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FRACTIONATION OF OLIGONUCLEOTIDE ISOPLITHS BY ELECTROPHORESIS ON POLYACRYLAMIDE GELS

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SUMMARY

A new procedure has been described for the analysis of an enzymatic digest of [^{32}P]RNA. It has been shown that oligonucleotides of equal chain length (isopliths) may be subfractionated into components of different composition by electrophoresis on polyacrylamide gels. Separation appears to be due primarily to differences in net charge. The distribution of components within the gel was determined by slicing the gel and counting ^{32}P by Cerenkov radiation in a scintillation counter. Since the method is simple and rapid it lends itself to the analysis of many samples and may be useful as a 'fingerprinting' procedure for RNA.

INTRODUCTION

We have recently described a method for comparing RNA molecules in which selected oligonucleotides derived from them were analyzed by column chromatography[1]. Although a limited number of compositional isomers* were separated by the technique employed at that time, the use of appropriate double isotope labels permitted the demonstration of significant differences between 18S and 28S ribosomal RNA molecules. This has established the usefulness of the experimental approach. We have now investigated a different method for separating compositional isomers which, because of its speed and increased resolution, may extend the number of RNA species which can be compared in this manner.

In the present study we have shown that oligonucleotides of equal size (isopliths) may be subfractionated into their compositional isomers by electrophoresis on polyacrylamide gels in acidic buffers. Other workers have shown that these gels are capable of separating high molecular weight RNAs[2–4], large RNA fragments[5] and oligonucleotides[6,7] on the basis of molecular size. Under the conditions which we have employed, the separation of compositional isomers appears to be due to differences in

* For purposes of this discussion, the term 'compositional isomer' is used to denote a class of oligonucleotides whose members have identical nucleotide compositions, but not necessarily identical sequences. No other generally accepted term is in use at the present time.

their net charge. Since the running time is short (2–3 h) and many samples can be analyzed concurrently, the procedure should be particularly useful for comparing RNA molecules by their oligonucleotide 'fingerprints'.

Techniques for separating oligonucleotides have been the subject of a recent review by RUSHIZKY AND SOBER[8].

MATERIALS AND METHODS

Preparation of ^{32}P-labeled ribosomal RNA

Ribosomal RNA was prepared from Ehrlich ascites tumor cells, grown in the peritoneal cavity of mice[9] for eleven days. Each of two animals was injected with 1.5 mCi of $[^{32}P]H_3PO_4$ in 0.5 ml of spinner culture medium (Grand Island Biological, neutralized with NaOH). After a period of 40 h the animals were sacrificed and the tumor cells collected. The cells were washed in cold saline and cytoplasmic extract was prepared by a method similar to that described for HeLa cells[10]. For 10 min prior to their disruption, the cells were allowed to swell in a hypotonic medium containing 0.01 *M* Tris-HCl, 0.01 *M* NaCl, 0.003 *M* $MgCl_2$, pH 7.4. The cells were disrupted with a stainless steel Dounce homogenizer, and nuclei removed by centrifugation. Ribosomal RNA was purified from the cytoplasmic extract by a hot phenol-detergent method[11]. Transfer RNA and any contaminating DNA was removed by precipitating the high molecular weight RNA with 2.5 *M* LiCl[12]. The precipitate was washed twice with 1 *M* LiCl. The initial specific activity of the purified RNA preparation was 6×10^6 d.p.m./mg.

Enzymatic hydrolysis of RNA

Seven milligrams of ^{32}P-labeled ribosomal RNA and 200 units of ribonuclease T1 (Sankyo, obtained from Calbiochem, Los Angeles) in 2 ml of 0.05 *M* Tris-HCl, pH 7.4, were incubated at 37° for 3 h. HCl was added (final concentration, 0.1 *N*) to hydrolyze cyclic phosphodiester bonds. After 30 min, the incubation mixture was neutralized with NaOH, and further maintained at 37° for 16 h. The digestion was terminated by the addition of three volumes of 10 *M* urea.

Chromatography of enzymatic digest of RNA at neutral pH

The products of digestion were separated into isopliths (fragments of equal chain length) by chromatography on DEAE-Sephadex at neutral pH. The conditions of chromatography and preparation of the ion exchanger have been described previously[1]; further details are given in the legend to Fig. 1.

Concentration and desalting of oligonucleotides

Appropriate fractions from the neutral pH chromatogram, corresponding to isopliths of the desired chain length, were pooled and the oligonucleotide material was concentrated as follows. Zinc chloride (1 ml of a 0.5 *M* solution) was added to 100 ml of pooled eluate. The pH of the solution was adjusted to 8–9 by the addition of NaOH. The solution was allowed to stand with occasional stirring for 20 min, during which time a zinc hydroxide precipitate formed. The suspension, combined with a water rinse of the container, was centrifuged at 1500 × *g* for 2 min. The pellet was suspended in 15 ml of water and centrifuged in a 15 ml graduated conical tube.

At this stage, the oligonucleotides were tightly bound to the zinc hydroxide gel. Nucleotides were released and simultaneously the zinc was removed in the following way. The gel was well dispersed in 2.5 ml water; 0.95 ml of oxalic acid (0.5 *M*) was quickly added, and the tube immediately mixed vigorously. The addition of the acid solubilized the gel, and after a few seconds delay, zinc oxalate precipitated. After 15 min, the zinc oxalate was removed by centrifugation (2000 × *g* for 5 min) leaving the oligonucleotides and approximately 10 m*M* oxalic acid (pH 2) in the supernatant fluid. Although satisfactory for many applications, the ionic strength was still too high for electrophoresis on polyacrylamide gels.

