 5, and 6, and about 10.5 μl 1 *N* HCl were added to samples 3 and 4 so that the solutions became slightly acidic. After addition of HCl all samples were taken to dryness in a stream of air at room temperature. HCl was removed completely (this was verified by examining the outcoming air with wet pH paper). The dry residues of samples 1, 2, 5, and 6 were taken up in 150 μl water and the residues of samples 3 and 4 in 100 μl water, so that the concentration of LiCl was 0.1 *M* (pH 5–6).

(*b*) *Chromatography of hydrolyzed samples.* The six hydrolyzed trinucleotides were applied to six 5 cm wide PEI-cellulose sheets in 10-μl portions without intermediate drying (starting line at 2 cm from the bottom edge). After the total sample had been applied, the starting area was dried in a stream of cool air. In order to remove residual LiCl, each sheet was soaked in 200 ml anhydrous methanol for 10 min.

The dry sheets were developed with 1 *N* acetic acid up to 4 cm above the starting line followed, without intermediate drying, by 0.3 *M* LiCl up to 14 cm[20]. After the chromatograms had been dried in a stream of cool air, they were treated with 200 ml anhydrous methanol for 10 min. The dry chromatograms could be stored at —20° overnight before elution.

(*c*) *Elution of nucleoside-2'- and -3'-monophosphates.* All hydrolysis products and corresponding blank areas were cut out; 2'-AMP and 3'-AMP (see below, Fig. 2) were treated as a single zone. Elution was carried out as described in the Section "Determination of recoveries", with 500 μl or, in the case of the two adenylic acids, 1000 μl 1 *M* LiCl, 0.02 *M* Tris–HCl, pH 7.4, for 1 h. The eluates were centrifuged for 5 min at 2000–3000 r.p.m. Spectra from 220 to 290 mμ of all compounds were measured against the corresponding blanks in cells of 2 mm inside width and 1 cm light path. Base compositions were calculated from the extinction values and the molar extinction coefficients at 260 mμ[21].

Sequential analysis of trinucleotide isomers by ribonuclease T1 degradation

(*a*) *Preparation of positional isomers.* The mixtures of the positional isomers GpApCp + ApGpCp and GpApUp + ApGpUp isolated by thin-layer chromatography as described above were separated into the individual isomers as follows.

Sixty μl of a solution of (ApGp)Cp* (about 0.35 mμmole/μl) and 60 μl of a solution of (ApGp)Up (about 0.45 mμmole/μl) were applied as 3 cm long streaks to a PEI-cellulose sheet, 25 × 12 cm, at a distance of 2 cm from the 12-cm edge. Descending continuous-flow chromatography was carried out as described above. The chromatograms were developed at 25° with 0.2 *M* Tris–CH_3COOH (molarity of free acid plus anion), pH 5.7, up to the folding line of the paper wick followed, without intermediate drying, by 1.7 *M* Tris–CH_3COOH, pH 5.7, for 18 h. Each mixture was resolved into two compounds: (ApGp)Cp_f + (ApGp)Cp_s, and (ApGp)Up_f + (ApGp)-Up_s (f = fast component, s = slow component). Isolation of the compounds was carried out as described in Section "Preparative isolation of trinucleotides". The residues of the samples were finally taken up in 50 μl water.

(*b*) *Enzymatic degradation of positional isomers.* Ammonium sulfate in the ribonuclease T1 preparation was removed by dialysis. Fifty μl of the enzyme solution were diluted with 150 μl water and dialyzed against 2 l distilled water at 4° overnight. After dialysis the enzyme solution was removed from the tubing as completely as possible with a pipette and was stored at —20°. This solution (250 μl) was used for digestion of the trinucleotides.

