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ANALYSIS OF BASE COMPOSITION OF RNA AND DNA HYDROLYSATES BY GAS–LIQUID CHROMATOGRAPHY*

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SUMMARY

A quantitative gas–liquid chromatographic method has been developed for the analysis of the base composition of ribonucleic acid and deoxyribonucleic acid hydrolysates at the macro (1.0 mg) and semimicro (100 μg) levels of total nucleic acids, and a semi-quantitative method for micro samples (500 ng of each base). An anion-exchange cleanup procedure to remove perchlorates, phosphates, and other interfering products released during hydrolysis of the sample is included. Silylation of the bases was conducted in a closed reaction vial with bis(trimethylsilyl)-trifluoroacetamide; with chromatography on a 10 w/w% SE-30 100/120 mesh Supelcoport column. Three different ribonucleic acids and two deoxyribonucleic acids were analyzed at the three levels. The results at each level were precise and in good agreement with literature values.

INTRODUCTION

Because of the importance of nucleic acids to the genetic code, many researchers have studied the biological function and chemical structure of the various nucleic acid components (purine and pyrimidine bases, nucleosides, and nucleotides). The biological function of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) depends on the base composition of the polymer and the sequence of the bases in the polymer.

Many methods for the detection of the nucleic acid components have been developed with varying degrees of quantitation obtained. These methods include ion-exchange chromatography by COHN[1], paper chromatography by VISCHER AND CHARGAFF[2], paper electrophoresis by GORDON AND REICHARD[3], thin-layer chromatography (TLC) by RANDERATH[4], paper chromatography and time of flight mass spectrometry by STUDIER *et al.*[5], and gas–liquid chromatography (GLC) by MACGEE[6],

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HASHIZUME AND SASAKI[7], GEHRKE AND RUYLE[8], and the authors of this paper[9].

Many of these methods have been used for the analysis of the base composition of RNA and DNA hydrolysates. However, before analysis, the nucleic acids must be hydrolyzed into bases, nucleosides, or nucleotides, depending on the type of hydrolysis employed. In 1960, JORDAN[10] presented the different types of hydrolysis used to obtain the various monomers. Sample plus 70 % $HClO_4$ heated in a closed tube at 100° for 40 min has been accepted as the best hydrolysis procedure to obtain the purine and pyrimidine bases. Concentrated formic acid or trifluoroacetic acid also has been employed to yield bases. To obtain the nucleosides or nucleotides much milder hydrolysis conditions are necessary, and complete conversion to either the nucleosides or nucleotides is often not achieved.

Many researchers, including HEDGCOTH AND JOCOBON[11], MONJARDINO[12], and LEECH *et al.*[13], have used TLC with spectrophotometric detection and quantitation for the analysis of the base composition. These methods are time consuming, semi-quantitative, and the amount of sample is fairly large (more than 100 μg). An electrophoretic method by BORKOWSKI *et al.*[14] gave a good analysis on a minimum of 50 μg of total DNA. HIBY AND KRÖGER[15] in 1967 used a combination of TLC and electrophoresis for nucleotide analysis of RNA, but did not obtain quantitative data.

GLC, because of its speed, sensitivity, and quantitative aspects, appeared to be the best method available for the analysis of nucleic acid base composition. In 1966, MACGEE[6] reported on the analysis of RNA by GLC. The samples were hydrolyzed with perchloric acid, followed by ion-exchange cleanup to remove the interfering compounds. MACGEE used the N-methyl derivative, which gave multiple peaks for some of the bases, but he was able to obtain quantitative data. HASHIZUME AND SASAKI[7], in 1968, reported the analysis of RNA using the trimethylsilyl (TMS) derivative. The nucleic acid was hydrolyzed with perchloric acid, and the excess perchloric acid was precipitated with KOH. Silylation was achieved with hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) in pyridine. Single peaks for all of the bases were obtained, and quantitation was good; but, 20 mg of RNA were needed for each analysis. GEHRKE AND RUYLE[8], in 1968, presented a method for the analysis of 5 mg of RNA. The nucleic acid was hydrolyzed with perchloric acid, followed by precipitation with KOH and ion-exchange cleanup to remove the phosphate and sugar. The TMS derivatives of the bases were formed with bis (trimethylsilyl)acetamide (BSA) and chromatographed on an SE-30 column. The method was quantitative, but 20 % of the bases were lost during cleanup. In 1969, JOCOBON *et al.*[16] reported on the analysis of RNA using the TMS derivative of the nucleosides. The method was quantitative, but the sample size was large.

Since many biological samples are available only in microgram quantities, these methods are not useful to determine the base composition. A method was needed that could determine the base composition of RNA or DNA at a biologically significant level. We reported a GLC method for the silylation and chromatography of the purine and pyrimidine bases at macro (100 μg @), semimicro (10 μg @), and micro (500 ng @) levels with good quantitation at all levels[9]. The purpose of this investigation was to develop a method for the analysis of nucleic acid hydrolysates at macro, semimicro, and micro levels. This would include the development of a cleanup procedure which would give quantitative recovery of the bases at these levels. This cleanup method, with the silylation and GLC method developed earlier,

would provide a simple, quantitative, and fast method for the analysis of the base composition of RNA and DNA in a sample size as small as 5 μg of nucleic acid.

CLEANUP AND ANALYSIS OF RNA AND DNA

Apparatus

An F and M 402 biochemical gas chromatograph (F and M Scientific, Division of Hewlett-Packard, Avondale, Pa.), equipped with dual hydrogen flame detectors, was used in this investigation.

For elevated temperature reactions, a sand bath with a variable temperature control ($\pm$ 2.0°) was constructed.

Standards and ion-exchange cleaned nucleic acid hydrolysates were dried on a 60° hot plate with an IR lamp or a 60° hot plate with a stream of pure nitrogen gas.

The ion-exchange columns used in this study were 4 × 150 mm, 9 × 150 mm, and 15 × 150 mm pyrex glass with teflon stopcocks and were obtained from Fischer and Porter Company, Warminster, Pa.

The other apparatus used in this study was described in a previous paper[9].

Reagents and materials

The micro reaction vials were obtained from Analytical Biochemistry Laboratories, Inc., Box 1097, Columbia, Mo.

The purine and pyrimidine bases (uracil, thymine, cytosine, adenine, and guanine) were obtained from Mann Research Laboratories, New York, N. Y., and were chromatographically pure. The bis(trimethylsilyl) trifluoroacetamide (BSTFA) was purchased from Regis Chemical Company, 1101 North Franklin Street, Chicago, Ill., in teflon-lined screw cap vials. Acetonitrile, methylene chloride, and dichloroethane were of "nanograde" purity and were purchased from Mallinckrodt Chemical Works, St. Louis, Mo., as well as formic acid, acetic acid, and perchloric acid. Dowex 1 X2 anion-exchange resin was obtained from Dow Chemical Company, Midland, Mich.; and AG 1-X2, Bio-Rex 9, and AG 3-X4 anion-exchange resins were purchased from Bio-Rad Laboratories, Richmond, Calif. Amberlite IR-4B and Amberlite CG-120 resins were obtained from Mallinckrodt Chemical Works. The water was triply distilled from an all glass distillation apparatus. The other reagents used were of the highest purity available.

The yeast RNA I was obtained from Dr. JAMES ROSS, Department of Plant Pathology, University of Missouri; yeast RNA II and tobacco mosaic virus RNA (TMV-RNA) were obtained from Dr. OM SEGHAL, Department of Genetics, University of Missouri. The salmon sperm DNA was purchased from Mann Research Laboratories, New York, N. Y., and the calf thymus DNA was purchased from Sigma Chemical Company, St. Louis, Mo.

Instrumental and chromatographic conditions

The chromatographic columns used were 10 w/w % GC SE-30 (straight-chained polymethylsiloxane) on 100/120 mesh Supelcoport (chromatographic W type). The column materials were packed in glass columns (borosilicate), which were 1 m long with an I. D. of 4 mm. The columns were held in the chromatographic

oven by front and back teflon ferrules on normal Swagelok fittings. For column preparation and conditioning procedures, see our previous paper[9].

