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GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF NUCLEIC ACID COMPONENTS*

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SUMMARY

The aim of this investigation was to develop a quantitative gas–liquid chromatographic method of analysis for the components of nucleic acids (*i.e.*, purine and pyrimidine bases, nucleosides, or nucleotides), and to apply this method to the analysis of biological material. The N-methyl derivatives of the purine and pyrimidine bases, prepared by thermal dissociation of their tetramethylammonium salts were found to be unsuited for quantitative analysis due to the formation of multiple chromatographic peaks for cytosine, adenine, and guanine. The trimethylsilyl (TMS) derivatives of the bases were found to be far superior to the N-methyl compounds. Bis(trimethylsilyl)acetamide (BSA) was evaluated and found to be a good reagent for silylation of the bases. The optimum derivatization conditions were heating the bases in a closed tube at 150° for 45 min, with a 100 molar excess of BSA to total bases, and a 3:1 v/v acetonitrile/BSA ratio. Calibration curves for the five main bases (U, T, C, A, and G) were prepared and found to be linear over a sample weight range of 25–2000 μg of base. The relative standard deviations ranged from 1.1 % for uracil to 3.1 % for cytosine. The minimum detectable amount (MDA) using the hydrogen flame ionization detector, was determined to be 3 to 5 $\times$ 10^{-9} g or *ca.* 3 $\times$ 10^{-11} moles of each base injected. Comparative studies were made of BSA as a silylation reagent with bis(trimethylsilyl)trifluoroacetamide (BSTFA), and reagent solutions of 0.1 v/v % TMCS in BSA, and 1.0 v/v % TMCS in BSA. There were no significant differences in regard to analytical derivatization yield with any of these reagents. Important advantages of BSTFA include "cleaner" chromatograms with fewer extraneous peaks and complete miscibility of BSTFA in the solvent. A column of 8 w/w % SE-30 on 100–120 mesh Supelcoport provided good resolution and stability for the TMS bases and was superior to all chromatographic columns investigated.

Application of the developed method to the analysis of biological materials was accomplished using perchloric acid hydrolysis and anion-exchange removal of the bases from biological background prior to derivatization and GLC analysis. The combined hydrolysis and purification procedures were shown to give a recovery of the

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bases of *ca.* 82 % with the recovery of the four bases being equivalent (± 1.5 %). Base ratio analysis of purified yeast RNA showed that the method was precise giving an average relative standard deviation of 3.0 %. Experiments are in progress on the analysis of microgram amounts of RNA and DNA.

INTRODUCTION

Many areas of biochemistry, molecular biology and other related fields of research are involved either directly or indirectly with the study of the nucleic acids.

Because of the importance of the genetic code it is mandatory to know as much as possible about the chemical compositions of these compounds, in order to study their functions. Determination of the base composition is made possible by hydrolysis of these polymeric compounds into the various monomers. Of particular interest in many areas is the determination of the purine and pyrimidine base ratios.

Methods of determining the bases and other nucleic acid components, including nucleosides and nucleotides, have already been developed using various analytical, chromatographic, and instrumental techniques. These include ion-exchange chromatography, COHN[1]; paper chromatography, VISCHER AND CHARGAFF[2]; paper electrophoresis, GORDON AND REICHARD[3]; and thin-layer chromatography, RANDERATH[4].

The development of gas–liquid chromatography (GLC) and its successful application to many similar analytical problems in the field of biochemistry as steroids, fatty acids and amino acids, suggested that a similar approach for the analysis of nucleic acid components might be developed.

The speed, accuracy, and sensitivity afforded by gas–liquid chromatography offers definite advantages over these other analytical techniques. However, before gas chromatographic analysis of the purine or pyrimidine bases, nucleosides, or nucleotides can be accomplished, these compounds must first be converted to volatile derivatives with suitable chromatographic properties.

In 1962, the first report of the analysis of nucleic acid components by GLC was published by MILES AND FALES[5]. They investigated only the nucleosides, and the derivatives they chose to study were acetyl, methyl, and/or isopropylidene. MACGEE[6] investigated the N-methyl derivatives of the purine and pyrimidine bases, and applied the method to the analysis of hydrolysates of nucleic acids. The procedure yielded useful data on as little as 2.5 nanomoles of each base, but only if the bases were present in an equimolar distribution. Further, only a few of the naturally occurring nucleic acids have an equimolar base ratio. The primary disadvantage of their method involved multiple derivatives, with as many as four chromatographic peaks derived from one particular purine, adenine. These multiple peaks required the use of correction factors in the calculations.

GLC of the trimethylsilyl (TMS) derivatives of nucleosides was also reported by HANCOCK AND COLEMAN[8]. The derivatives were synthesized using hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) in pyridine. HANCOCK[9] reported later the successful gas chromatography of various adenosine derivatives which included 5-adenosyl-methionine and the nucleotides adenosine monophosphate and adenosine diphosphate.