Two μl buffer (0.2 *M* Tris–HCl, 0.005 *M* EDTA, pH 7.6) and 4 μl enzyme solution were added to 30 μl of the above solutions of (ApGp)Cp_f, (ApGp)Cp_s, (ApGp)-Up_f, and (ApGp)Up_s respectively. The samples were kept at 37° for 30 min. They could be stored at —20° or chromatographed immediately.

(*c*) *Chromatography of ribonuclease T1 digests of trinucleotide isomers.* The four samples obtained according to Section (b) were applied directly to four 5 cm wide PEI-cellulose sheets in 10-μl portions without intermediate drying at 2 cm from the bottom edge. Corresponding reference substances were also applied (about 4 mμmoles each): 2'- + 3'-GMP, 2'- + 3'-CMP, and ApCp** beside the digests of (ApGp)Cp_f and (ApGp)Cp_s; and 2'- + 3'-GMP, 2'- + 3'-UMP, and ApUp** beside the digests of (ApGp)Up_f and (ApGp)Up_s. After the starting areas had been dried with cool air, the sheets were treated with 300 ml anhydrous methanol for 10 min.

Chromatograms of ribonuclease T1 digests of (ApGp)Cp_f and (ApGp)Cp_s were developed by ascending irrigation with water up to the starting line followed, without intermediate drying, by 0.75 *M* LiCl up to 11 cm.

Chromatograms of ribonuclease T1 digests of (ApGp)Up_f and (ApGp)Up_s were developed similarly with water up to the starting line followed by 1 *M* Tris–HCl (pH 8.5 at 25°) up to 11 cm.

After chromatography each sheet was desalted with 500 ml methanol for 10 min. Mono- and dinucleotide areas were marked under U.V.-light and cut out.

All mononucleotides and corresponding blanks were eluted with 500 μl 1 *M* LiCl, 0.02 *M* Tris–HCl, pH 7.4, for 1 h at room temperature and spectra of the centrifuged eluates were recorded.

---

* The parentheses indicate a mixture of positional isomers. (ApGp)Cp thus represents a mixture of ApGpCp and GpApCp.

** Dinucleotides were prepared in an analogous way as described for trinucleotides[17].

(*d*) *Analysis of dinucleotides obtained from trinucleotide isomers.* The dinucleotides were eluted with 600 μl 0.5 *M* ammonium carbonate, pH 8.6, for 15 min at room temperature. The eluates were centrifuged and 500 μl aliquots were lyophilized. The residues were taken up in 40 μl water and alkaline hydrolysis was carried out by treatment with 10 μl 1 *N* LiOH for 19 h at 37°.

Further treatment of the samples, chromatography, and elution were performed as described in Section "Base analysis of trinucleotides". Spectra of nucleoside monophosphates were recorded.

RESULTS

Properties of PEI-cellulose sheets

The advantages of the plastic backing for the anion-exchange layer have been discussed in a previous paper[18]. The paper-like sheets have proven to be particularly valuable in our work with oligonucleotides, because they can be cut to any size desired, and quantitative and preparative procedures are greatly facilitated by the possibility of cutting the substance areas from the sheets. More complex oligonucleotide mixtures, which require longer development distances, may be resolved on long sheets, and the compounds may then be isolated on an analytical or preparative scale directly from the cut-outs.

Different chromatographic procedures for trinucleotides

Three chromatographic procedures for the separation of the trinucleotides ApApCp, ApApUp, (ApGp)Cp, (ApGp)Up, GpGpCp, and GpGpUp were studied:

(a) Dry start; ascending continuous-flow development.

(b) Dry start; descending continuous-flow development.

(c) Pre-equilibration; wet start; descending continuous-flow development.

Procedure c is analogous to column chromatography, where the ion-exchanger is pre-equilibrated with the solvent to be used for subsequent development, whereas procedures a and b are similar to techniques described for mononucleotides on PEI-cellulose[13–16,18]. In the case of the dry start procedures it is, however, essential to develop the sheet briefly with water (or a very dilute buffer) prior to development with the concentrated buffer. This results in more compact substance areas and allows the compounds to migrate only after the right pH has been established in the layer.