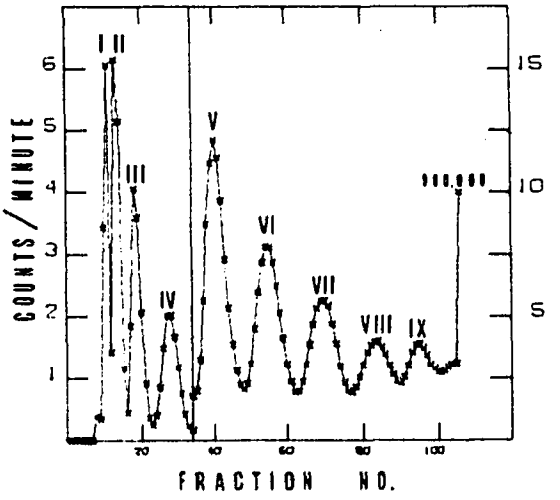


Fig. 1. Chromatography at neutral pH of a ribonuclease T_1 digest of ^{32}P-labeled ribosomal RNA. The digest was applied to a DEAE-Sephadex A-25 column (0.9 × 20 cm) and eluted with a linear gradient of NaCl (0.17–0.28 *M*) in 7 *M* urea–0.02 *M* Tris-HCl (pH 7.4) at 0.5 ml/min. Total volume of gradient was 1 l. Left ordinate: counts per min × 10^{-5}; right ordinate: (× 10^{-4}) refers to fractions to the right of the vertical line. The final fraction combines counts eluted from the column by 0.5 *M* NaCl.

The remaining oxalic acid was removed by dialysis. The solution was transferred to Visking dialysis tubing (8/32) and dialysed for a total of 3 h against three changes of water (2 l each). Dialysis tubing was prepared by soaking it in water for 5 min and rinsing it immediately before use. At the completion of dialysis, the pH of the sample was 4–5. The sample was dried in an Evapo-mix (Buchler Instruments, Fort Lee, N.J.) at a temperature below 40°. The nucleotides were dissolved in 50–100 μl of 5 *M* urea solution.

Electrophoresis of oligonucleotides on polyacrylamide gels

Polyacrylamide gels were prepared using the following reagents: acrylamide (recrystallized from chloroform as described by LOENING[2]); N,N'-methylenebisacrylamide; N,N,N',N'-tetramethylethylenediamine (TEMED); these were obtained from Eastman Organic Chemicals. Stock solutions contained: (a) acrylamide, 12.5%, bisacrylamide, 0.125% (both w/v); (b) TEMED-acetate, 10% (TEMED adjusted to pH 7 with acetic acid); (c) ammonium persulfate, 10% (w/v), freshly prepared. For 40 ml of 8% acrylamide gel mixture, 25.6 ml of (a), 0.2 ml of (b), and 14.0 ml of water were combined. The mixture was degassed for several seconds under vacuum,

then 0.2 ml of (c) was added and the preparation gently mixed. Acrylic plastic tubes (0.625 cm I.D. by 18 cm) were filled to a height of 15 cm. After 1 h, polymerization was complete and the gels were electrophoretically equilibrated with the required buffer (see RESULTS) for 16 h at 250 V.

For electrophoresis, 20–50 μl of oligonucleotide solution was layered above a vertical gel. Upper and lower reservoirs were filled with buffer and the whole apparatus was air-cooled with a 10 in. fan in a 4° room. At an applied voltage of 400 V, the current flow was 2–6 mA/gel, depending upon the particular buffer used. Other details are given in the legends to the figures.

Following electrophoresis, gels were fractionated into 1.4 mm thick disks using a semi-automatic slicer[13]. Removal of the gel from the plastic tube was facilitated by using a stream of water from a fine steel tube connected to a syringe. The gel was transferred to a trough filled with 50% glycerol for lubrication, then to the slicer. Disk-shaped slices were collected in plastic scintillation vials. The slices were immersed in 1 ml of 0.02 *M* NH_4OH, and the vials counted directly in a Beckman LS-250 scintillation counter. Efficiency of counting ^{32}P by Cerenkov radiation[14] was about 45% under these conditions.

Composition of oligonucleotides

The composition of oligonucleotides separated by gel electrophoresis was determined after eluting them from the gel slices. Eighty to 90% of the counts were recovered in the NH_4OH which had been added to the scintillation vials. Appropriate eluates were pooled and 0.1 ml of zinc chloride (0.5 *M*) was added per 10 ml. The zinc hydroxide precipitate which formed was allowed to stand for 20–30 min, then collected by centrifugation. The zinc was removed with oxalic acid as described above. The supernatant fluid was made 0.3 *N* with respect to KOH, and oligonucleotides were hydrolyzed for 18 h at 37°. The solution was diluted to 5 ml with water and acidified by the addition of 0.2 ml of 3 *M* formic acid. It was slowly filtered through a bed of activated charcoal (0.2 ml of 30%, supported on a 1 in. Whatman GF/A glass fiber filter). The charcoal was washed with water and the nucleotides eluted with 5 ml of ethanol–1 *M* NH_4OH (1:1). The ethanol–ammonia was removed under vacuum on a Buchler Evapo-mix, following which the mononucleotides were dissolved in 100 μl of ethanol–ammonia.

Mononucleotides were separated by high voltage paper electrophoresis on a flat-plate apparatus (Savant Instruments, Hicksville, N.Y.). The samples, to which were added 100 μg of each of the four ribonucleotides, were applied to Whatman No. 3 MM paper as 2 cm streaks. The buffer system was 0.1 *M* sodium citrate, pH 3.9 (measured at 23°). Voltage gradients of 40–50 V/cm were applied for 2–2.5 h. Under these conditions, the nucleotides were well separated. Using UV absorbance as a guide, the nucleotide spots were cut out. The paper was cut into 3 mm squares and counted in glass vials containing 15 ml of scintillator (Liquifluor, 160 ml to 3.8 l of toluene, Pilot Chemicals).

