CHROM. 4806

THE DETERMINATION OF THE BASE COMPOSITION OF RNA BY HIGH-PRESSURE CATION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

A method has been developed to determine the base composition of micro- and nanogram quantities of RNA in 4 min. The RNA's were hydrolyzed (either chemically or enzymatically) to their nucleoside level and separated by cation-exchange chromatography. The nanogram quantities of ribonucleosides were separated in less than 4 min by eluting with formate buffer at a flow rate and pressure of 50 ml/h and 3000 p.s.i., respectively. The separation time is reduced to 2 min by eluting at 80 ml/h and 4600 p.s.i. The column was monitored with a sensitive UV detector which permitted absorbance to be recorded linearly and allowed analysis of nanomole to picomole quantities of nucleosides with a relative standard error of 2–3%. Data from four RNA samples are presented. The method is also applicable to the determination of the base composition of DNA since the deoxynucleosides are also separated by the procedure.

INTRODUCTION

The determination of the base sequence of a nucleic acid or oligonucleotide requires hydrolysis (either chemically or enzymatically) followed by separation and quantitation of the resultant products. With the increased interest in the sequencing of nucleic acids has come the need for a rapid and sensitive method for base composition analysis.

Previously, the most widely employed method for the determination of base composition required hydrolysis of the nucleic acid or oligonucleotide to the nucleotide level, followed by separation and quantitation of the resultant mixture by either anion-exchange[1–8] or cation-exchange chromatography[9–13]. An alternative method has been hydrolysis to the nucleoside or N-base level followed by separation and quantitation by cation-exchange chromatography[8,13,14–17] although anion-exchange chromatography[5–6] has been used in some instances.

Recently, UZIEL *et al.*[16] have demonstrated the advantages of enzymatically hydrolyzing nucleic acid to the nucleoside level followed by separation and quantitation by cation-exchange chromatography. Using an 0.6 × 20 cm column filled

with an efficient cation-exchange resin, and by operating at a low flow rate and pressure, they were able to separate and quantitate nanomole quantities of nucleosides in less than 1 h. With this technique they were able to avoid both the isomeric peaks that arise from alkaline hydrolysis of nucleic acids and the more drastic hydrolytic procedures required to realize bases.

To decrease the time required for the nucleoside separation, we have employed a high-resolution liquid chromatograph that utilizes a column filled with a cation-exchange resin of smaller particle size than that used in the method of UZIEL *et al.*[16] By monitoring the system with a sensitive UV photometer and by operating at high linear velocities (1 cm/sec) and pressures (3000–5000 p.s.i.), we demonstrate the separation and quantitation of nano- and picomole quantities of nucleosides in less than 4 min. Using this separation a method is described for the determination of the base composition of transfer and ribosomal RNA hydrolysates.

EXPERIMENTAL

Materials

Chemicals. The ribonucleosides were purchased from either Calbiochem or Sigma Chemicals and the deoxyribonucleosides from Schwarz Bioresearch, Inc. Isopentenyladenosine was kindly supplied by M. SCHWEIZER, Nucleic Acid Institute, International Chemical & Nuclear. Alkaline phosphatase from *Escherichia coli* and venom phosphodiesterase *(Crotalus adamanteus)* were purchased from Schwarz Bioresearch Inc. Yeast tRNA and *E. coli* B tRNA were purchased from General Biochemicals, and *E. coli* K-12 tRNA and *E. coli* Q-13 ribosomal RNA were kindly supplied by Z. B. EGAN and A. D. KELMERS, Oak Ridge National Laboratory.

Resins. The cation-exchange resin VC-10, having a particle size range of 7–14 μ, was kindly supplied by Sondell Scientific Instruments, Inc. The cross-linking in this resin (*i.e.*, % of divinylbenzene in the polymer bead) was 10%. The resin, as received from the manufacturer, was swollen for 24 h in 0.4 *M* ammonium formate, pH 4.50, before column packing.

Methods

Column preparation. A 0.24 × 25 cm stainless steel column was "dynamically packed" with the VC-10 resin as described by SCOTT AND LEE[18]. A short extension (0.54 × 10 cm) was added to the column and both filled with a 50 : 50 slurry of the resin and 0.4 *M* ammonium formate, pH 4.50. The column was packed under a pressure of 1000 p.s.i. using a Milton Roy Minipump, and equilibrated for several hours with buffer. The extension was then removed and the column placed in the chromatographic system.