The instrumental conditions were a 7.5°/min temperature program with a 4-min initial hold. The program ran from 90° to 250°. The detector was operated in the 280° to 320° region, and the injection heater was at approximately 200°. The gas flow rates were as follows: nitrogen, 40 ml/min; hydrogen, 30 ml/min; air, 300 ml/min. The attenuation for macro samples was 10×32 (1.2×10^{-9} a.f.s., for semimicro samples it was 10×8 (3.2×10^{-10} a.f.s.), and for micro samples it was 10×2 (8×10^{-11} a.f.s.).

Development of a cleanup procedure for the analysis of the purine and pyridine bases from a nucleic acid hydrolysate

Before the analysis of the base composition of a nucleic acid could be performed, it was necessary to hydrolyze the nucleic acid into its various monomers. To hydrolyze a nucleic acid to the bases, MACGEE used 70% perchloric acid in a closed tube at 100° for 40 min. HASHIZUME AND SASAKI[7] and GEHRKE AND RUYLE[8] also used this procedure in their investigations with good success.

Because the perchlorate interfered with the silylation of the bases, it was necessary to first remove it from the sample. HASHIZUME AND SASAKI precipitated the perchlorate with KOH before silylation with HMDS and TMCS, and they were able to obtain good results for macro samples. GEHRKE AND RUYLE also precipitated the perchlorate with KOH, but because BSA was a more powerful silylating reagent, an ion-exchange cleanup was needed to completely remove the interfering compounds from the sample, including the phosphate and degradation products of ribose. They found that approximately 20% of each base was non-selectively lost during precipitation, transfer, and ion-exchange cleanup. The development of a quantitative method for the analysis of the base composition of RNA and DNA would require 95 to 100% recovery of the bases, thus it was necessary to develop a cleanup method that would give this recovery without adding impurities and that would remove the perchlorate and phosphate from the sample.

Two-column method

The perchlorate used for the hydrolysis and the phosphate can be removed by anion-exchange chromatography. The perchlorate and phosphate free bases can then be dried, made alkaline with KOH, and placed on another anion-exchange column for the removal of neutral (sugar) compounds.

With this outline, the following two-column method was developed for the cleanup of a nucleic acid hydrolysate. The method was developed with standards of each base, ribose, and H_3PO_4.

TWO-COLUMN METHOD

1. Hydrolysis

(a) Add 0.5 ml of 70% perchloric acid to 0.1 mg each of U, T, C, A, G, ribose, and H_3PO_4 in a 16 × 75 mm culture tube.
(b) Seal the tube with a teflon-lined cap and heat it at 100° for 40 min.
(c) Cool the tube to room temperature and dilute the sample to 3 ml with triply distilled water.

2. Removal of anions

(a) Place the hydrolyzed sample on a 15 × 70 mm anion-exchange column of Dowex 1-X2 formate (50/100 mesh).
(b) Wash the column with 7 × 3 ml triply distilled water at a rate of 1 ml/min.

(c) Discard the first 3 ml.
(d) Collect the next 6 × 3 ml in a culture tube (8.5 ml total capacity), evaporating while collecting with a 90° sand bath and a stream of pure nitrogen gas.
(e) Evaporate to dryness.

3. Removal of neutral molecules
(a) Dissolve the dried sample in 2 ml of 0.5 *N* KOH.
(b) Place the sample on a 9 × 60 mm anion-exchange column of Dowex 1-X2 formate (50/100 mesh).
(c) Wash the column with 12 × 4 ml triply distilled water at a rate of 1 ml/min and discard.
(d) Elute the column with 6 × 3 ml of 1 *N* formic acid.
(e) Discard the first 3 ml.
(f) Collect the next 5 × 3 ml in a culture tube (4.5 ml total capacity), evaporating while collecting with a 90° sand bath and a stream of pure nitrogen gas.
(g) Evaporate the sample to complete dryness.
Note: methylene chloride was used to azeotrope the last traces of water.

4. Silylation
(a) Add 0.2 ml BSTFA and 0.2 ml acetonitrile containing 100 μg phenanthrene (internal standard).
(b) Tightly seal the vial and silylate at 150° for 15 min.
(c) Cool and inject 3 to 4 μl into the GC.

To evaluate the procedure, each column was analyzed independently, and as a two-column method. For these analyses, twelve standards of 100 μg of each base were pipetted from a stock solution (3.3 mg of each base/100 ml 0.1 *N* HCl ≃ 0.10 mg of each base/3 ml) and dried on a 60° hot plate with a stream of pure nitrogen gas. Four of the samples were hydrolyzed with 70% perchloric acid and cleaned by the first anion-exchange column only. Another four of the samples were dissolved in 2 ml of 0.5 *N* KOH and cleaned by the second anion-exchange column only. Then, 0.4 mg ribose and 0.4 mg H_3PO_4 were added to four of the samples, which were carried through the total two-column anion-exchange cleanup method.

The dried samples were silylated and analyzed by GLC (for development of the silylating conditions and instrumental settings, refer to our previous paper[9]). The chromatographic peak areas were integrated, and the $RMR_{B/IS}$ values were calculated as follows:

$$RMR_{B/I.S.} = \frac{\dfrac{\text{Area}_{Base}}{g_B/MW_B}}{\dfrac{\text{Area}_{I.S.}}{g_{I.S.}/MW_{I.S.}}}$$

$$RMR_{B/I.S.} = \frac{A_B}{A_{I.S.}} \times \frac{g_{I.S.}}{g_B} \times \frac{MW_B}{MW_{I.S.}}$$

where

Area_{Base} = area of the chromatographic peak for the base
$\text{Area}_{I.S.}$ = area of the chromatographic peak for the internal standard
MW = molecular weight

Cytosine gave two chromatographic peaks, and the $RMR_{C/I.S.}$ was calculated by integrating each peak separately and adding the areas to obtain the total area.

From the $RMR_{B/I.S.}$ values for the twelve samples and the $RMR_{B/I.S.}$ values for standards not carried through the cleanup steps, the % recovery was calculated as

$$\% \text{ recovery} = \frac{RMR_{B/I.S.}\text{ (after cleanup)}}{RMR_{B/I.S.}\text{ (standards)}} \times 100$$

The average % recoveries for the four samples for each experiment are presented in Table I.

One-column method

As the two-column method presented was time consuming, modifications in the procedure were made to speed up the cleanup for simplification, without affecting the recovery of the bases.

Drying of the sample. The evaporation of the sample while collecting the eluate from the anion-exchange column was very time consuming. A faster method for drying the eluate, which did not affect the sample, was needed. Thus, to determine the best drying method for the column eluate, the following drying methods were evaluated: rotary evaporation, lyophilization, a hot plate with an IR lamp, and a hot plate with a stream of pure nitrogen gas. Each method was studied by diluting four aliquots (100 μg of *A* base) in 3 ml of 0.1 *N* HCl with 20 ml of triply distilled water. The samples were concentrated to approximately 2 ml and then transferred to a culture tube before evaporation to complete dryness, derivatization, and chromatography.

Ion-exchange columns. Even with modifications of the drying step, the total method was still fairly time consuming. To further modify the method, each anion-exchange column was evaluated with ribose and phosphoric acid added to all samples. It appeared that the second anion-exchange column was not needed as the analysis of standards with added ribose and H_3PO_4 showed that the ribose was completely degraded by the hydrolysis, and the phosphate and perchlorate were removed by the first anion-exchange column. Thus, the following one-column method was developed.

ONE-COLUMN METHOD

1. Hydrolysis
Perchloric acid plus sample at 100° for 40 min.

2. Anion-exchange cleanup
(a) Place the hydrolyzed sample on a 100 × 15 mm anion-exchange column of Dowex 1-X2 formate (50/100 mesh).
(b) Wash the column with 10 × 3 ml of triply distilled water at 1 ml/min.
(c) Discard the first 3 ml.
(d) Collect the next 15 × 3-ml fractions in a 50-ml beaker.
(e) Concentrate the sample to 2 ml on a 60° hot plate with an IR lamp.
(f) Transfer the sample to a culture tube (4.5 ml total capacity), washing the beaker with 2 × 1 ml of 0.1 *N* HCl.
(g) Evaporate the sample to dryness on a 60° hot plate with a stream of pure nitrogen gas.

3. Silylation
The samples were derivatized with BSTFA at 150° for 15 min.

Using this procedure, four 100-μg standards of each base with 0.4 mg ribose and 0.4 mg H_3PO_4 were carried through the entire cleanup method. The % recoveries are given in Table II.