Other materials

Urea solutions used for column chromatography were purified to remove an insoluble residue which otherwise contaminated the desalted oligonucleotide solutions. To 1 l of a 10 *M* solution of urea (J. T. Baker Chemicals), 10 ml of 0.5 *M* zinc chloride,

2 ml of 5 *M* NaOH and 2 ml of Norit (30%) were added. Zinc hydroxide precipitated and the suspension was permitted to stand overnight. The urea solution was filtered through a Whatman GF/A glass fiber filter, then through a Millipore membrane (0.45 μ pore size). The final product had an absorbance of 0.01 units at 260 nm and 0.10 units at 230 nm; a 7 *M* solution did not interfere with the precipitation of oligonucleotides by zinc hydroxide (Table I), and little insoluble material accompanied the concentrated oligonucleotides.

Graphs were plotted by a Calcomp Incremental Plotter with a CDC G-20/3100 computer system.

RESULTS

Concentration and desalting of oligonucleotides

Oligonucleotides eluted from a DEAE-Sephadex column were concentrated and desalted as described in METHODS prior to analysis by gel electrophoresis. The recovery at each stage of the purification scheme is shown in Table I, expressed as percent recovered at each step. The overall recovery is the product of the individual recoveries. In two separate experiments the overall recovery ranged from 66–90%. A critical step in the procedure is the addition of oxalic acid. The zinc hydroxide gel must be well dispersed in water in order to obtain its complete dissolution before the zinc oxalate precipitates. Losses associated with dialysis seemed to be due predominately to handling and not through the membrane itself.

TABLE I

RECOVERY OF OLIGONUCLEOTIDES DURING DESALTING (%)

Procedure	*Oligonucleotide size*			
	Tetra	*Penta*	*Hexa*	*Hepta*
Pooled sample	100	100	100	100
Zinc hydroxide precipitation	99	99	98	99
Zinc oxalate precipitation	87	81	88	89
Dialysis	85	82	85	88
Overall recovery	73	66	73	78
Overall recovery (in a separate experiment)	90	80	87	

Stability of oligonucleotides

Two experiments were carried out to assess the stability of oligonucleotide isopliths during their purification and analysis. The results are shown in Fig. 2. In one experiment, tetranucleotides were concentrated as described above and, after dialysis, they were mixed with cold marker oligonucleotides and re-chromatographed on DEAE-Sephadex. The results shown in the lower panel of Fig. 2 indicate that almost all the material co-chromatographed with marker tetranucleotides. In another experiment, pentanucleotides were eluted from a polyacrylamide gel after electrophoresis under conditions similar to Fig. 4A, then re-chromatographed with marker oligonucleotides. As shown in Fig. 2 (upper panel), approximately 95% of the nucleotide material chromatographed with pentanucleotides.