Table I lists R_{ApApCp} values obtained for trinucleotides by the three different chromatographic procedures.

As can be seen from Table I, the mixture of the eight trinucleotides is separated into 6–7 components by each of the three procedures. The positional isomers GpApUp and ApGpUp are partially resolved. The same sequence of R_{ApApCp} values applies in each case. The sequential isomers GpApCp and ApGpCp could not be resolved at this pH, whereas the isomers GpApUp and ApGpUp are almost completely resolved by each system, a fact which demonstrates a comparable effectiveness of the three systems.

Preparative isolation of trinucleotides

Preliminary experiments indicated that for preparative chromatography of trinucleotides, wet start development was not well suited. It was, for example, difficult

TABLE I

R_{ApApCp} VALUES OF TRINUCLEOTIDES IN TRIS–HCl SOLVENT SYSTEMS AT pH 8.5

Procedures: (a) Dry start; ascending continuous-flow development; solvents: water followed by 1.3 *M* Tris–HCl (pH 8.5); 4 h.
(b) Dry start; descending continuous-flow development; solvents: water followed by 1.2 *M* Tris–HCl (pH 8.5); 16 h.
(c) Pre-equilibration with 1 *M* Tris–HCl (pH 8.5); wet start; descending continuous-flow development; solvent: 1 *M* Tris–HCl (pH 8.5); 16 h.

Trinucleotide	*Procedure a*	*Procedure b*	*Procedure c*
ApApCp	1.0	1.0	1.0
ApApUp	0.89	0.83	0.75
GpApCp + ApGpCp	0.75	0.68	0.55
GpApUp	0.64	0.54	0.37
ApGpUp	0.58	0.48	0.34
GpGpCp	0.44	0.35	0.22
GpGpUp	0.31	0.24	0.19

to obtain straight bands when large volumes of samples were applied to wet sheets, presumably because of uneven displacement of buffer around the starting area. Dry start development was preferred therefore. The sample streaker described by BACON[19] fitted with a modified syringe made possible an even application of large sample volumes.

Fig. 1 shows a preparative chromatogram of the trinucleotide mixture; development was carried out using a descending continuous-flow technique with water followed by 1.2 *M* Tris–HCl (pH 8.5) as described in Table I (procedure b).

Beside the main components several weaker bands were visible when larger amounts were applied. These compounds probably represent nucleoside-3′,5′-diphosphates and trinucleotides containing minor bases.

The loading capacity of the PEI-cellulose layers is quite high. A total of 110 mμmoles trinucleotides/cm, *i.e.* about 100 μg/cm, was applied in the chromatogram of Fig. 1. Because there is still ample space between the substance zones, except for the (ApGp)Up isomers, it is possible to separate a total of about 400 μg trinucleotides/cm.

For elution, volatile buffers were used, after the solvent electrolytes had been removed with methanol. We found 0.5 *M* ammonium carbonate (pH 8.6) well suited to elute all trinucleotides quantitatively from PEI-cellulose. In this procedure small amounts of soluble cellulose and of poly(ethyleneimine) are eluted also. Such impurities impair the recoveries of oligonucleotides. When the residues after lyophilization are treated with water, part of the nucleotides stays adsorbed to an insoluble residue, which consists of aggregated cellulose and poly(ethyleneimine). Trinucleotides in the presence of poly(ethyleneimine) alone are completely soluble in water and cellulose alone adsorbs only negligible amounts of the compounds. Recoveries can therefore be greatly improved by removing poly(ethyleneimine) with a cation exchanger, *e.g.*, cellulose phosphate paper.