Anion-exchange resin. A small amount of interference, due to "fines" from the Dowex 1-X2 formate anion-exchange resin passing through the porous glass disc, was noticed on the chromatograms of the cleaned standards. A regeneration procedure was developed for the preparation of the resins to prevent further contamination by "fines", and another anion-exchange resin was used, *viz.* AG 1-X2 (a purified and sized Dowex 1-X2) resin in the acetate form.

REGENERATION OF RESIN

(1) Place 300 ml of the dry resin (AG 1-X2) in a 500 ml graduated cylinder.
(2) Add 3 *N* KOH (to the 500-ml mark).
(3) Invert the cylinder, mixing the resin and 3 *N* KOH.
(4) Allow the larger resin particles to settle, then pour off the excess KOH and resin "fines".
(5) Wash the resin five times with distilled water, mixing each time, allowing to settle, then pouring off the "fines".
(6) Repeat steps 2 to 5 at least three times.
(7) Wash the resin to neutral with distilled water.
(8) Add 3 *N* acetic acid (to the 500-ml mark).
(9) Thoroughly mix, allow to settle.
(10) Pour off the excess acetic acid and "fines".
(11) Wash five times with distilled water as in step 5.
(12) Repeat steps 8 to 11 at least three times.
(13) Wash the resin to neutral with distilled water.

Using this resin regeneration procedure and AG 1-X2 anion-exchange resin, the one-column method was again evaluated by cleaning four 100-μg standards of each base plus 0.4 mg ribose and 0.4 mg H_3PO_4. The dried samples were silylated and analyzed. The % recovery for each sample is given in Table II.

Cleanup of semimicro standards

Using the one-column cleanup method described above and the silylation conditions and instrumental settings developed for semimicro standards in our previous paper[9], four semimicro standards (10 μg each base) plus 40 μg ribose and 40 μg H_3PO_4 were carried through the cleanup procedure. A further refinement of the one-column method was employed: a 1/4-in. plug of glass wool was placed in the ion-exchange column on the coarse-porosity fritted disc, then the anion-exchange resin was added. The glass wool prevented the resin particles from passing through the glass disc and causing extraneous peaks on the chromatograms.

Analysis of RNA and DNA at macro and semimicro levels

Using this technique for sample cleanup and the silylating conditions presented in our previous paper[9], several types of RNA and DNA samples were analyzed at the macro (*ca.* 100 μg of each base) and the semimicro (*ca.* 10 μg of each base) levels. For macro samples, 1 mg and for semimicro samples 100 μg of nucleic acid were used. The mole % composition for each nucleic acid was calculated as follows:

$$\text{Mole \% composition} = \frac{\mu\text{moles}_{\text{Base}}}{\mu\text{moles}_{\text{Total}}} \times 100$$

where

$$\mu\text{moles}_{\text{B}} = \mu g_{\text{B}}/\text{MW}_{\text{B}}$$

$$\mu g_{\text{B}} = \frac{A_{\text{B}}}{A_{\text{I.S.}}} \times \frac{\text{MW}_{\text{B}}}{\text{MW}_{\text{I.S.}}} \times \frac{\mu g_{\text{I.S.}}}{RMR_{\text{B/I.S.}}}$$

$$RMR_{\text{B/I.S.}}\ (\text{standard}) = \frac{\text{Area}_{\text{B}}/\text{Moles}_{\text{B}}}{\text{Area}_{\text{I.S.}}/\text{Moles}_{\text{I.S.}}}$$

The average mole % composition for each nucleic acid and standard deviations are presented in Tables III and IV. Fig. 1–8 show typical chromatograms for RNA and DNA samples at the macro and semimicro levels.

Micro sample cleanup procedure

Initial experiments showed that, as in the silylation and chromatography of micro standards, the cleanup of micro sample hydrolysates would require changes from the cleanup method developed for macro and semimicro sample hydrolysates. Many types of ion-exchange cleanup were available, but since the one-column method using AG 1-X2 acetate anion-exchange resin developed for macro samples had worked satisfactorily it was fully evaluated first.

One-column method

Using the one-column method, eight micro (*ca.* 500 ng @ base) standards were analysed. The dried samples were silylated with 50 μl BSTFA, 25 μl acetonitrile, and 25 μl dichloroethane at 150° for 30 min (for development of silylating conditions, refer to our previous paper[9]), and analyzed by GLC. The chromatograms obtained from these analyses contained many extraneous peaks and had bad bleed rates. To remove as much contamination as possible, the AG 1-X2 acetate resin was again regenerated, but greater care was used to remove all of the resin "fines". The reagents used for the sample preparation were redistilled to remove contamination, and the BSTFA was evaluated to determine the best lot, *i.e.* the lot that gave the best chromatograms. The chromatographic column was conditioned at 270° until there was little column bleed at 240°. Using these refinements and very careful techniques to prevent sample contamination, the 500 ng of each base standard were again carried through the one-column method and analyzed by GLC.

The purines were not quantitatively recovered from the anion-exchange column, thus modifications in the procedure were made. One modification was elution of the purines from the column with dilute acid, another was to place the standards on an acidic column, and a third was to evacuate the resin before sample application to remove trapped air because the air could form pockets which would prevent the bases from passing through the resin at a uniform rate.

Evaluation of other ion-exchange resins

Since the one-column method with AG 1-X2 acetate anion-exchange resin did not give good recoveries for the purines, adenine and guanine, other ion-exchange resins were studied which would possibly give better recovery. The following resins were carefully regenerated and evaluated as possible replacements for AG 1-X2: AG 3-X4, Bio-Rex 9, Amberlite IR-4B, and Amberlite CG-120. These resins were chosen because they have different polymer lattices or functional groups from AG 1-X2. Four standards (10 μg, 2 μg, and two 500 ng @ base) were cleaned by each resin, and the samples were silylated and chromatographed.

Evaluation of a charcoal column cleanup procedure

Since ion-exchange chromatography gave poor recoveries for adenine and guanine, a different type of cleanup was attempted. SAXINGER[17] used a charcoal column cleanup for the purine and pyrimidine bases and reported good recoveries on 200 ng of radioactive adenine by counting the adenine before and after cleanup.

The charcoal column cleanup method was evaluated for nucleic acid hydrolysates at the micro level. The charcoal column was initially prepared by refluxing

a 1:1 w/w mixture of Norit A charcoal and Celite 545 for 2 h with the following solvents: 2 *N* HCl, 6 *N* NH_4OH, pyridine–water (1:1), pyridine, water, methanol, benzene–methanol–water, benzene, formic acid, and water. The charcoal–Celite mixture was thoroughly washed with each reagent before refluxing with that reagent. The prepared charcoal was placed in a 9 × 150 mm ion-exchange column, column size 9 × 9 mm. The hydrolyzed standard of the purine and pyrimidine bases was placed on the column and washed with 12 × 4 ml of triply distilled water and 12 × 4 ml 1 *N* HCl. The bases were then eluted with 6 × 4 ml of concentrated formic acid. The samples were dried, silylated, and analyzed by GLC. Semimicro standards (*ca.* 10 μg @ base) were used to evaluate this cleanup method. Initial experiments showed that the background from the charcoal procedure was not satisfactory. After the charcoal was exhaustively washed with formic acid, it was then evaluated at the semimicro and micro levels.

Evaluation of small ion-exchange column

Since the sample size for micro samples had been greatly reduced (*ca.* 500 ng @ base), the large anion-exchange column (100 × 15 mm) had prevented the purines from being recovered. The AG 1-X2 acetate anion-exchange resin or the glass wool used to prevent resin "fines" from contaminating the sample were probably adsorbing the purines, thus preventing their recovery. By using a smaller amount of resin and a "silanized" glass wool plug, the purines would have less sites for adsorption and would be recovered at least semi-quantitatively.

To evaluate the small column and "silanized" glass wool plug, four micro standards were hydrolyzed with 50 μl 70% perchloric acid, placed on the anion-exchange column (column size 100 × 4 mm), and washed through the column with 25 × 1 ml of triply distilled water. The experiment was repeated a number of times, and the average % recovery for each of the bases was calculated.