Chromatographic system. The system used was the Varian Aerograph Model 4100 liquid chromatograph. A schematic of the system is seen in Fig. 1. The chromatograph utilizes a 5000 p.s.i., pulseless, constant-displacement pump capable of delivering solvents at flow rates from 1 to 200 ml/h. It has a reservoir capacity of 250 ml. The pressure generated by the pump is monitored by means of a pressure-sensitive transducer and displayed on an optical meter. A safety circuit is incorporated into the system which allows the operator to select any pressure limit up to 5000 p.s.i. Additional protective devices automatically shut down the pump if the pressure

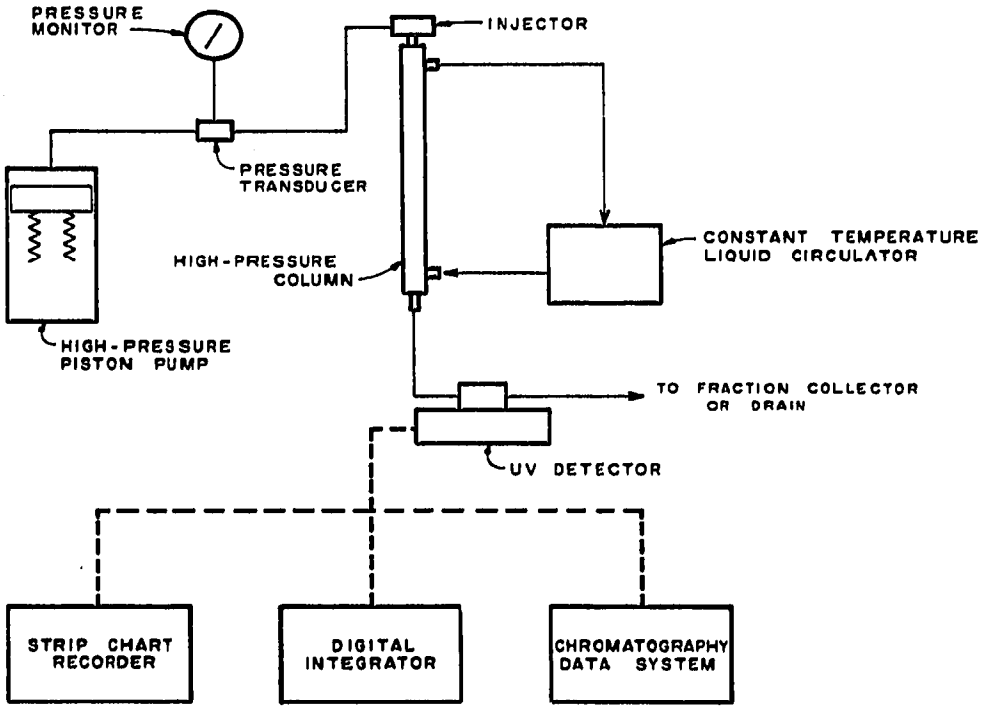


Fig. 1. Schematic of the Varian Aerograph Model 4100 liquid chromatograph.

exceeds 5050 p.s.i. and 6000 p.s.i., respectively. The sample is introduced into the column by a Hamilton syringe through a septumless injector which is attached to the chromatographic column. Water-jacketed columns are constructed of 0.24 cm I.D. 316 stainless steel tubing. A 0.5 μ stainless steel frit supports the column bed and prevents resin from contaminating the column detector. The temperature of the column was maintained within $\pm$ 0.01° of the desired temperature by means of a liquid bath. The column effluent is monitored by a sensitive UV photometer, operating at 254 nm, which is equipped with a cylindrical flow cell having a 1-mm diameter and 10-mm path length (*i.e.*, 8-μl cell volume). The detector output is linear in absorbance units, therefore, linear with respect to solute concentration in accordance with Beer's Law. Full-scale absorbance ranges from 0.005 up to 0.64 are provided in binary steps. In addition, a non-linear high absorbance range is provided for qualitative monitoring of highly absorbing samples. The photometer output (10 mV) is displayed on a strip chart recorder or fed into a digital integrator or a chromatography data handling system for data acquisition and processing. The column effluent, after passing through the detector, is routed either to a drain or to a fraction collector.