For a semiquantitative determination of the PEI concentration in solutions containing ammonium carbonate, a modified eosin staining test based on the procedure of POSCHMANN[22] was used[23]. The amount of PEI eluted from the layer with 0.5 *M*

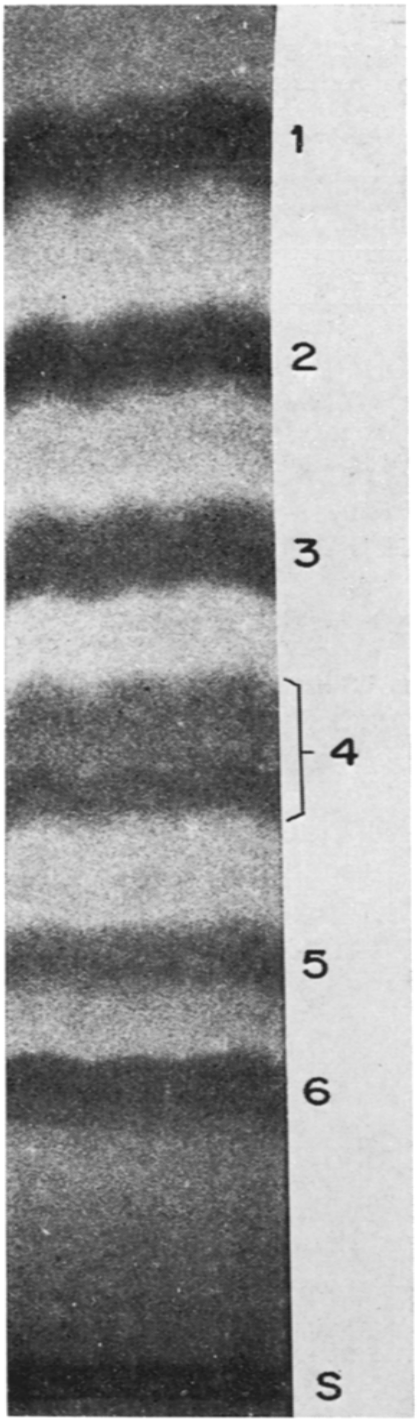

Fig. 1. Preparative chromatogram (section) of a trinucleotide mixture. Conditions, see procedure b in Table I. S = starting line; 1 = ApApCp; 2 = ApApUp; 3 = (ApGp)Cp; 4 (double zone) = (ApGp)Up; 5 = GpGpCp; 6 = GpGpUp. Photographed by short-wave ultraviolet light.

ammonium carbonate (pH 8.6) at 25° in 15 min was found to be about 3 μg/cm^2 layer. The preparative samples isolated from zones of 15 cm^2 thus contained about 45 μg PEI. These amounts of PEI can be removed quantitatively by passing the sample through several layers of phosphate paper. Even amounts in excess of 2 mg PEI were removed almost quantitatively under these conditions.

Total recoveries of trinucleotides determined by thin-layer chromatographic comparison of aliquots from the isolated compounds with aliquots from the initial mixture were usually between 65 and 70 %. Elution with 1.5 *M* LiCl, 0.02 *M* Tris–HCl, pH 7.4, was found to be quantitative. Absolute amounts of trinucleotides were calculated by using the molar extinction coefficients at 260 mμ of these compounds[24].

Base analysis

Hydrolysis with LiOH resulted in quantitative degradation of the oligonucleotides to a mixture of nucleoside-2′- and -3′-phosphates. After neutralization with HCl the monophosphates were further analyzed on PEI-cellulose layers. It was not necessary to remove LiCl prior to application. The sample had only to be diluted to give a LiCl concentration $\leqslant$ 0.1 *M*. Even large volumes of such samples (200 μl and more) could be applied, because the salt concentration is low enough to allow a concentration of the nucleotides in the center of the starting area. LiCl was then removed with methanol[13]. Development with a stepwise acetic acid/LiCl system separated the nucleoside monophosphates[20].