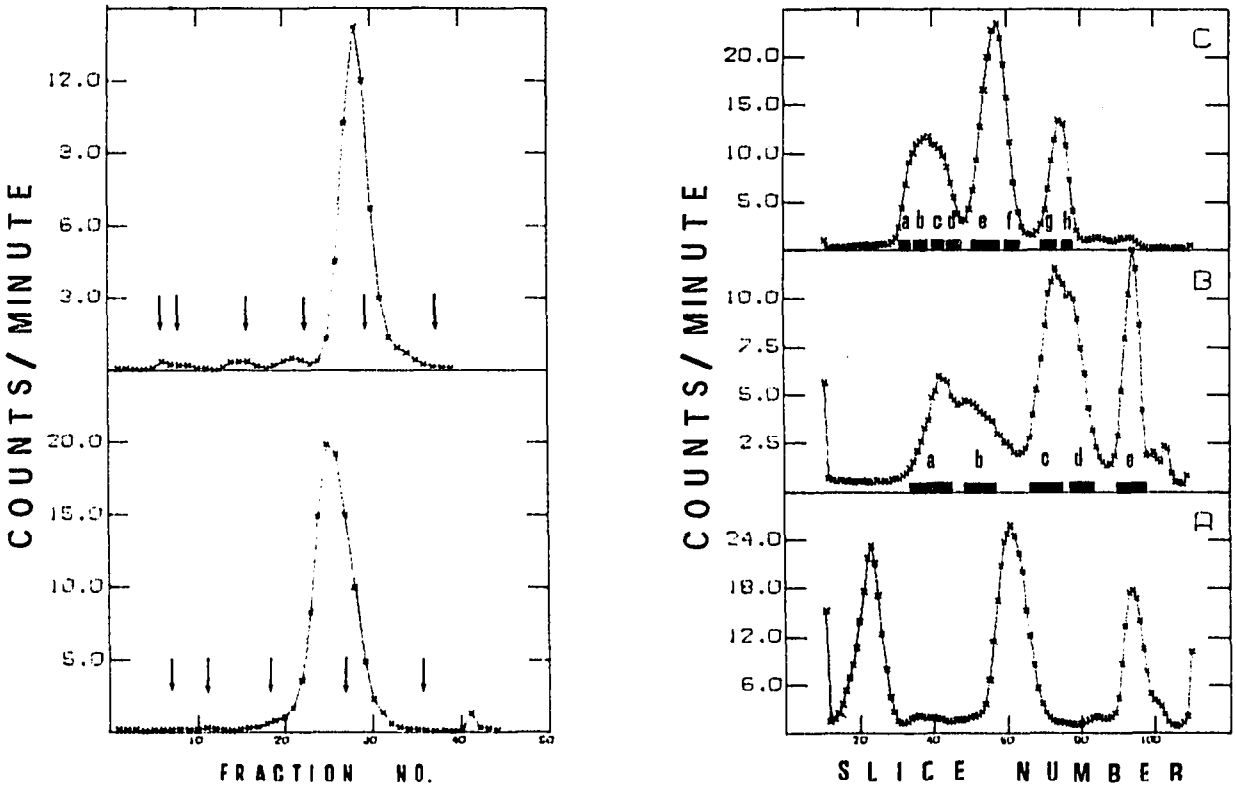


Fig. 2. Re-chromatography of recovered tetra- and pentanucleotides. Lower panel: tetranucleotides from a ribonuclease T_1 digest of ^{32}P-labeled rRNA were separated by chromatography as in Fig. 1, then concentrated and desalted as described in METHODS. Following the dialysis step, the nucleotides were combined with an unlabeled mixture of oligonucleotides (prepared by limited alkaline hydrolysis of rRNA) and re-chromatographed on DEAE-Sephadex. Upper panel: a similar experiment in which pentanucleotides were electrophoresed on polyacrylamide gels as described in METHODS, eluted from the sliced gel with 0.1 *M* formic acid, and chromatographed as above. In both figures, the arrows indicate the positions of the marker oligonucleotide peaks. Ordinate: counts per min $\times 10^{-2}$.

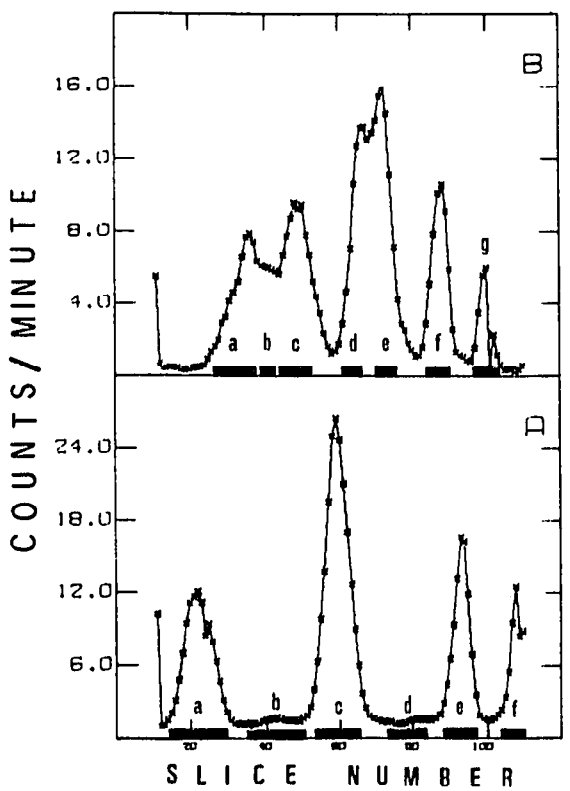
Fig. 3. Electrophoresis of tetranucleotides on polyacrylamide gels. Tetranucleotides (peak IV of Fig. 1) were concentrated and desalted as described in METHODS. One hundred micrograms of oligonucleotide, dissolved in 40–50 μl of 5 *M* urea, were layered over each of three polyacrylamide gels and subjected to electrophoresis. Running times and buffers used were as follows: (A) 120 min, 0.1 *M* formic acid; (B) 100 min, 0.05 *M* Na citrate, pH 3.2; (C) gel was modified to contain 4% acrylamide, 0.04% bisacrylamide, 6.7 *M* urea, and 0.15 *M* formic acid to give a final pH of 3.3; the lower buffer reservoir contained urea and formic acid, the upper only formic acid; running time was 110 min. In this and subsequent figures, the applied voltages were 400 V; current flows were 2–6 mA/gel; anode is on the right; following electrophoresis, the gel was sectioned into disk-shaped slices, starting at the top of the gel; where gels are plotted as starting at slice No. 11, this indicates that slices 1–11 have been counted together; similarly, any gel slices beyond No. 110 have been pooled; horizontal bars indicate fractions which have been pooled for nucleotide composition analysis (see Table II); all ordinates, counts per min $\times 10^{-2}$.

Electrophoresis of oligonucleotide isopliths

The results of a series of experiments designed to test the use of polyacrylamide gels for electrophoresis of oligonucleotides are shown in Figs. 3–8, and the composition of selected peaks is listed in Table II. Tetra- to nonanucleotide peaks from the chromatogram shown in Fig. 1 were selected for analysis on gels. Several acidic buffer systems were compared, ranging from 0.1 *M* formic acid (pH 2.3 at 23°) to Na citrate, pH 3.4. The effect of chain length on mobility may be estimated by comparing Figs.

3A, 4A, 6A, 7A, 8A and 8B. These are tetra- to nonanucleotides, respectively, which have been electrophoresed in 0.1 *M* formic acid, 8% acrylamide gels. Fairly small differences in overall mobilities were observed. For example, if we compare components with one uridylate residue, we see that these are recovered at approximately slice No. 60. The mobilities may therefore be estimated from their running times, which range from 120 min for tetranucleotides to 155 min for heptanucleotides. This suggests that chain length plays a relatively minor role in separations under these conditions.

The most striking feature of these electropherograms is the differences in mobilities within isopliths, in some cases greater than five-fold. These differences are most distinct in gels run in 0.1 *M* formic acid. From inspection of the nucleotide compositions (Table II), it is clear that the predominant factor governing mobility is the number of uridylate residues per chain. Adenylate and cytidylate residues contribute