Sample preparation. The RNA samples were hydrolyzed to their nucleoside level by either (I) alkaline hydrolysis with 1 *N* sodium hydroxide followed by enzymatic hydrolysis with alkaline phosphatase, or (II) a dual enzymatic hydrolysis using a combination of venom phosphodiesterase and alkaline phosphatase. *Procedure I.* A 20-μl aliquot containing 6 μg of RNA was placed in a small glass test tube and diluted with 10 μl of 1 *N* sodium hydroxide and incubated for 40 min at 80°. UZIEL *et al.*[16] have reported this time sufficient for quantitative hydrolysis with minimal loss of major components. The sample was partially neutralized with

9 μl of 1 *N* formic acid and adjusted to pH 8.8 with 20 μl 0.2 *M* ammonium acetate. Approximately 0.15 units of alkaline phosphatase in 4 μl of H_2O were added and the solution incubated for 4 h at 37°. A 5-μl aliquot representing 0.5 μg of hydrolyzed RNA was analyzed. *Procedure II*. Three micrograms of RNA were incubated for 4 h at 45° in 20 μl of 0.2 *N* sodium acetate, pH 8.8, containing 0.023 *M* magnesium acetate, 0.03 unit/ml of venom phosphodiesterase, and 7.4 units/ml of alkaline phosphatase. Two to five microliters of the hydrolysate representing 0.3 to 0.7 μg of RNA were then analyzed.

Calibration. Stock solutions of the four common ribonucleosides were prepared by dissolving 6 mg of each nucleoside in 10 ml of distilled water. Each stock solution was diluted 1 : 100 with distilled water and precisely calibrated by UV spectrophotometry. A working nucleoside mixture, containing 0.06 mg/ml of each nucleoside, was prepared by pipetting 1 ml of each nucleoside stock solution into a common vial and diluting the resultant mixture to 10 ml with water. Aliquots of this mixture were then chromatographed and the nucleoside peak areas determined from the resultant chromatograms by peak area integration. Response factors for each nucleoside were obtained by dividing the quantity of each nucleoside by its respective peak area.

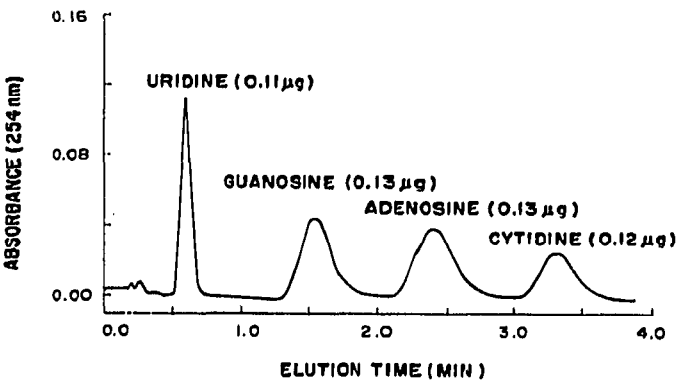
Cation-exchange chromatography. To inject a sample the flow was stopped, pressure reduced to atmospheric by means of a valve, and the sample injected through the septumless injector directly into the column bed with a 10-μl Hamilton syringe. The syringe was withdrawn, the injector closed, and the flow rate and pressure quickly (<30 sec) brought to the desired level by means of a fast pump switch on the pump control module. Since the separation requires only a single eluent, no regeneration of the column was necessary between chromatographic runs.

Calculation of base composition. Response factors for each nucleoside were obtained as described under "calibration". The nucleoside peak areas from the RNA hydrolysates were determined by peak area integration. The quantity of nucleoside that each peak represented was obtained by multiplying each peak area by its respective response factor. Within each sample the nucleoside quantities may be normalized to obtain a base composition on a weight % basis or converted to moles and normalized to a mole % basis. The data presented here have been calculated on the mole % basis.

RESULTS

Fig. 2 illustrates a typical chromatogram obtained from the analysis of an aliquot of the standard nucleoside mixture. An aliquot of the standard mixture containing 0.1 μg of each nucleoside was separated in less than 4 min by eluting with 0.4 *M* ammonium formate at a flow rate and pressure of 50 ml/h and 3000 p.s.i., respectively. The separation time was reduced to less than 2 min by increasing the flow rate and pressure to 80 ml/h and 4600 p.s.i., respectively (Fig. 3). The 4-min, rather than the 2-min separation, was used for the quantitation of the RNA hydrolysates since the increased resolution, obtained at the lower flow rate, facilitated the integration of the individual peak areas.