Fig. 2 is a schematic representation of the chromatograms of the trinucleotide hydrolysates. The base composition of each compound can be inferred directly from the respective chromatogram. Elution of each nucleoside monophosphate with 1 *M* LiCl, 0.02 *M* Tris–HCl, pH 7.4, and subsequent recording of the U.V.-spectra provided an unequivocal identification of the compounds.

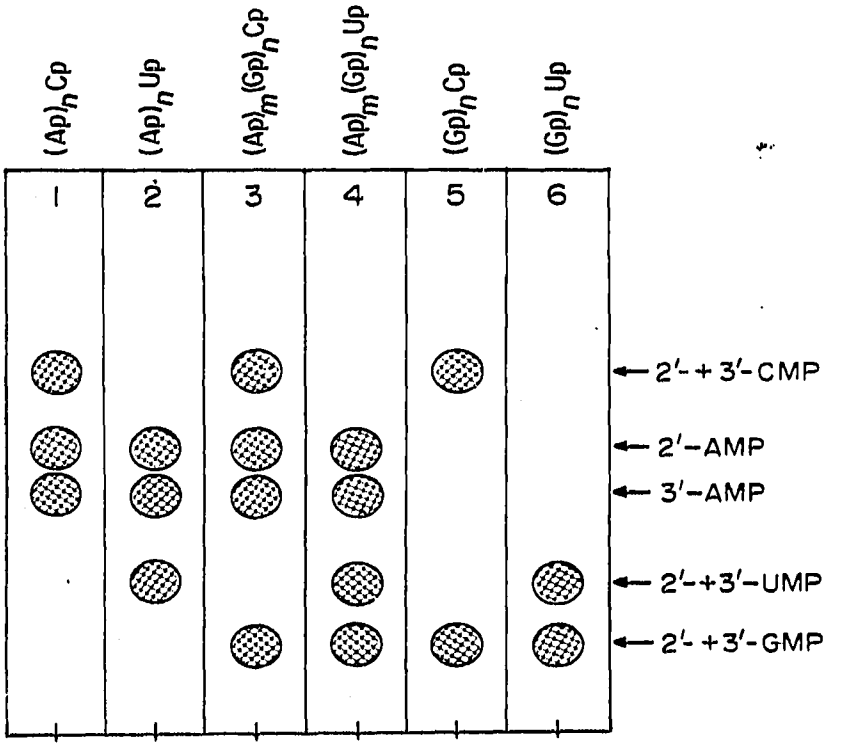

Fig. 2. Schematic representation of chromatograms of LiOH hydrolysates of oligonucleotides. The numbers 1–6 correspond to the numbers of the compounds in Fig. 1. The general structure of the compounds is indicated at the top of each chromatogram.

Table II lists the base ratios found for the compounds of Fig. 1. It can be seen that the results are in close agreement with theory.

TABLE II

BASE RATIOS OF TRINUCLEOTIDES[a]

No. of compound zone[b]	*Trinucleotide (20–30 mμmoles)*	*2'- + 3'-CMP*	*2'- + 3'-AMP*	*2'- + 3'-UMP*	*2'- + 3'-GMP*
1	ApApCp	1	2.02		
2	ApApUp		1.90	1	
3	(ApGp)Cp	1	1.03		1.06
4 (double zone)	(ApGp)Up		1.06	1	0.99
5	GpGpCp	1			2.16
6	GpGpUp			1	1.99

[a] Base ratios are expressed relative to the 3'-terminal pyrimidine nucleotide. The following molar extinction coefficients ($\varepsilon \times 10^{-3}$ at 260 mμ, pH 7) were employed: 7.3 for CMP, 9.9 for UMP, 15.4 for AMP, and 11.6 for GMP.

[b] See Fig. 1.

Base analysis of the dinucleotides ApCp, ApUp, GpCp, and GpUp, isolated by thin-layer chromatography, gave similar results.