Analysis of RNA and DNA at the micro level

RNA analysis

The three types of RNA, yeast RNA I and II and tobacco mosaic virus (TMV-RNA), analyzed at the macro level were analyzed at the micro level by the procedure developed above. Approximately 5 μg of RNA were used for each analysis. Fifty microliters 70% perchloric acid were used for the hydrolysis and the hydrolysate was cleaned by anion-exchange chromatography using a 100 × 4 mm column of AG 1-X2 acetate anion-exchange resin. The samples were dried, silylated with BSTFA, and analyzed by GLC. The results of these analyses are presented in Table V. Fig. 9 shows a typical chromatogram of yeast RNA II at the micro level.

DNA analysis

As with the macro samples, a formic acid hydrolysis was used for the salmon sperm and calf thymus DNA samples to prevent thymine degradation. The anion-exchange columns were washed with 25 × 1 ml of 0.5 *N* acetic acid in place of the water wash. The results of these analyses are presented in Table VI. Fig. 10 shows a typical chromatogram of a 5 μg salmon sperm DNA hydrolysate carried through the procedure.

RESULTS AND DISCUSSION

Anion-exchange cleanup methods

Two-column method

The evaluation of the two-column cleanup method showed that a nucleic acid hydrolysate at the macro level could be cleaned by anion-exchange chromatography without significant loss of any of the bases and with complete removal of the interfering compounds, perchlorate, phosphate, and ribose. The average % recovery of four macro standards for each column and for the total method are presented in Table I. The first anion-exchange column gave good recoveries for all the bases, but the second anion-exchange column showed a slight loss for the purines, adenine and guanine. The average % recovery for the total procedure was very good, indicating that the method would be acceptable as a cleanup step for the analysis of the base composition of RNA and DNA hydrolysates.

TABLE I

GLC ANALYSIS AND RECOVERY OF THE PURINE AND PYRIMIDINE BASES AT THE MACRO LEVEL

Two-column ion-exchange cleanup method.

Column	*Recovery* (%)[a]				
	U	*T*	*C*[b]	*A*	*G*
First column[c]	93	—	90	97	97
Second column[c]	96	95	93	88	82
Total method[c]	94	—	88	90	99

[a] $\text{Recovery (\%)} = \frac{RMR_{B/I.S.}\ \text{(after cleanup)}}{RMR_{B/I.S.}\ \text{(standard)}} \times 100$

$$RMR_{B/I.S.} = \frac{\text{Area}_B/\text{Moles}_B}{\text{Area}_{I.S.}/\text{Moles}_{I.S.}}$$

[b] Cytosine gave two chromatographic peaks, each was integrated separately and the areas were added to obtain the total area.

[c] Average of four independent runs containing 100 μg of each base.

It is considered that the inconsistancies among the values for the single columns and the total method were due to deterioration of the stock solution as described[9].

One-column method

Even though the two-column cleanup method gave excellent recoveries of the bases, it was time consuming and thus a more efficient one-step column cleanup method was desired.

Drying of samples. Various drying methods were evaluated which would eliminate possible contamination of the sample and shorten analysis time. Four drying methods were evaluated, *viz.* rotary evaporation, lyophilization, and a hot plate with an IR lamp or a stream of pure nitrogen gas, and all gave good results with no apparent loss of the bases. The hot plate with an IR lamp was the easiest, fastest, and least likely to add contamination to the sample so it was used to dry large volumes, such as the wash from the anion-exchange columns. Also, the hot

plate with a stream of pure nitrogen gas was used to dry small volumes because of its speed and simplicity.

Ion-exchange columns. Further evaluation of each column in the two-column method indicated that the second anion-exchange column was not needed. The perchlorate and phosphate were removed by the first column, and the ribose was completely degraded in the perchloric acid hydrolysis step. The one-column cleanup method thus developed was evaluated with four 100-μg standards of each base plus ribose and H_3PO_4. The % recovery is given in Table II. No interfering peaks were found on the chromatograms, due to the ribose or phosphate, but a few resin "fines" caused some extraneous peaks that could interfere with the bases at a lower sample level.

TABLE II

GLC ANALYSIS AND RECOVERY OF THE PURINE AND PYRIMIDINE BASES AT THE MACRO LEVEL

One-column ion-exchange cleanup method.

Sample	*Dowex 1-X2 formate and recovery (%)*[a]				*AG 1-X2 acetate and recovery (%)*[a]			
	U	*C*	*A*	*G*	*U*	*C*	*A*	*G*
1	95	95	95	92	94	94	92	95
2	95	101	98	102	96	98	98	96
3	99	100	97	95	94	97	94	92
4	96	97	96	95	95	100	92	101
Average	96	98	96	96	95	95	94	96

$$\text{Recovery (\%)} = \frac{RMR_{B/I.S.}\text{ (through cleanup)}}{RMR_{B/I.S.}\text{ (standard)}} \times 100$$

where

$$RMR_{B/I.S.} = \frac{\text{Area}_B/\text{Moles}_B}{\text{Area}_{I.S.}/\text{Moles}_{I.S.}}$$

$RMR_{B/I.S.}$ (standard) is the average of four independent results.

Anion-exchange resin. To achieve quantitative recovery and efficiency of cleanup of the purine and pyrimidine bases, the anion-exchange resin was changed from Dowex 1-X2 formate to AG 1-X2 acetate, and a regeneration procedure of the resin was developed. To evaluate the AG 1-X2 acetate anion-exchange resin, four 100-μg standards of each base plus ribose and H_3PO_4 were analyzed, and the % recovery is presented in Table II. No resin "fines" were observed in the samples, and the chromatograms were free of extraneous peaks.

Semimicro samples

Analysis of semimicro (*ca.* 10 μg @ base) standards using the single-column procedure with AG 1-X2 showed that all of the bases were quantitatively recovered. The following average % recoveries were obtained from four independent semimicro standards plus ribose and phosphate: U 85 %, T 93 %, C 92 %, A 85 %, and G 86 %. No extraneous peaks due to ribose degradation products, phosphate, or resin "fines" were found on the chromatograms.

RNA analysis

Macro samples

Three types of RNA (yeast RNA I, yeast RNA II, and TMV-RNA) were analyzed at the macro level (1 mg of RNA or *ca.* 100 μg @ base) using the single-column procedure described above. The mole % composition for each type of RNA was calculated by the formula presented earlier, and these values, along with the standard deviations, are presented in Table III. The literature values for the yeast RNA II and TMV-RNA were obtained from TLC data by Dr. SEHGAL, Department of Genetics, University of Missouri. The values obtained by the GLC procedure were in excellent agreement with those obtained by TLC. Figs. 1–3 show typical chromatograms of the RNA's analyzed. Each peak represents approximately 1 μg injected.

TABLE III

MOLE PER CENT COMPOSITION OF RNA SAMPLES

Weight[a] (μg)	*RNA*	*Mole % composition*[b] ± *S.D.*[c]			
		U	*C*	*A*	*G*
100	Yeast RNA I	20.6 ± 1.4	24.2 ± 1.3	26.5 ± 0.9	28.7 ± 1.4
10	Yeast RNA I	20.8 ± 2.3	23.9 ± 1.0	26.3 ± 1.7	28.0 ± 1.2
100	Yeast RNA II	21.2 ± 2.0	25.1 ± 0.9	26.6 ± 0.6	27.0 ± 1.1
10	Yeast RNA II	21.7	26.5	27.0	24.8
Literature value[d]		20.1	24.6	25.0	30.1
100	TMV-RNA	26.5 ± 2.3	20.2 ± 0.8	30.2 ± 2.7	23.1 ± 1.0
10	TMV-RNA	25.8 ± 1.5	20.0 ± 2.2	31.3 ± 1.2	22.3 ± 0.7
Literature value[d]		26.3	18.5	29.8	25.3

[a] Approximate weight of each base in sample of RNA analyzed.
[b] Mole % composition = μmoles$_B$/μmoles$_{Total}$ × 100.
[c] S.D. = Standard deviation. Four or more independent analyses.
[d] Literature values obtained from TLC data by Dr. SEHGAL, Department of Genetics, University of Missouri.

Semimicro samples

For the analysis of RNA at the semimicro level, 100 μg of RNA (*ca.* 10 μg @ base) were carried through the single-column procedure. The mole % composition plus standard deviations for each type of RNA are given in Table III. The results agree quite well with those obtained from macro samples and with TLC literature values. Typical chromatograms for semimicro samples of RNA are shown in Figs. 4 and 5. Each peak represents approximately 250 ng injected. Because of impurities in the RNA, some samples contained as little as 5 μg of each base. No problems were encountered due to this decrease in total RNA or from the impurities in the sample analyzed.