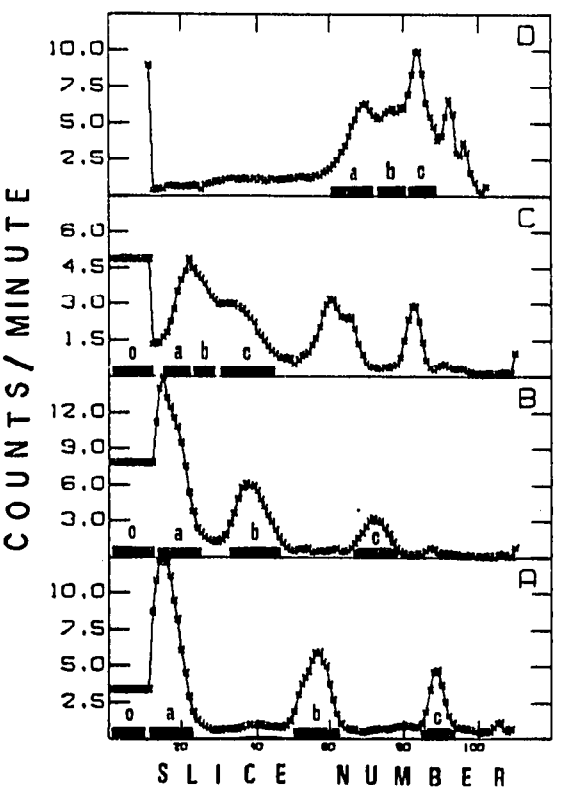


Fig. 4. Electrophoresis of pentanucleotides. Approximately 80 μg of desalted pentanucleotides (from peak V of Fig. 1) were layered over polyacrylamide gels and subjected to electrophoresis. Running times and buffers used were as follows: (A) 130 min, 0.1 *M* formic acid; (B) 105 min, 0.05 *M* Na citrate, pH 3.2. See also the legend to Fig. 3.

Fig. 5. Electrophoresis of dephosphorylated pentanucleotides. The 3'-terminal phosphates of desalted pentanucleotides (peak V of Fig. 1) were removed with alkaline phosphatase. Two hundred micrograms of pentanucleotides were incubated with 5 μg of Worthington phosphatase in 50 μl of 0.2 *M* Tris, pH 7.4. The sample was incubated at 37° for 3.5 h, following which it was dialysed against 2 l of water for 1 h. The nucleotide solution was concentrated under vacuum and dissolved in 100 μl of 5 *M* urea. Twenty-five micrograms of the dephosphorylated pentanucleotides were layered over each of four polyacrylamide gels and subjected to electrophoresis. Buffers and running times used were as follows: (A) 0.1 *M* formic acid, 130 min; (B) 0.05 *M* Na citrate, pH 2.9, 105 min; (C) 0.05 *M* Na citrate, pH 3.2, 105 min; (D) 0.05 *M* Na citrate, pH 3.4, 120 min. See also the legend to Fig. 3.

TABLE II

NUCLEOTIDE COMPOSITIONS OF OLIGONUCLEOTIDES

The two sets of numbers refer to duplicate nucleotide analyses performed on each oligonucleotide peak.

Peak	*Distribution of* ^{32}P				*Composition*[a]
	G	*C*	*A*	*U*	
(i) Tetranucleotides, pH 3.2, Fig. 3B					
(a)	0.32	0.48	0.15	0.05	GC_2A
	0.24	0.60	0.12	0.04	
(b)	0.27	0.32	0.34	0.06	GC_2A; GCA_2
	0.23	0.33	0.38	0.06	
(c)	0.26	0.38	0.12	0.24	GC_2U; GCAU
	0.26	0.39	0.10	0.26	
(d)	0.25	0.16	0.33	0.26	GCAU; GA_2U
	0.24	0.15	0.35	0.25	
(e)	0.25	0.16	0.10	0.48	GCU_2; GAU_2
	0.23	0.18	0.10	0.48	
(ii) Tetranucleotides, urea, Fig. 3C					
(a)	0.22	0.58	0.13	0.07	GC_3; GC_2A
	0.22	0.63	0.11	0.04	
(b)	0.22	0.50	0.22	0.06	GC_2A
	0.23	0.53	0.21	0.03	
(c)	0.23	0.30	0.41	0.05	GCA_2
	0.23	0.31	0.41	0.05	
(d)	0.23	0.20	0.48	0.09	GCA_2
	0.24	0.17	0.47	0.12	
(e)	0.23	0.39	0.11	0.27	GC_2U; GCAU
	0.24	0.36	0.16	0.24	
(f)	0.24	0.19	0.29	0.28	GCAU
	0.25	0.20	0.31	0.24	
(g)	0.24	0.17	0.10	0.49	GCAU; GCU_2 (?)
	0.26	0.21	0.17	0.36	
(h)	0.26	0.13	0.18	0.43	GCAU; GAU_2 (?)
	0.27	0.12	0.22	0.39	
(iii) Pentanucleotides, 0.1 M formic acid, Fig. 4A					
(a)	0.20	0.49	0.29	0.02	GC_3A; GC_2A_2
	0.23	0.47	0.28	0.03	
(b)	0.21	0.41	0.27	0.11	GC_2A_2; GC_2AU
(c)	0.19	0.39	0.22	0.21	GC_2AU
	0.19	0.39	0.21	0.21	
(d)	0.21	0.25	0.18	0.36	$GCAU_2$
(e)	0.19	0.24	0.15	0.42	$GCAU_2$
	0.19	0.25	0.15	0.40	
(f)	0.18	0.12	0.10	0.60	GCU_3; GAU_3
	0.21	0.13	0.11	0.55	
(iv) Pentanucleotides, pH 3.2, Fig. 4B					
(a)	0.20	0.65	0.12	0.04	GC_3A
	0.20	0.66	0.12	0.02	
(b)	0.20	0.41	0.36	0.03	GC_2A_2
	0.19	0.46	0.32	0.03	
(c)	0.22	0.26	0.48	0.05	GCA_3; GC_2A_2
	0.22	0.28	0.47	0.03	
(d)	0.21	0.46	0.11	0.22	GC_2AU; GC_3U
	0.21	0.46	0.11	0.22	
(e)	0.18	0.25	0.36	0.21	GCA_2U
	0.20	0.25	0.35	0.20	
(f)	0.19	0.24	0.16	0.40	$GCAU_2$
	0.19	0.25	0.19	0.37	
(g)	0.19	0.12	0.12	0.56	GCU_3; GAU_3
	0.19	0.13	0.14	0.55	