Fig. 4 illustrates the four chromatograms obtained from the analysis of the alkaline hydrolysates of RNA. Separation of the four ribonucleosides was complete



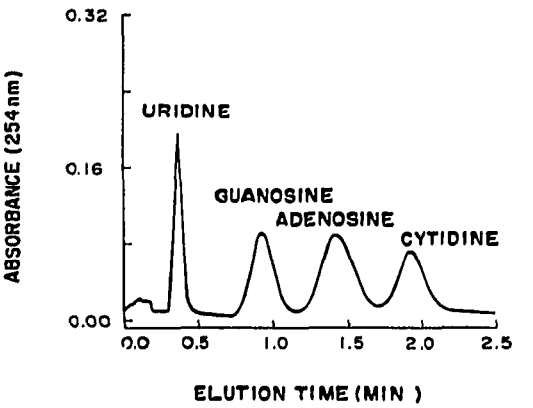


Fig. 2. Separation of the ribonucleosides by high-pressure, cation-exchange chromatography. Conditions: column, 0.24 × 25 cm; resin, 7–14 μ VC-10 (10X); eluent, 0.4 *M* ammonium formate, pH 4.50; flow rate, 50 ml/h; pressure, 3000 p.s.i.; column temperature, 75°; sample, mixture containing 0.1 μg of each nucleoside.

Fig. 3. Rapid separation of the ribonucleosides by high-pressure, cation-exchange chromatography. Conditions: column, 0.24 × 25 cm; resin, 7–14 μ VC-10 (10X); eluent, 0.4 *M* ammonium formate, pH 4.50; flow rate, 80 ml/h; pressure 4600 p.s.i.; column temperature 75°; sample, mixture containing 0.2 μg of each nucleoside.

in less than 4 min and the nucleoside peak areas were used to determine the base composition of the RNA's as described in METHODS. Since unhydrolyzed RNA and nucleotides were found to elute at the same time as the large peak that preceded the uridine peak, the column eluent containing this peak was collected and tested for its RNA and nucleotide content. Complete hydrolysis to the nucleoside level was indicated in both procedures as this fraction contained neither unhydrolyzed RNA, as determined by the procedure of MILLER *et al.*[19], nor nucleotides, as determined by the procedure of HORVATH *et al.*[7]. Chromatography of a sample blank indicated that this peak was due to the enzyme and neutralization salt of the hydrolytic procedure. Since this peak was resolved from those of the nucleosides and did not contain material of nucleic acid origin, it did not interfere with the quantitation.

The base compositions, as determined from their alkaline hydrolysates, of the four RNA samples are found in Table I. The base composition of both the B and K-12 strains of *E. coli* transfer RNA were similar. The yeast tRNA contained similar quantities of guanosine and adenosine when compared with the *E. coli* strains, but was characterized by a decreased cytidine and increased uridine content. As expected, the ribosomal RNA had a different base composition than did the tRNA's as it had a lower guanosine and cytidine content and a higher uridine and adenosine content. In all of the samples the guanosine content was highest, representing 30–34 mole % of the individual RNA samples.

To minimize decomposition of alkaline labile components or to hydrolyze alkali-resistant linkages, a dual enzymatic hydrolysis may be performed with the RNA first being hydrolyzed to the nucleotide level by venom phosphodiesterase and then to the nucleoside level by alkaline phosphatase. The four RNA samples were therefore enzymatically hydrolyzed (Procedure II) and the chromatograms obtained from analysis of the hydrolysates are seen in Fig. 5. In general, the chromatograms are similar to those obtained from the alkaline hydrolysates with the four nucleosides