DNA analysis

Hydrolysis

Initial experiments showed that the hydrolysis of DNA with 70 % perchloric acid was unsatisfactory. Only 20 % of the thymine was recovered under the hydrolysis conditions used. JORDAN[10] indicated that perchloric acid was not the reagent

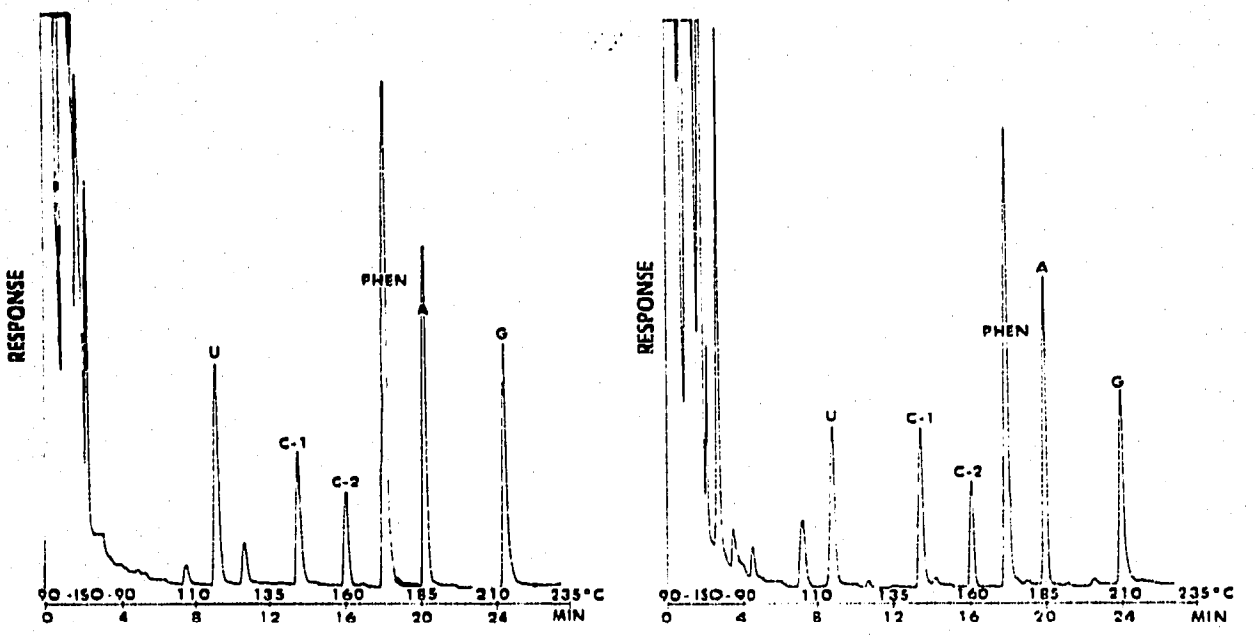

Fig. 1. GLC chromatogram of TMS derivatives of bases in yeast RNA I — macro analysis. Column: 10 w/w% GC SE-30 on 100/120 mesh Supelcoport, 1 m × 4 mm I.D. glass. Instrumental conditions: 7.5°/min temperature program from 90°–250° with a 4-min initial hold. Attenuation: 1.2×10^{-9} a.f.s. RNA sample: 1 mg; each peak represents *ca.* 1 μg injected. Internal standard: phenanthrene. Hydrolysis with 70% perchloric acid at 100° for 40 min. Ion-exchange cleanup by the one-column method on a 100 × 15 mm AG 1-X2 acetate column. Silylation with 200 μl BSTFA and 200 μl acetonitrile at 150° for 15 min.

Fig. 2. GLC chromatogram of TMS derivatives of bases in yeast RNA II — macro analysis. For further information, see the legend to Fig. 1.

of choice for DNA, but that concentrated formic acid plus DNA heated in a closed tube at 175° for 2 h gave good results. The formic acid hydrolysis method was found to be satisfactory for DNA, but not for RNA as uridylic acid was not completely hydrolyzed to uracil.

Also, the one-column cleanup method had to be slightly modified for analysis of DNA because the formic acid was not strong enough to keep all the bases as cations. The purines tended to be partially retained by the anion-exchange resin with water as wash, but when 1 *N* acetic acid was used to elute the bases, all were quantitatively recovered.

Macro samples

Two types of DNA (Salmon sperm DNA and calf thymus DNA) were analyzed by the method proposed above. One milligram of DNA was hydrolyzed with 0.5 ml of 98 to 100% formic acid at 175° for 2 h. The samples were carried through the one-column method using 1 *N* acetic acid to elute the column. The average mole % composition for each DNA was calculated and these values plus the standard deviations are given in Table IV. The literature values were obtained from SOBER's *Handbook of Biochemistry*. The values obtained for the salmon sperm DNA agreed closely with the literature values, but the calf thymus DNA values for thymine were low and for cytosine were high as compared to the literature. It is possible

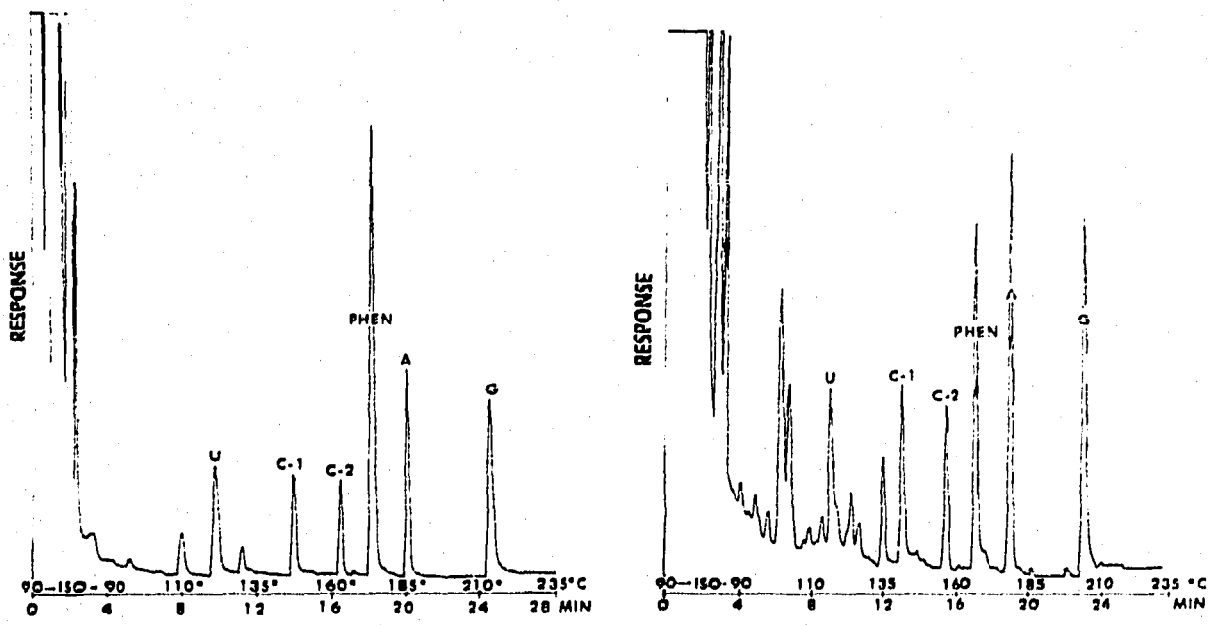

Fig. 3. GLC chromatogram of TMS derivatives of bases in TMV-RNA — macro analysis. For further information, see the legend to Fig. 1.

Fig. 4. GLC chromatogram of TMS derivatives of bases in yeast RNA I — semimicro analysis. Column: 10 w/w% GC SE-30 on 100/120 mesh Supelcoport, 1 m × 4 mm I.D. glass. Instrumental conditions: 7.5°/min temperature program from 90°–250° with a 4-min initial hold. Attenuation: 3.2×10^{-10} a.f.s. RNA sample: 100 μg; each peak represents *ca.* 250 ng injected. Internal standard: phenanthrene. Hydrolysis with 70% perchloric acid at 100° for 40 min. Ion-exchange cleanup by the one-column method on a 100 × 10 mm AG 1-X 2 acetate column. Silylation with 100 μl BSTFA and 100 μl acetonitrile at 150° for 15 min.