Sequential analysis of trinucleotide isomers by ribonuclease T1 degradation

Positional trinucleotide isomers were resolved in a Tris–acetic acid system at pH 5.7 and isolated as described above in Section "Methods". Sequential analysis was carried out by degradation with endoribonuclease T1, which cleaves polyribonucleotides specifically between 3'-guanylic residues and the 5'-hydroxy residues of the adjacent nucleotides[25]. These trinucleotides yield the following degradation products:

$$\text{GpApCp} \xrightarrow{\text{RNase T1}} \text{Gp} + \text{ApCp}$$

$$\text{ApGpCp} \xrightarrow{\text{RNase T1}} \text{ApGp} + \text{Cp}$$

$$\text{GpApUp} \xrightarrow{\text{RNase T1}} \text{Gp} + \text{ApUp}$$

$$\text{ApGpUp} \xrightarrow{\text{RNase T1}} \text{ApGp} + \text{Up}$$

Under appropriate conditions, *i.e.* in the presence of relatively high enzyme concentrations[5], the intermediate 2',3'-cyclic nucleoside phosphates (2',3'-cyclo-GMP and ApG-cyclic-p) are converted quantitatively to nucleoside-3'-phosphates. Optimum reaction conditions were determined with the dinucleotides GpUp and GpCp as substrates. These conditions were then used in the trinucleotide degradations (see Section "Methods").

The solvents described in Section "Chromatography of ribonuclease T1 digests of trinucleotide isomers", were capable of separating all reaction products. The following orders of R_F values were found:

(a) in the water/LiCl system: 3'-CMP > 3'-GMP > ApCp > ApGp*,

* In the reference chromatograms 3'-GMP preceded 2'-GMP, and 3'-CMP preceded 2'-CMP.

(b) in the water/Tris–HCl system: 2′- + 3′-UMP > 2′- + 3′-GMP > ApUp > ApGp.

As expected, two major components were obtained in each degradation, the compounds with the higher R_F values being nucleoside monophosphates, which were identified spectrophotometrically after elution:

$$(ApGp)Cp_f^* \xrightarrow{\text{RNase T1}} 3'\text{-GMP} + \text{dinucleotide 1}$$

$$(ApGp)Cp_s \xrightarrow{\text{RNase T1}} 3'\text{-CMP} + \text{dinucleotide 2}$$

$$(ApGp)Up_f \xrightarrow{\text{RNase T1}} 3'\text{-GMP} + \text{dinucleotide 3}$$

$$(ApGp)Up_s \xrightarrow{\text{RNase T1}} 3'\text{-UMP} + \text{dinucleotide 4}$$

Identification of the nucleoside monophosphates combined with the results obtained by alkaline hydrolysis established the nucleotide sequence:

$(ApGp)Cp_f$ = GpApCp
$(ApGp)Cp_s$ = ApGpCp
$(ApGp)Up_f$ = GpApUp
$(ApGp)Up_s$ = ApGpUp

For further confirmation, the dinucleotides obtained by enzymatic digestion of the isomers were eluted, hydrolyzed with LiOH and rechromatographed in the acetic acid/LiCl system (see above). Nucleoside monophosphates were identified on the basis of their mobilities and spectra. The following mononucleotides were obtained:

$$\text{Dinucleotide 1} \xrightarrow{\text{LiOH}} 2'\text{-} + 3'\text{-AMP} + 3'\text{-CMP}$$

$$\text{Dinucleotide 2} \xrightarrow{\text{LiOH}} 2'\text{-} + 3'\text{-AMP} + 3'\text{-GMP}$$

$$\text{Dinucleotide 3} \xrightarrow{\text{LiOH}} 2'\text{-} + 3'\text{-AMP} + 3'\text{-UMP}$$

$$\text{Dinucleotide 4} \xrightarrow{\text{LiOH}} 2'\text{-} + 3'\text{-AMP} + 3'\text{-GMP}$$

The amounts of trinucleotides analyzed in this way were about 3 mμmoles of GpApCp, and about 7 mμmoles each of ApGpCp, GpApUp and ApGpUp.