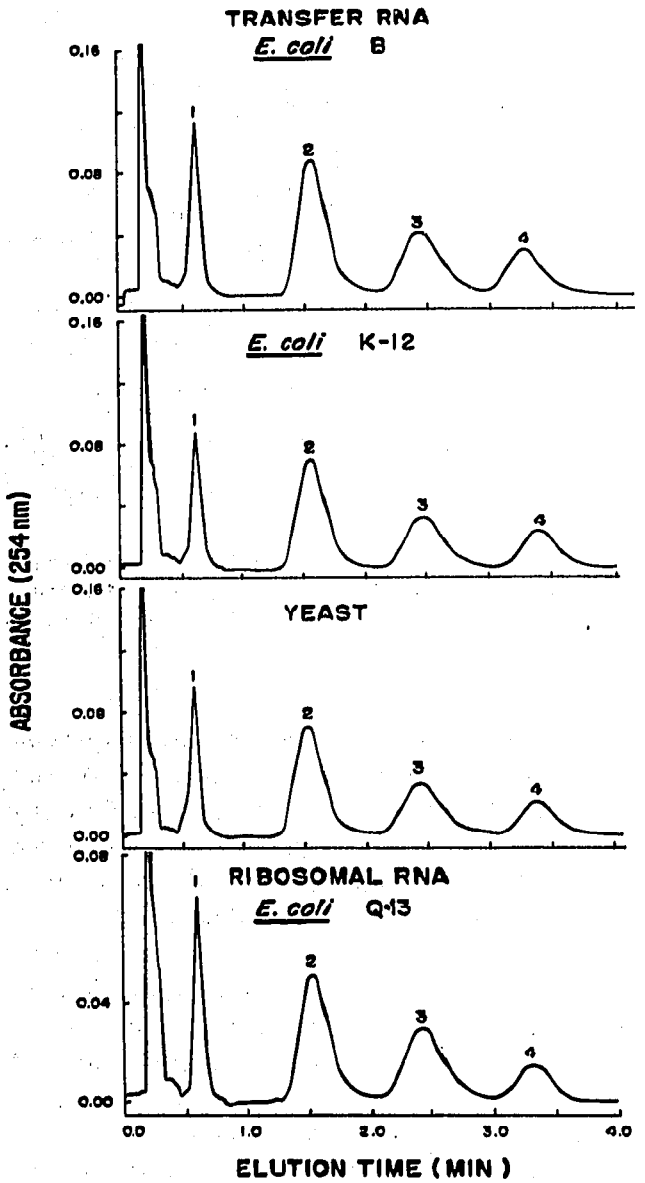


Fig. 4. Separation of the ribonucleosides obtained from the alkaline hydrolysis of various RNA samples. Conditions: column, 0.24 × 25 cm; resin, 7–14 μ VC-10 (10X); eluent, 0.4 *M* ammonium formate, pH 4.50; flow rate, 50 ml/h; pressure, 3000 p.s.i.; column temperature, 75°; sample, aliquots representing 0.5 μg of RNA that had been hydrolyzed by a combination of NaOH and alkaline phosphatase hydrolysis (see text for hydrolysis conditions). 1 = Uridine; 2 = guanosine; 3 = adenosine; 4 = cytidine.

being separated in less than 4 min. A noticeable difference is the reduction in the large peak eluting before uridine. Analysis of this peak for its unhydrolyzed RNA and nucleotide content proved negative. The subsequent chromatography of an aliquot of a sample blank indicated that this peak was due to the enzymes used in the hydrolysis. The smallness of this peak, when compared with the large peak in the alkaline hydrolysate chromatograms, indicates that the size of the latter was due to the sodium formate salt arising from the neutralization of the NaOH with the formic acid. It is probable that the detector response to this salt peak is not due to

TABLE I

MOLAR BASE COMPOSITION OF ALKALINE HYDROLYZED[a] RNA

Chromatographic conditions: column, 0.24 × 25 cm; resin VC-10 (7–14 μ); flow rate, 50 ml/h; pressure, 3000 p.s.i.; monitored at 254 nm, full scale either 0.08 or 0.16 absorbance units; eluent, 0.4 *M* ammonium formate, pH 4.50.

RNA type	*Mole %*			
	Uridine	*Guanosine*	*Adenosine*	*Cytidine*
Transfer				
E. coli B	19.6[b]	33.5	20.9	26.1
E. coli K-12	19.9	33.6	21.0	25.7
Yeast	22.1	33.9	21.1	23.0
Ribosomal				
E. coli Q-13	23.5	30.7	23.9	21.9

[a] Samples hydrolyzed to their nucleoside level by a combination alkaline (NaOH) and alkaline phosphatase hydrolysis.

[b] Numbers represent the mean ± relative standard error of 2–3% for nine determinations.

the UV absorption of the salt, since sodium formate has a low molar absorptivity at 254 nm. Instead, it is probably dependent upon a change in refractive index since it has been previously demonstrated that the small dead volume UV detectors are sensitive to changes in the refractive index[17] of the solvent.

The base composition was determined for the enzyme hydrolysates of the four RNA samples (Table II). Again, a close similarity was observed for the base composition of the two strains of *E. coli* tRNA. Yeast tRNA had similar adenosine and guanosine content, but had a decreased cytidine and increased uridine content when compared to the *E. coli* samples. The ribosomal RNA had lower guanosine and cytidine and higher adenosine and uridine content than did the transfer RNA's.