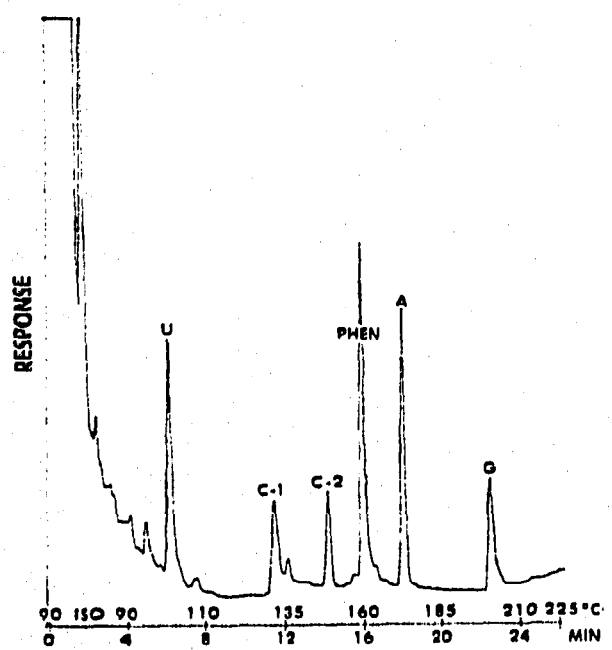

Fig. 5. GLC chromatogram of TMS derivatives of bases in TMV-RNA — semimicro analysis. For further information, see the legend to Fig. 4.

TABLE IV

MOLE PER CENT COMPOSITION OF DNA SAMPLES

Weight[a] (μg)	DNA	Mole % composition[b] ± S.D.[c]			
		T	C	A	G
100	Salmon sperm DNA	28.9 ± 1.4	21.9 ± 1.6	28.8 ± 0.7	20.6 ± 0.5
10	Salmon sperm DNA	26.8 ± 1.1	20.5 ± 1.7	31.2 ± 0.2	22.3 ± 1.4
Literature value[e]		28.5 ± 1.5[d]	21.1 ± 0.8[d]	28.7 ± 0.9[d]	22.3 ± 0.7[d]
100	Calf thymus DNA	23.6	24.4	30.4	21.6
10	Calf thymus DNA	24.6	22.8	28.9	23.5
Literature value[e]		28.2	21.2	28.4	21.8

[a] Approximate weight of each base in sample of DNA analyzed.
[b] Mole % composition = $\mu moles_{B}/\mu moles_{Total} \times 100$.
[c] S.D. = Standard deviation. Four or more independent analyses.
[d] S.D. = Standard deviation of four literature values.
[e] Literature value = average of eighteen results.

that some deamination of cytosine occurred during hydrolysis giving low cytosine data for the literature values, and incomplete hydrolysis of the DNA would give low thymine values. Figs. 6 and 7 show typical chromatograms obtained for DNA at the macro level. Each peak represents *ca.* 1 μg injected.

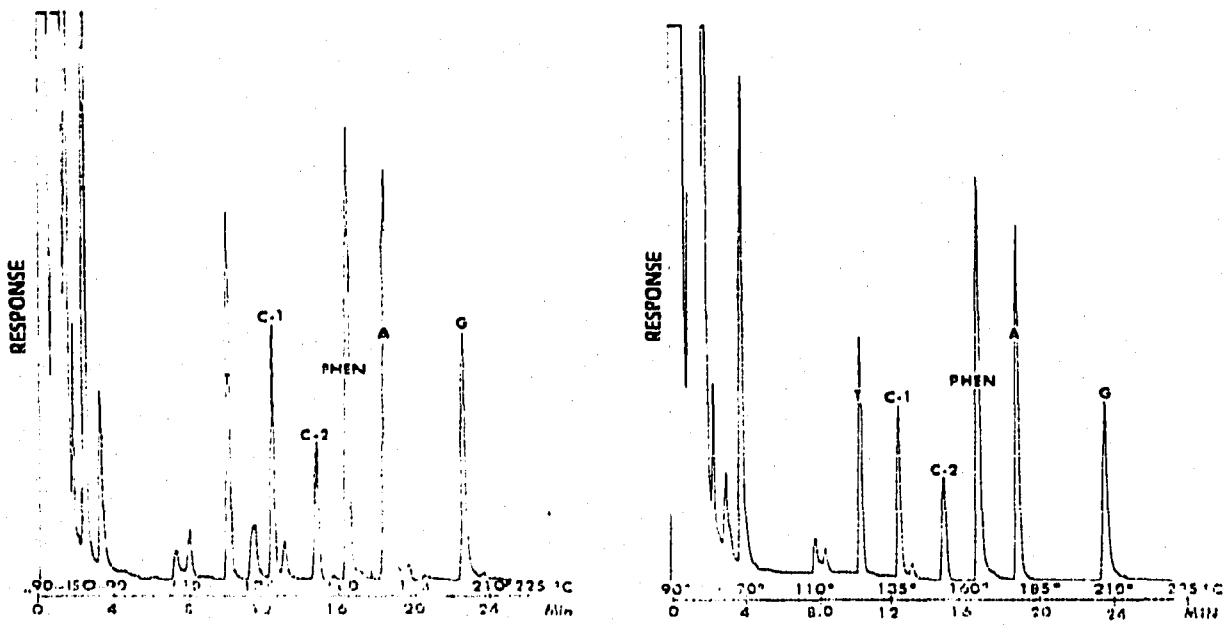

Fig. 6. GLC chromatogram of TMS derivatives of bases in salmon sperm DNA — macro analysis. Column: 10 w/w% GC SE-30 on 100/120 m. Supelcoport, 1 m × 4 mm I.D. glass. Instrumental conditions: 7.5°/min temperature program from 90°–250° with a 4-min initial hold. Attenuation: 1.2×10^{-8} a.f.s. DNA sample: 1 mg; each peak represents *ca.* 1 μg injected. Internal standard: phenanthrene. Hydrolysis with formic acid at 175° for 2 h. Ion-exchange cleanup by the one-column method on a 100 × 15 mm column washed with 1 *N* acetic acid. Silylation with 200 μl BSTFA and 200 μl acetonitrile at 150° for 15 min.

Fig. 7. GLC chromatogram of the bases in calf thymus DNA — macro analysis. For further information, see the legend to Fig. 6.

Semimicro samples

Analysis of 100 μg of DNA (*ca.* 10 μg of base) using formic acid hydrolysis gave good results. The mole % composition (Table IV) agreed very well with the values obtained from macro samples of DNA. Fig. 8 shows a typical chromatogram of a semimicro sample of salmon sperm DNA. Each peak represents 250 ng injected.

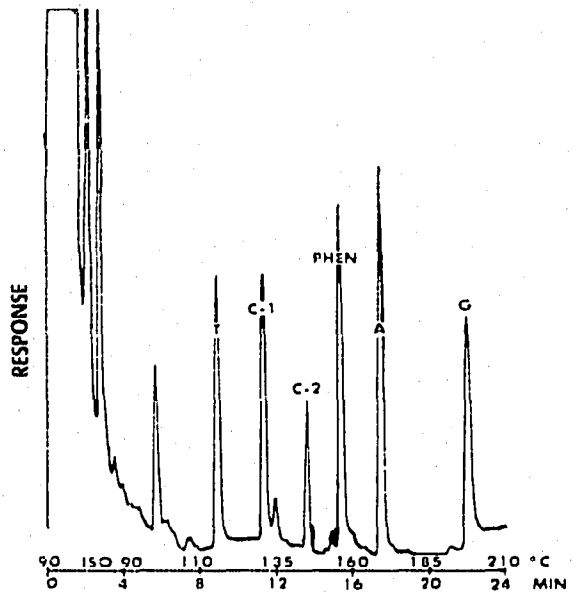

Fig. 8. GLC chromatogram of TMS derivatives of bases in salmon sperm DNA — semimicro analysis. Column: 10 w/w% GC SE-30 on 100/120 mesh Supelcoport, 1 m × 4 mm I.D. glass. Instrumental conditions: 7.5°/min temperature program from 90°–250° with a 4-min initial hold. Attenuation: 3.2×10^{-10} a.f.s. DNA sample: 100 μg; each peak represents 250 ng injected. Internal standrad: phenanthrene. Hydrolysis with formic acid at 175° for 2 h. Ion-exchange cleanup by the one-column method on a 100 × 15 mm AG 1-X2 acetate column washed with 1 *N* acetic acid. Silylation with 100 μl BSTFA and 100 μl acetonitrile at 150° for 15 min.