(*continued on next page*)

TABLE II (*continued*)

Peak	*Distribution of* ^{32}P				*Composition*[a]
	G	*C*	*A*	*U*	
(v) Dephosphorylated pentanucleotides, 0.1 M formic acid, Fig. 5A					
(o)	0.05	0.51	0.20	0.24	GC_2AU
	0.04	0.57	0.19	0.20	
(a)	0.04	0.43	0.31	0.22	GC_2AU
	0.02	0.43	0.31	0.24	
(b)	0.03	0.33	0.18	0.45	$GCAU_2$
	0.02	0.34	0.18	0.46	
(c)	0.02	0.16	0.14	0.68	GCU_3; GAU_3
	0.04	0.18	0.15	0.63	
(vi) Dephosphorylated pentanucleotides, pH 2.9, Fig. 5B					
(o)	0.08	0.43	0.21	0.28	GC_2AU
(a)	0.04	0.40	0.32	0.23	GC_2AU
	0.03	0.41	0.30	0.25	
(b)	0.06	0.27	0.25	0.42	$GCAU_2$
	0.00	0.34	0.18	0.47	
(c)	0.08	0.14	0.18	0.61	(?)
	0.04	0.14	0.16	0.67	
(vii) Dephosphorylated pentanucleotides, pH 3.2, Fig. 5C					
(o)	0.07	0.38	0.48	0.07	GC_2A_2
	0.08	0.46	0.37	0.09	
(a)	0.05	0.56	0.18	0.21	GC_2AU
	0.10	0.44	0.24	0.22	
(b)	0.04	0.49	0.24	0.24	GC_2AU
	0.06	0.47	0.23	0.25	
(c)	0.05	0.30	0.39	0.27	GCA_2U
	0.07	0.27	0.39	0.26	
(viii) Dephosphorylated pentanucleotides, pH 3.4, Fig. 5D					
(a)	0.01	0.62	0.16	0.21	GC_2AU
	0.01	0.48	0.24	0.27	
(b)	0.01	0.44	0.30	0.26	GC_2AU
	0.02	0.35	0.34	0.29	
(c)	0.04	0.31	0.28	0.37	$GCAU_2$
	0.03	0.26	0.31	0.40	
(ix) Hexanucleotides, 0.1 M formic acid, Fig. 6A					
(a)	0.15	0.68	0.14	0.04	GC_4A
	0.17	0.62	0.17	0.04	
(b)	0.19	0.40	0.38	0.03	GC_3A_2; GC_2A_3
	0.16	0.37	0.42	0.04	
(c)	0.15	0.57	0.11	0.18	GC_3AU
	0.19	0.53	0.12	0.17	
(d)	0.17	0.30	0.34	0.19	GC_2A_2U
	0.18	0.29	0.35	0.18	
(e)	0.20	0.30	0.20	0.31	GC_2AU_2
	0.20	0.27	0.20	0.33	
(f)	0.16	0.17	0.15	0.52	$GCAU_3$
	0.18	0.18	0.15	0.50	
(x) Hexanucleotides, 0.1 M acetic acid, Fig. 6B					
(a)	0.16	0.56	0.24	0.04	GC_3A_2; GC_4A
	0.16	0.56	0.24	0.04	
(b)	0.18	0.40	0.25	0.17	GC_2A_2U; GC_3AU
	0.16	0.36	0.33	0.15	
(c)	0.19	0.28	0.20	0.33	GC_2AU_2
	0.19	0.26	0.21	0.33	
(d)	0.20	0.18	0.26	0.37	GCA_2U_2
	0.20	0.17	0.26	0.37	
(e)	0.15	0.15	0.10	0.59	GCU_4; $GCAU_3$

(*continued on next page*)

TABLE II (*continued*)

Peak	*Distribution of* ^{32}P				*Composition*[a]
	G	*C*	*A*	*U*	
(*xi*) *Hexanucleotides, 0.2 M acetic acid, Fig. 6C*					
(a)	0.21	0.68	0.07	0.04	(?)
(b)	0.18	0.37	0.37	0.09	(?)
(c)	0.17	0.57	0.08	0.18	GC_3AU; GC_4U
	0.17	0.55	0.10	0.18	
(d)	0.18	0.27	0.37	0.18	GC_2A_2U
	0.18	0.27	0.35	0.20	
(f)	0.19	0.26	0.21	0.34	GC_2AU_2
	0.20	0.26	0.22	0.32	
(g)	0.19	0.17	0.17	0.48	$GCAU_3$
	0.19	0.18	0.13	0.50	
(*xii*) *Hexanucleotides, pH 3.2, Fig. 6D*					
(a)	0.14	0.65	0.16	0.05	GC_4A
(b)	0.14	0.46	0.33	0.07	GC_3A_2
(c)	0.15	0.45	0.21	0.18	GC_3AU
	0.17	0.47	0.19	0.17	
(d)	0.16	0.29	0.34	0.21	GC_2A_2U
	0.18	0.28	0.36	0.18	
(e)	0.16	0.18	0.17	0.48	$GCAU_3$
	0.17	0.18	0.17	0.48	
(*xiii*) *Heptanucleotides, 0.1 M formic acid, Fig. 7A*					
(a)	0.09	0.37	0.36	0.17	GC_2A_3U; GC_3A_2U
	0.15	0.37	0.33	0.15	
(b)	0.14	0.17	0.12	0.02	GC_5A
	0.12	0.72	0.12	0.04	
(c)	0.15	0.43	0.39	0.03	GC_3A_3
(d)	0.15	0.58	0.14	0.15	GC_4AU
	0.12	0.57	0.13	0.16	
(e)	0.16	0.27	0.28	0.29	$GC_2A_2U_2$
	0.13	0.30	0.28	0.29	
(f)	0.15	0.23	0.21	0.40	GC_2AU_3; GCA_2U_3
	0.11	0.27	0.21	0.41	
(*xiv*) *Octanucleotides, 0.1 M formic acid, Fig. 8A*					
(a)	0.12	0.62	0.22	0.04	GC_5A_2
	0.12	0.62	0.22	0.04	
(b)	0.12	0.46	0.27	0.14	GC_4A_2U
(c)	0.11	0.56	0.16	0.16	GC_5AU
(d)	0.12	0.46	0.27	0.15	GC_4A_2U
	0.13	0.47	0.25	0.15	
(e)	0.10	0.36	0.36	0.18	GC_3A_3U
(f)	0.12	0.35	0.28	0.25	$GC_3A_2U_2$
	0.13	0.35	0.27	0.25	
(g)	0.13	0.22	0.23	0.42	$GC_2A_2U_3$
	0.17	0.23	0.24	0.36	