In general, the base compositions of the tRNA's were similar using either hydrolysis procedure. The slight increase in the uridine content of the alkaline

TABLE II

MOLAR BASE COMPOSITION OF ENZYMATIC HYDROLYZED[a] RNA

Chromatographic conditions: the same as in Table I.

RNA type	*Mole %*			
	Uridine	*Guanosine*	*Adenosine*	*Cytidine*
Transfer				
E. coli B	18.3[b]	34.0	21.7	26.0
E. coli K-12	17.9	34.6	21.7	25.8
Yeast	19.3	34.9	21.9	23.9
Ribosomal				
E. coli Q-13	20.4	32.9	22.0	23.8

[a] Samples hydrolyzed to their nucleoside level by enzymatic hydrolysis using venom phosphodiesterase and alkaline phosphatase.

[b] Numbers represent the mean ± relative standard error of 2–3% for nine determinations.

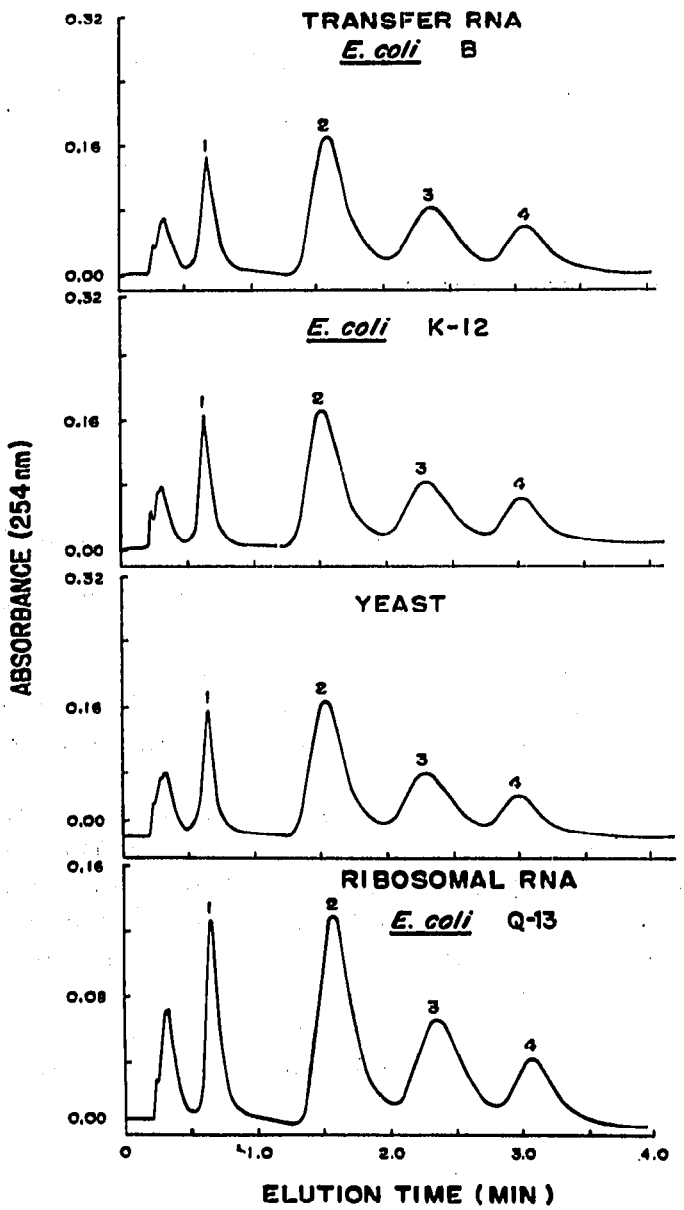


Fig. 5. Separation of the ribonucleosides obtained from the enzymatic hydrolysis of various RNA samples. Conditions: column, 0.24 × 25 cm; resin, 7–14 μ VC-10 (10X); eluent, 0.4 M ammonium formate, pH 4.50; flow rate, 50 ml/h; pressure, 3000 p.s.i.; column temperature, 75°; sample, aliquots representing 0.3–0.7 μg of RNA that had been hydrolyzed by a combination venom phosphodiesterase and alkaline phosphatase (see text for hydrolysis conditions). 1 = Uridine; 2 = guanosine; 3 = adenosine; 4 = cytidine.

hydrolysates, when compared to the enzymatic hydrolysates, is probably due to deamination from the alkaline conditions of Procedure I. The resultant deaminated products (*i.e.*, xanthosine, inosine and uridine) would elute with the uridine peak which, upon quantitation, would result in an increase in the uridine content of the sample. However, the observed increase was small, which confirms the work of UZIEL *et al.*[16], who found minimal deamination under the conditions of Procedure I.