DISCUSSION

The results presented indicate that oligonucleotides in enzymatic digests of nucleic acids can be separated by anion-exchange thin-layer chromatography on PEI-cellulose. The method combines advantages of both column ion-exchange techniques and "sheet" separations by partition chromatography or electrophoresis on

* For abbreviations of the unidentified trinucleotide isomers, see Section "Methods", under "Sequential analysis of trinucleotide isomers by ribonuclease T1 degradation".

paper or thin layers. It is equally well suited for preparative isolation as for subsequent analysis of the oligonucleotides. The high resolution obtainable makes possible two-dimensional "mapping" of nucleic acid digests either with or without a preliminary fractionation into compound groups (see the following paper[17]).

Our results further indicate that, similar to mononucleotide separations on PEI-cellulose[13–16,18], wet start chromatography on pre-equilibrated layers is not required in the majority of cases. In contrast to most mononucleotides, oligonucleotides tend to "streak", however, if a single buffer is used. Although elution with continuous electrolyte gradients can be carried out on ion-exchange layers[26], discontinuous, "stepwise" elution[13] suffices in most instances. For the separation of the members of an oligonucleotide group, *e.g.*, the trinucleotide group, in a digest, a brief preliminary development with water or dilute buffer followed by concentrated buffer results in similar resolution as the technically more difficult wet start development on a sheet that has been preconditioned with buffer. The preliminary development apparently serves to establish the buffer pH in the layer prior to migration of the compounds. (This appears to be mainly due to diffusion effects; electrolyte concentration and pH of the mobile phase change gradually in such a discontinuous elution procedure).

PEI-cellulose layers, due to their high ion-exchange capacity, are capable of separating compounds on a milligram scale. For example, on a 20–25 cm wide sheet, up to 10 mg of the trinucleotide mixture obtained by pancreatic ribonuclease digestion of ribosomal RNA can be separated into six zones of different base composition. Isolation of the compounds is more convenient from thin layers than from columns, where each eluate has to be desalted by passing it over a fresh column.

Nucleotides obtained by the preparative extraction procedure described in the present paper contain small amounts of soluble cellulose. In our experience, this material did not interfere with chromatography and enzymatic reactions performed with the isolated substances.

Our results demonstrate also that ion-exchange thin-layer chromatography is well suited for rapid structural analysis of oligonucleotides after digestion by chemical and enzymatic methods.

The novel LiOH/HCl procedure was chosen for chemical degradation rather than the conventional KOH/$HClO_4$ procedure[27], because neutralization and removal of salt were easier to carry out on a microscale this way. In view of the small relative standard deviations of mononucleotide assays on PEI-cellulose layers[18,20], it can be anticipated that higher oligonucleotides can be analyzed also by this technique.

To establish the sequence of the trinucleotides, it was necessary to analyze further the positional isomers (ApGp)Cp and (ApGp)Up only, because one can derive the sequence of the four other compounds from the results of alkaline degradation. The isomers were further characterized by degradation with ribonuclease T1[25]. The products obtained in this reaction suffice to establish the nucleotide sequence in this case. For the characterization of higher oligonucleotides, however, additional enzymes, such as phosphomonoesterases and phosphodiesterases, will be required. Direct analysis of these reactions on PEI-cellulose layers appears to be possible[23]. The fact that these enzymatic reactions can be followed directly on the layers will certainly facilitate a rapid identification of longer oligonucleotides.

Although only results pertaining to smaller oligonucleotides have been presented, preliminary experiments show that ion-exchange thin-layer techniques may

be adapted for larger oligonucleotides in nucleic acid digests as well[23]. The combination of great sensitivity and high loading capacity characteristic for this technique will greatly contribute to its usefulness as an analytical tool in sequential analysis of nucleic acids.