[a] Composition of an oligonucleotide peak was deduced from the distribution of ^{32}P amongst the four nucleotides, taking chain length and a single guanylate residue per molecule (except the dephosphorylated pentanucleotides) into consideration. In those cases where peaks appear to be a mixture of compositional isomers the two major species are identified. (?) = a complex mixture whose composition cannot be deduced.

proportionately less to mobility, and hence are less well resolved. In order to increase the separation of cytidylate and adenylate-containing oligonucleotides, buffers of higher pH were utilized. This is shown in Fig. 3B for tetranucleotides, which appears to be an improvement over Fig. 3A. Definite increase in resolution of some penta-

nucleotide peaks was obtained in citrate buffer, pH 3.2 (Fig. 4B) as compared to 0.1 *M* formic acid (Fig. 4A). However, amongst hexanucleotides, buffers of higher pH caused crowding together of components (Fig. 6D *vs.* 6A). Unusually sharp peaks were observed with acetic acid buffers (Figs. 6B, 6C and 7B). The explanation for this is not known.

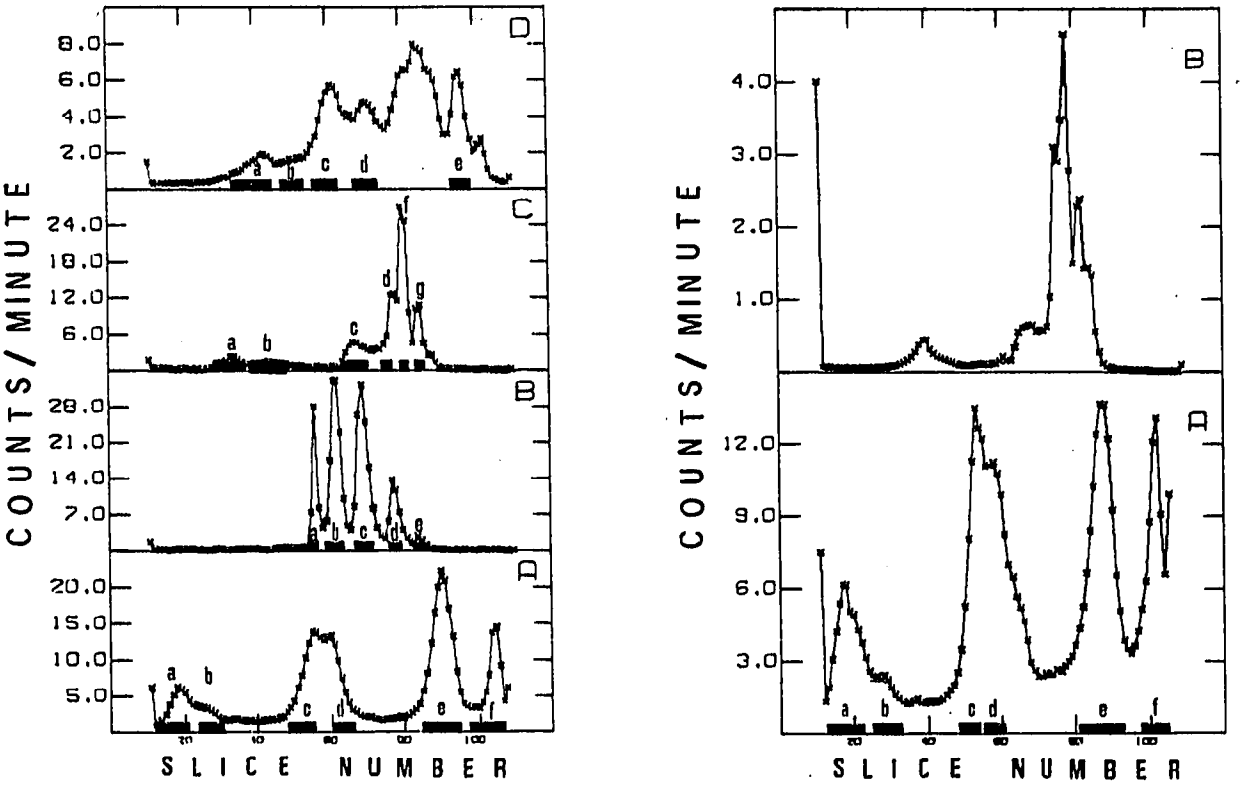


Fig. 6. Electrophoresis of hexanucleotides. Approximately 80 μg of desalted hexanucleotides (peak VI of Fig. 1) were layered over each of four gels and subjected to electrophoresis. Running times and buffers used were as follows: (A) 140 min, 0.1 *M* formic acid; (B) 125 min, 0.1 *M* acetic acid; (C) 130 min, 0.2 *M* acetic acid; (D) 110 min, 0.05 *M* Na citrate, pH 3.2. See also the legend to Fig. 3.