The largest difference in the base composition as determined by the two

TABLE III

ELUTION PARAMETERS OF THE NUCLEOSIDES OF THE UNUSUAL BASES

Chromatographic conditions: column, 0.24 × 25 cm; resin, Sondell VC-10; flow rate, 25 ml/h; pressure, 1400 p.s.i.; temperature, 75°; eluent, 0.4 *M* ammonium formate, pH 4.5.

Nucleoside	*Elution time (min)*	*Elution volume (ml)*
Dihydrouridine	1.2	0.50
Pseudouridine	1.4	0.58
Uridine	1.5	0.62
5-Hydroxyuridine	1.6	0.67
Thymine riboside	1.6	0.67
Deoxyuridine	1.9	0.79
Thymidine	1.9	0.79
Xanthosine	2.4	1.00
Inosine	2.4	1.00
7-Methylxanthosine	2.4	1.00
Guanosine	3.3	1.37
Deoxyguanosine	4.5	1.87
Adenosine	4.7	1.96
Cytosine arabinoside	5.7	2.37
Cytidine	6.1	2.54
6-Methyladenosine	6.3	2.62
Deoxyadenosine	6.5	2.71
Deoxycytidine	6.8	2.83
6-Dimethyladenosine	11.8	4.92
Isopentenyladenosine	15.1	6.29
3-Methylcytidine	26.0	10.83
7-Methylinosine	30.0	12.49
1-Methyladenosine	30.5	12.70
7-Methylguanosine	52.8	22.0

hydrolysis procedures was observed with ribosomal RNA. A 2% increase in both guanosine and cytidine and a 4% decrease in uridine content was observed when the enzymatic hydrolysis is compared to the alkaline hydrolysis. Again, this is probably due to deamination of the guanosine and cytidine to xanthosine and uridine under the alkaline conditions of Procedure I. Since the possibility of deamination does exist with alkaline hydrolysis, it is probable that the milder enzyme hydrolysis gives a more accurate measurement of the actual base composition of an RNA sample.

Since tRNA is known to contain "unusual bases", samples of the nucleosides of several of these bases were obtained and aliquots of solutions of them were chromatographed. Table III indicates the elution times and volumes of these nucleosides. The elution times and volumes of the eight common ribo- and deoxynucleosides are also included in this table for comparative purposes. To obtain increased resolution, the flow rate and pressure for the separation of the nucleosides of the unusual bases to the separation was decreased to 25 ml/h and 1400 p.s.i., respectively. Under these conditions, nucleosides of the unusual bases were found in the three tRNA hydrolysates with the yeast tRNA hydrolysate containing the highest number of these compounds. The enzymatic hydrolysis (Procedure II) was preferred, when analyzing for the nucleosides of the unusual bases, since many of these compounds are labile to the alkaline condition of Procedure I. The nucleosides of the unusual bases were not found in the ribosomal RNA hydrolysates.

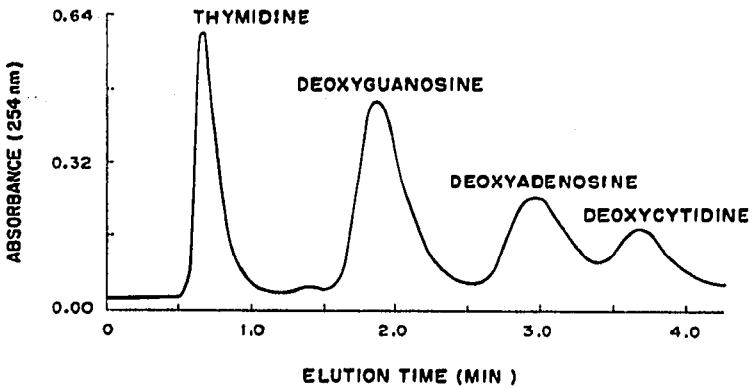


Fig. 6. Separation of deoxyribonucleosides by high-pressure, cation-exchange chromatography. Conditions: column 0.24 × 25 cm; resin, 7–14 μ VC-10 (10X); eluent, 0.4 *M* ammonium formate, pH 4.50; flow rate, 50 ml/h; pressure, 3000 p.s.i.; column temperature, 75°; sample, mixture containing 0.4 μg of each deoxynucleoside.