SUMMARY

The present paper reports the separation, isolation, and analysis of oligonucleotides on anion-exchange (PEI-cellulose) thin layers. Detailed procedures are presented for separation, preparative isolation, and further chemical and enzymatic characterization of trinucleotides obtained by digestion of RNA with pancreatic ribonuclease.

REFERENCES

1 G. M. TENER, H. G. KHORANA, R. MARKHAM AND E. H. POL, *J. Am. Chem. Soc.*, 80 (1958) 6223.
2 M. STAEHELIN, in J. N. DAVIDSON AND W. E. COHN (Editors), *Progress in Nucleic Acid Research*, Vol. 2, Academic Press, New York, London, 1963, p. 169.
3 R. V. TOMLINSON AND G. M. TENER, *J. Am. Chem. Soc.*, 84 (1962) 2644.
4 G. W. RUSHIZKY AND C. A. KNIGHT, *Virology*, 11 (1960) 236.
5 F. SANGER, G. G. BROWNLEE AND B. G. BARRELL, *J. Mol. Biol.*, 13 (1965) 373.
6 G. G. BROWNLEE AND F. SANGER, *J. Mol. Biol.*, 23 (1967) 337.
7 R. W. HOLLEY, J. APGAR, G. A. EVERETT, J. T. MADISON, M. MARQUISEE, S. H. MERRILL, J. R. PENSWICK AND A. ZAMIR, *Science*, 147 (1965) 1462.
8 H.G. ZACHAU, D. DÜTTING AND H. FELDMANN, *Z. Physiol. Chem.*, 347 (1966) 212.
9 H. FELDMANN, D. DÜTTING AND H. G. ZACHAU, *Z. Physiol. Chem.*, 347 (1966) 236.
10 D. DÜTTING, H. FELDMANN AND H. G. ZACHAU, *Z. Physiol. Chem.*, 347 (1966) 249.
11 J. T. MADISON, G. A. EVERETT AND H. KUNG, *Science*, 153 (1966) 531.
12 K. RANDERATH, *Angew. Chem.*, 74 (1962) 780; *Angew. Chem., Intern. Ed. Engl.*, 1 (1962) 553.
13 K. RANDERATH AND E. RANDERATH, *J. Chromatog.*, 16 (1964) 111.
14 E. RANDERATH AND K. RANDERATH, *J. Chromatog.*, 16 (1964) 126.
15 J. NEUHARD, E. RANDERATH AND K. RANDERATH, *Anal. Biochem.*, 13 (1965) 211.
16 K. RANDERATH AND E. RANDERATH, *Anal. Biochem.*, 13 (1965) 575.
17 K. RANDERATH AND E. RANDERATH, *J. Chromatog.*, 31 (1967) 500.
18 K. RANDERATH AND E. RANDERATH, *J. Chromatog.*, 22 (1966) 110.
19 M. F. BACON, *Chem. Ind. (London)*, (1965) 1692.
20 E. RANDERATH AND K. RANDERATH, *Anal. Biochem.*, 12 (1965) 83.
21 *Circular OR-10*, Pabst Laboratories, Milwaukee, Wisc., 1956; see also ref. 24.
22 F. J. POSCHMANN, *Tappi*, 40 (1957) 487.
23 E. RANDERATH AND K. RANDERATH, unpublished experiments, 1966–1967.
24 W. M. STANLEY, JR. AND R. M. BOCK, *Anal. Biochem.*, 13 (1965) 43.
25 F. EGAMI, K. TAKAHASHI AND T. UCHIDA, in J. N. DAVIDSON AND W. E. COHN (Editors), *Progress in Nucleic Acid Research*, Vol. 3, Academic Press, New York, London, 1964, p. 59.
26 R. G. STICKLAND, *Anal. Biochem.*, 10 (1965) 108.
27 J. N. DAVIDSON AND R. M. S. SMELLIE, *Biochem. J.*, 52 (1952) 594.