Fig. 7. Electrophoresis of heptanucleotides. Approximately 60 μg of desalted heptanucleotides (peak VII of Fig. 1) were layered over each of two gels and subjected to electrophoresis. Running times and buffers used were as follows: (A) 150 min, 0.1 *M* formic acid; (B) 140 min, 0.2 *M* acetic acid. See also the legend to Fig. 3.

The effect on mobility and resolution of removing the 3'-terminal phosphate from pentanucleotides was examined (Fig. 5). The pentanucleotide-tetraphosphates migrated more slowly than the corresponding pentanucleotides, but no marked increase in resolution was detected. Such a statement must be qualified, however, since the nucleotide compositions suggest that the dephosphorylation may not have gone to completion, and some components seem to have been lost at the origin of the gels. In another experiment, the effect of urea in the buffer system was tested. The result, shown in Fig. 3C, is little different than in the absence of urea (Fig. 3B).

DISCUSSION

We have explored the usefulness of polyacrylamide gels as a supporting medium for the electrophoretic separation of oligonucleotides. Although (deoxy)oligonucleotides have been successfully fractionated on such gels[6], the separations were based upon size and utilized the 'sieving' action of the gels. Our method was designed to subfractionate oligonucleotide isopliths into their compositional isomers on the basis of differences in net charge. Initially, a discontinuous buffer system was used to concentrate oligonucleotides for application to the gels, but this was unsatisfactory. This led to the development of the zinc hydroxide–oxalic acid dialysis procedure which has proven to be rapid and reliable.

Amongst the electrophoresis buffer systems tested, 0.1 *M* formic acid has been the simplest and most useful. Many of the compositional isomers were resolved in this system. We have no information about separation on the basis of sequence. The incomplete separation of isomers containing cytidylate from those containing adenylate residues led to an investigation of other buffer systems. Some increase in resolution with higher pH was obtained in the case of pentanucleotides (and probably tetranucleotides) but not with hexanucleotides. The inclusion of high concentrations of urea to suppress non-ionic binding of purine nucleotides to the polyacrylamide gave no detectable increase in resolution. We have adopted the formic acid system for most purposes.

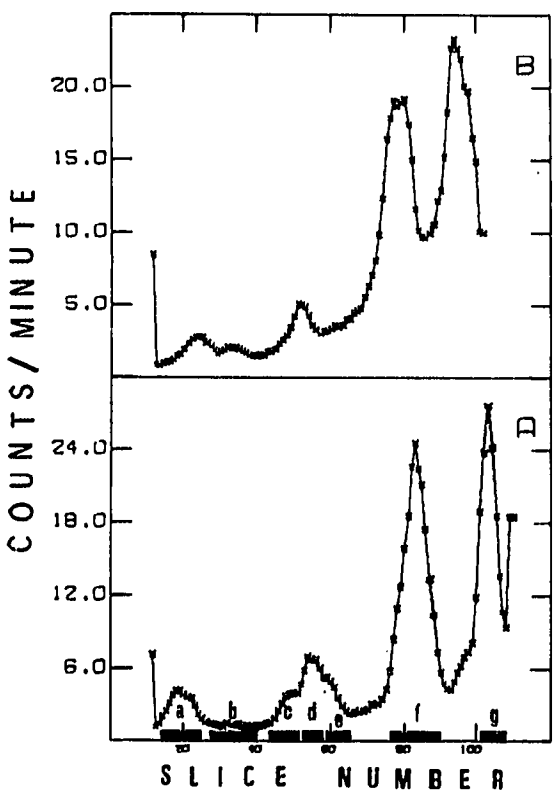


Fig. 8. Electrophoresis of octa- and nonanucleotides. Eighty-five micrograms of octanucleotides and 70 μg of nonanucleotides (peaks VIII and IX Fig. 1, respectively) were electrophoresed on polyacrylamide gels equilibrated with 0.1 *M* formic acid. (A) octanucleotides for 155 min; (B) nonanucleotides for 160 min. See also the legend to Fig. 3.

The present procedure was developed as part of a method for characterizing complex RNA molecules by their oligonucleotide 'fingerprints'[1,15]. Because of the short time required for electrophoresis and the simplicity of fractionating and counting gels, the method seems well suited to this purpose. Although ^{32}P has been used in this study, the ease of elution of labeled oligonucleotides from the gel should permit the use of ^{3}H and ^{14}C for double-label experiments where two RNA species are to be compared directly[1].

These data can be examined to see if they provide any evidence for heterogeneity of the ribosomal RNA molecules. For this analysis, the combined molecular size of 18S + 28S RNA is assumed to be equivalent to 7000 nucleotides. A specified heptanucleotide would therefore be 7/7000 = 1/1000 of the weight of the RNA if it occurred only once per 18S + 28S RNA molecule. If a heptanucleotide peak could be identified that was less than 1/1000 of the weight of the total, unfractionated RNA, this would mean that it occurred less than once per 18S + 28S RNA molecule and would imply heterogeneity in these molecules. When the appropriate corrections for recoveries and isotope decay are applied to peak b of Fig. 7A, the counts in this peak are close to 1/1000 of the total. Thus, this peak probably has a unique sequence, but does not provide evidence for heterogeneity amongst ribosomal RNA molecules. Similar calculations carried out on the minor octa- and nonanucleotide peaks (Figs. 8A and B) lead to the same conclusion. Such evidence, of course, does not rule out the possibility that some heterogeneity exists.

In conclusion, a method has been described for subfractionating oligonucleotides. It is rapid and lends itself to the analysis of several samples simultaneously. Ready recovery of the separated isomers from the gel should permit counting of double-labeled (^{3}H and ^{14}C) samples, and further analysis of the oligonucleotides where necessary.

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