Although the base composition of DNA was not determined in this study, the method should be applicable for such an analysis since a mixture of the four deoxynucleosides commonly found in DNA was found to separate in less than 4 min (Fig. 6). Therefore, a DNA sample, hydrolyzed to its nucleoside level, could be separated and quantitated as previously described. It should be noted, however, that the enzymatic hydrolysis (Procedure II) would have to be used due to the resistance of DNA to alkaline hydrolysis.

DISCUSSION

The advantage of operating columns, filled with an efficient cation-exchange resin of small particle size, at high flow rates and pressures is obvious. The time required to separate a mixture of the four common ribonucleosides has been reduced to less than 4 min. Previously, one was only able to attain these speeds of analysis with gas chromatography[20–22]. However, the gas chromatography analysis of the nucleosides requires derivatization of the nucleosides, which consists of several reaction steps. Using liquid chromatography, we have shown rapid separations of the nucleosides without the need for a time-consuming derivatization procedure.

The RNA base compositions determined in this study, using either hydrolysis procedure, agree with published data[11,23–34]. For example, the base composition of *E. coli* B tRNA, expressed as nucleoside mole %, was reported[11,24] to be uridine 20.0–21.4%, guanosine 31.0–33.7%, adenosine 18.2–19.6%, and cytidine 28.0–28.1%. For *E. coli* ribosomal RNA nucleoside mole % values of 21.1–21.4% uridine, 31.5–33.8% guanosine, 23.6–25.0% adenosine, and 21.4–22.1% cytidine have been reported[11,23]. These values are similar to those reported here (Tables I and II) but the techniques used to obtain them are characterized as being time consuming and lacking in sensitivity. In addition, the precision of these techniques ranged from 2–8% as compared to the 2–3% obtained with the method presented here.

Using a sensitive UV photometer as a column monitor, the method was found

to be very sensitive. The results presented here were obtained by hydrolyzing a 3–6 μg sample of RNA, of which a 0.5-μg aliquot was chromatographed and quantitated with a relative error of 2–3%. For this size of sample, the detector was operated at either 0.16 or 0.32 absorbance units full scale. Since the detector can be further attenuated, the method could be used for base composition determination on nanogram samples of RNA. However, by using smaller sample sizes and operating at the higher sensitivities, the accuracy and precision of the method would be expected to decrease due to the difficulties arising in the handling and analysis of these small quantities.

A note as to the operation and stability of conventional cation-exchange resins at high pressures: When a new column is operated at high pressures, we have observed an initial settling of the column bed. However, after the initial settling, the column will operate routinely at pressures up to 5000 p.s.i. with no further compression of its bed. For example, we have operated the column used in this study for 6 months, without encountering difficulty. We attribute the stability of this resin to high pressure to the fact that we have used resin of 10% cross-linkage* which contributes to the mechanical stability of the resin. The compression of columns filled with exchange resin was previously reported[25] but 4% cross-linked resins were used in these studies. In addition, the VC-10 resin was a narrow cut of small particle size which, when "dynamically packed", has been shown by SCOTT AND LEE[18] to yield a uniformly packed column bed that is operable at high pressures. In addition to the 10% cross-linked cation resin used in this study, we have used 8% cross-linked cation resin from two manufacturers and have observed no difficulty in operation and stability of these resins at pressures up to 5000 p.s.i.[26]. The feasibility of using conventional anion-exchange resin at high pressures has been previously demonstrated by SCOTT and co-workers[27–29].

In conclusion, this study has shown that base composition analysis of RNA may be determined rapidly, precisely and accurately by liquid chromatography. Using conventional cation-exchange resin as the chromatographic support and by operating at high flow rates and pressures, the four ribonucleosides are separated in less than 4 min. Since a single solvent is used, no equilibration between analyses is required. A sensitive UV detector used as a column monitor allows analysis of nanomole to picomole quantities of nucleosides with a precision of 2–3%. The method is also applicable to DNA base composition analysis since the four deoxynucleosides are also separated in less than 4 min.

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* %cross-linking indicates the % of divinylbenzene that is incorporated into the polymer beads prior to attaching ionic groups.

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