Micro sample cleanup method

One-column method

The single-column procedure using AG 1-X2 acetate anion-exchange resin developed for cleaning macro and semimicro hydrolysates was fully evaluated as a cleanup method for micro sample analysis. Initial experiments showed that extreme care in handling samples was necessary to prevent contamination. The resin "fines" must be completely removed, the reagents free of impurities, and the BSTFA of excellent quality. Even with these careful techniques, the purines adenine and guanine were not recovered from the anion-exchange column.

In experiments to recover the purines, the anion-exchange columns were eluted with dilute acid (HCl or acetic acid). The purine recovery was slightly better (approximately 20 % for adenine and guanine), but still too low for a semi-quantitative analysis. An acidic anion-exchange column was evaluated and the recoveries were improved, but still low. When the resin column was evacuated to remove trapped air, the resulting chromatograms were cleaner, but the recovery was not improved. These experiments indicated that the single-column macro or semimicro procedure, even with modifications, would not serve as a cleanup method for the analysis of nucleic acid hydrolysates at the micro level.

Other ion-exchange resins

The above experiments showed that adenine and guanine at the micro level were lost on passing through the anion-exchange column. The AG 1-X2 acetate resin could have active sites on which the purines were being held, thus preventing them from passing through the column.

Other ion-exchange resins, with a different polymer lattice or a different functional group from AG 1-X2, might give the desired recovery of these bases. The following resins were evaluated as possible replacements for AG 1-X2: AG 3-X4, Bio-Rex 9, Amberlite IR-4B, and Amberlite CG-120. To evaluate each resin, it was carefully regenerated, and the resin "fines" removed. Four standards (10 μg, 2 μg, and two 500 ng *a* base) were then cleaned by each resin. The AG 3-X4 resin gave very clean chromatograms and good recoveries of all the bases for the 10-μg standard, but the recoveries of adenine and guanine at the 500-ng level were again low. Other experiments with modifications gave similar results at the micro level. Bio-Rex 9 and Amberlite IR-4B gave poor recoveries at the 10-μg of each base level, and the chromatograms contained many extraneous peaks. Even with exhaustive regeneration, the chromatograms were unacceptable. Amberlite CG-120, a cation-exchange resin, gave purine recovery of 50 % at the micro level, but the pyrimidines were completely lost. Additional experiments did not improve the purine recoveries, and the pyrimidines were not detected.

Charcoal column cleanup

The ion-exchange methods gave poor recoveries for the purines, adenine and guanine, at the micro level. A method of cleaning up micro samples of the bases had been developed by Dr. CARL SAGINGER, Ames Research Center-NASA, but this method had not been used in conjunction with GLC so the method was fully studied. Initial experiments at the semimicro (10 μg *a* base) level showed the regeneration procedure developed by Dr. SAGINGER did not remove all of the impurities from the charcoal, and these impurities interfered with the chromatography of the bases by GLC. Thus, the charcoal was exhaustively washed with concentrated formic acid (the eluting reagent) and with water to remove as much of the impurities as possible. With this prepared charcoal, the semimicro experiment was rerun with good results. The average % recovery for twelve semimicro standards containing H_3PO_4 and ribose were: U 92 %, T 92 %, C 105 %, A 93 %, and G 90 %. No extraneous peaks were observed on the chromatogram due to the perchloric acid, H_3PO_4, or ribose; and only minor peaks were detected as coming from the charcoal.

With this method and the formic acid washed charcoal, experiments were then made to determine the recovery of the bases at the micro (500 ng *a* base) level. The contamination from the charcoal observed in the initial semimicro experiment was again present so the recovery could not be ascertained. Further regeneration of the charcoal did not remove the impurities so the method could not be used for micro analysis of nucleic acid hydrolysates, however, the method could be used for semimicro analyses with good success.

Small ion-exchange column

Since the amount of sample in micro analyses was greatly reduced, the amount of perchloric acid needed for hydrolysis was also reduced, and the amount

of resin needed to remove the interfering compounds could be lowered. The less resin employed for cleanup would offer fewer active sites for possible adsorption of the purines. Also, a "silanized" glass wool plug was used in place of normal glass wool to decrease active sites. With these ideas, experiments were conducted with 50 μl perchloric acid for hydrolysis, an AG 1-X2 acetate anion-exchange column of 100 × 4 mm, and 25 × 1 ml of triply distilled water as wash. Initial experiments showed promise with adenine and guanine being recovered at approximately 60 %. Using very careful techniques, twelve micro (500 ng *ea.* base) standards were carried through the method with an average recovery of U 66 %, T 53 %, C 92 %, A 70 %, and G 65 %. In general, these values were too low for good quantitative analysis, but could be used for semi-quantitative data. The mole % composition of RNA or DNA at the micro level could be semi-quantitatively determined.

Micro analysis of RNA and DNA

RNA analysis

The three types of RNA (yeast RNA I and II, and TMV-RNA) analyzed at the macro (1 mg RNA) and semimicro (100 μg RNA) levels were analyzed at the micro (5 μg RNA) level by the method outlined above. Four independent samples of each type of RNA were analyzed, and the average mole % composition is given in Table V. The values compare well with the mole % composition obtained at the macro level, even though the micro analyses were only semi-quantitative. Fig. 9 shows a typical chromatogram for yeast RNA II at the micro level. Each peak represents approximately 30 ng injected.

TABLE V

COMPARISON OF GLC MICRO AND MACRO ANALYSES OF RNA SAMPLES

500 ng of each base standard were carried through cleanup with the RNA's to correct losses of the bases on the anion-exchange column.

Weight[a]	*RNA*	*Mole % composition*[b]			
		U	*C*	*A*	*G*
5 μg	Yeast RNA I[c]	22.8	22.8	26.8	27.7
1 mg	Yeast RNA I[d]	20.6	24.2	26.5	28.7
5 μg	Yeast RNA II[c]	26.1	21.6	28.6	23.2
1 mg	Yeast RNA II[d]	26.5	20.2	30.2	23.1
5 μg	TMV-RNA[c]	23.3	26.5	24.2	26.2
1 mg	TMV-RNA[d]	21.2	25.1	26.0	27.6

[a] Weight = weight of RNA sample.
[b] Mole % composition = $\text{nmoles}_{\text{B}}/\text{nmoles}_{\text{Total}} \times 100$.
[c] Four independent analyses for each RNA. The standard deviation ranged from ± 0.5 to ± 1.7.
[d] Average value for each RNA at the macro level.

DNA analysis

Two types of DNA (salmon sperm DNA and calf thymus DNA) that were analyzed at the macro and semimicro levels were analyzed at the micro level. To hydrolyze the DNA, 100 μl of concentrated formic acid were used to prevent thymine

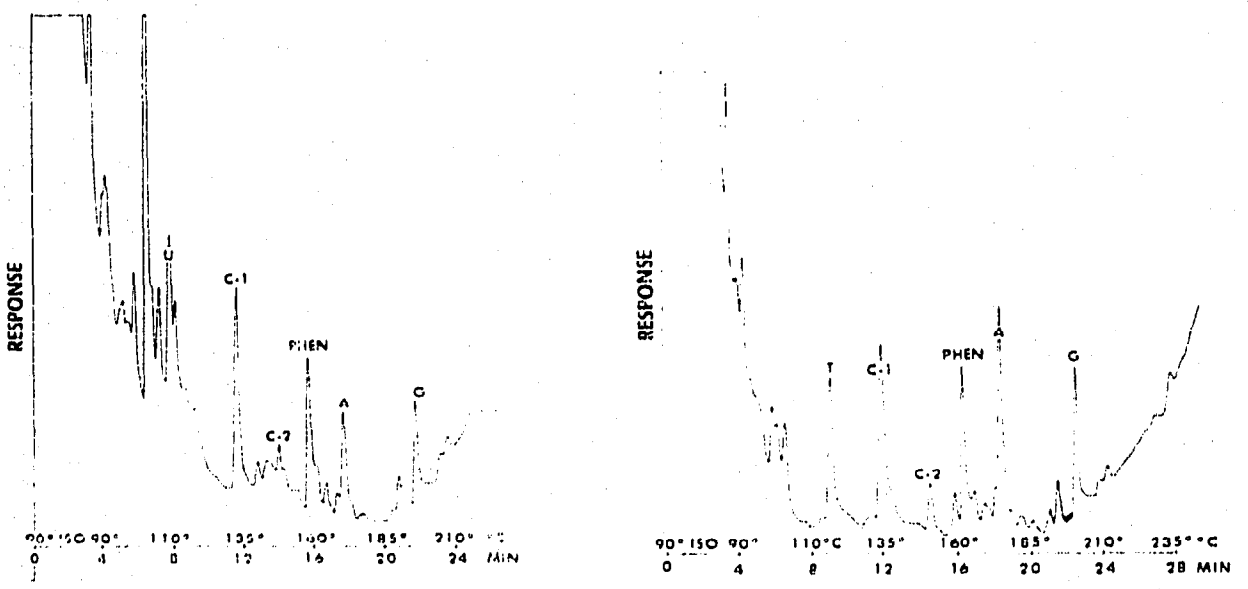

Fig. 9. GLC chromatogram of TMS derivatives of bases in yeast RNA II — micro analysis. Column: 10 w/w % GC SE-30 on 100/120 mesh Supelcoport, 1 m × 4 mm I.D. glass. Instrumental conditions: 7.5°/min temperature program from 90°–250° with a 4-min initial hold. Attenuation: 8×10^{-11} a.f.s. RNA sample: 5 μg; each peak represents *ca.* 30 ng injected. Internal standard: phenanthrene. Hydrolysis with 70% perchloric acid at 100° for 40 min. Ion-exchange cleanup by the one-column method on a 100 × 4 mm AG 1-X2 acetate column. Silylation with 50 μl BSTFA, 25 μl acetonitrile and 25 μl dichloroethane at 150° for 30 min.

Fig. 10. GLC chromatogram of TMS derivatives of bases in salmon sperm DNA — micro analysis. Column: 10 w/w % GC SE-30 on 100/120 mesh Supelcoport, 1 m × 4 mm I.D. glass. Instrumental conditions: 7.5°/min temperature program from 90°–250° with a 4-min initial hold. Attenuation: 8×10^{-11} a.f.s. DNA sample: 5 μg; each peak represents *ca.* 30 ng injected. Internal standard: phenanthrene. Hydrolysis with 100% formic acid at 175° for 2 h. Ion-exchange cleanup by the one-column method on a 100 × 4 mm AG 1-X2 acetate column. Silylation with 50 μl BSTFA, 25 μl acetonitrile and 25 μl dichloroethane at 150° for 30 min.

TABLE VI

COMPARISON OF GLC MICRO AND MACRO ANALYSES OF DNA SAMPLES

500 ng of each base standard were carried through cleanup with the DNA's to correct losses of the bases on the anion exchange column.

Weight[a]	*DNA*	*Mole % composition*[b]			
		T	*C*	*A*	*G*
5 μg	Salmon sperm DNA[c]	27.0	20.4	28.0	22.4
1 mg[d]	Salmon sperm DNA[c]	28.0	21.0	28.8	20.6
5 μg	Calf thymus DNA[c]	29.4	23.0	28.5	19.1
1 mg[d]	Calf thymus DNA[c]	23.0	24.4	30.4	21.6
Literature value		28.2	21.2	28.2	21.8

[a] Weight = weight of DNA sample.
[b] Mole % composition = $\text{nmoles}_{\text{n}}/\text{nmoles}_{\text{total}} \times 100$.
[c] Four independent analyses for each DNA. The standard deviation ranged from ± 0.3 to ± 2.7.
[d] Average value for each DNA at the macro level.

degradation, which occurs on hydrolysis with perchloric acid. The anion-exchange column was washed with 0.5 *N* acetic acid in place of water to prevent the bases from being held on the resin. Standards were recovered using this method at U 68 %, T 80 %, C 89 %, A 74 %, and G 66 %. Four independent samples of each DNA were analyzed and the average mole % composition is given in Table VI. The values for micro analysis of salmon sperm DNA compared well with those obtained at the macro level, and the calf thymus DNA mole % composition at the micro level compared well with literature values, but not with the values obtained at the macro level for thymine. The hydrolysis at the macro level may have been incomplete due to the composition of DNA, giving low thymine values. Fig. 10 shows a typical chromatogram for 5 μg of salmon sperm DNA hydrolyzed with formic acid. Each peak represents *ca.* 30 ng injected.

SUMMARY AND CONCLUSIONS

A quantitative GLC method has been developed for the analysis of hydrolysates of nucleic acids at the macro (1 mg) and semimicro (100 μg) levels of total nucleic acids and a semi-quantitative method for micro samples (5 μg of nucleic acid). An anion-exchange cleanup procedure, which gave quantitative recovery of the purine and pyrimidine bases, removed the phosphate and ribose released during hydrolysis of the sample, and did not add interfering compounds which would affect the silylation and chromatography of the bases, was needed. The final cleanup method consisted of a single-column procedure using AG 1-X2 acetate anion-exchange resin. GLC analysis of the bases was accomplished by first converting them to their TMS derivative with BSTFA followed by chromatography on a 10 w/w % CG SE-30 100/120 mesh Supelcoport column.

First, a two-column anion-exchange cleanup procedure was developed which gave good recovery of the bases without added contamination. However, this procedure was time consuming and thus was modified. The modifications gave rise to a single column cleanup procedure using AG 1-X2 acetate anion-exchange resin, which gave 95 to 100 % recovery of the bases at the macro level (100 μg (*a* base) and 85 to 90 % recovery at the semimicro level (10 μg (*a* base).

Using the one-column cleanup method, and the silylating and chromatographic conditions developed earlier, three types of RNA (yeast RNA I and II and TMV-RNA) and two types of DNA (salmon sperm DNA and calf thymus DNA) were analyzed at the macro and semimicro levels. The RNA's were hydrolyzed with 70 % perchloric acid and the DNA's with 98 to 100 % formic acid. DNA could not be quantitatively hydrolyzed with perchloric acid because thymine was partially destroyed, and formic acid did not completely hydrolyze uridylic acid in RNA to uracil. The mole % composition of each RNA and DNA was determined and found to be in good agreement with values reported in the literature.

A number of problems were encountered in the development of a micro method for analysis of nucleic acids (5 μg). The one-column method used for macro and semimicro samples was unsatisfactory, because the purines, adenine and guanine, were not recovered from the ion-exchange column. Some changes, including eluting with dilute acid, using an acidic column, evacuating the resin to remove trapped air, and changing the ion-exchange resin, were tried without success. Recovery

at the semimicro level with some of the modifications was good, but at the micro (500 ng @ base) level, the purines were not recovered.

A charcoal column cleanup procedure was evaluated as a possible alternative for micro analysis. Excellent recoveries at the semimicro level (10 μg @ base) were obtained, but at the micro level, impurities from the charcoal column interfered with the silylation and chromatography of the bases. Even with exhaustive regeneration of charcoal and very careful techniques, the impurities could not be removed.

In micro analyses, the amount of nucleic acid sample used was considerably reduced, thus the amount of perchloric acid needed for hydrolysis and the anion-exchange column size used in the one-column method could be correspondingly reduced. Using a 100 × 4 mm AG 1-X2 acetate anion-exchange column with a small plug of "silanized" glass wool above the fritted glass disc and 50 μl of perchloric acid for hydrolysis, all of the bases were semi-quantitatively recovered. Little interference from the anion-exchange resin was observed on the chromatograms so the procedure could be used for semi-quantitative determination of the mole % composition of RNA or DNA.

With this method, the three RNA's and two DNA's were analyzed at the micro level (5 μg of total nucleic acid). Hydrolysis with perchloric acid was used for RNA and with formic acid for DNA. The mole % composition for all samples was in good agreement with the values obtained at the macro level (1 mg of total nucleic acid) and with literature values.

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