In 1966, HASHIZUME AND SASAKI[10] reported the gas chromatographic separation

of the ribonucleotides utilizing the trimethylsilyl derivatives. Tris (TMS) phosphate was synthesized initially by reacting trisodium or tripotassium phosphate with a mixture of HMDS and TMCS. This led to the successful preparation of the TMS nucleotides by refluxing 10 mg of the alkali salts of the nucleotides for one hour in a solution of 0.2 ml anhydrous pyridine, 0.1 ml HMDS and 0.05 ml TMCS. Later the same year, SASAKI AND HASHIZUME[11], demonstrated the applicability of their procedure for preparing the TMS derivatives of the purine and pyrimidine bases and nucleosides. Pure TMS derivatives of selected bases and nucleosides were prepared in macro amounts and purified by vacuum distillation. These derivatives were characterized by elemental analysis, N.M.R., and I.R. spectra. Although quantitation was not demonstrated, the relative error of the individual molar responses was within $\pm 4\%$.

In July of 1966, KLEBE *et al.*[12] reported silylation studies with compounds other than nucleic acid components with a new reagent, bis(trimethylsilyl)acetamide (BSA). This reagent was demonstrated to be a more potent trimethylsilyl donor than previously known silylating reagents.

In 1968, GEHRKE and coworkers[13] reported a new silylating reagent for amino acids, bis(trimethylsilyl)trifluoroacetamide (BSTFA), which is an analog of BSA. BSTFA reacts similarly to BSA, is more volatile, and with some compounds reaction is faster and more complete.

The purpose of this investigation was to develop a method for the quantitative analysis of nucleic acid components using gas–liquid chromatography with particular emphasis on chemistry of derivatization, chromatography, quantitative analysis, and application to biological samples. GEHRKE *et al.*[14] in late 1967 reported a communication on some of the initial phases of this investigation.

EXPERIMENTAL

(1) *Apparatus*

An F and M Model 402 Biomedical Gas Chromatograph (F and M Scientific, Division of Hewlett Packard, Avondale, Pa.) equipped with dual hydrogen flame ionization detectors was used.

Area determinations of the chromatographic peaks were made with a Disc Integrator Model 228-A (Disc Instruments Inc., Santa Ana, Calif.).

For elevated temperature reactions, a magnetically stirred, high temperature oil bath (100–200°), with a variable temperature control system ($\pm 2°$), was constructed in this laboratory. The oil bath was placed behind a safety shield.

A freeze-dry apparatus, or lyophilizer, made by the Scientific Instrument Shop, University of Missouri, was used to dry the nucleic acid samples following hydrolysis and ion-exchange clean-up.

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

(2) *Reagents*

All of the purine and pyrimidine bases, nucleosides and nucleotides were obtained from Mann Research Laboratories, and were chromatographically pure. Bis-(trimethylsilyl)acetamide (BSA) was purchased from Aldrich Chemical Co., and bis-(trimethylsilyl)trifluoroacetamide (BSTFA) from Regis Chemical Co. The other

silylating reagents, hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were obtained from Pierce Chemical Co. Acetonitrile was of "nanograde" purity and was obtained from Mallinckrodt Chemical Works. The ion-exchange resin used was Dowex 1-x2 (100–200 mesh) and was purchased from J. T. Baker Chemical Co. The yeast RNA was obtained from Dr. James Ross, Department of Plant Pathology, University of Missouri.

(3) Instrumental and chromatographic conditions

The chromatographic columns used in the initial phases of this investigation were 4 w/w % SE-30 (straight chained polymethylsiloxane) on 80–100 mesh High Performance Chromosorb G (Johns Manville Inc., New York, N.Y.). The columns used in the latter part of the study were 8 w/w % SE-30 on 100–120 mesh Supelcoport (Chromosorb W type). These column materials were packed in glass columns (borosilicate) which were 1 m long, with an inside diameter of 4 mm.

The instrumental conditions were varied using both 7.5 and 10°/min temperature programs. These programs usually ran from 100° to 250°, however, nucleosides or nucleotides demanded higher final temperatures, approximately 300°. The detector was operated in the 280–320° region, and the injection heater was at approximately 180°. The gas flow rates were as follows with line pressures of 40 p.s.i.g. for all three gases: N_2, 80 ml/min; H_2, 40 ml/min; air, 300 ml/min.

The oven heaters were not capable of following the control setting for the temperature rate of increase. It was determined that the average rate of increase at a setting of 7.5°/min was 6.7°/min.

(4) Determination of optimum reaction conditions for silylation of the purine and pyrimidine bases using BSA

The determination of the reaction conditions necessary to achieve complete silylation involved a study of time, temperature, and reagent concentration. Initial investigations on the five main bases, uracil, thymine, cytosine, adenine, and guanine, demonstrated that guanine was the most difficult base to silylate. Therefore, guanine was chosen to further define the critical reaction conditions.

Molar excess of silylating reagent. The molar excess of BSA required for maximum yield of silylated product was determined as follows:

Samples containing guanine (5.0 mg) and internal standard (phenanthrene, 5.0 mg) were weighed into 16 × 75 mm screw-top culture tubes containing 8 × 15 mm teflon covered magnetic stirring bars. After the addition of acetonitrile and 0.10, 0.50, 1.00, or 2.00 ml of BSA, the tubes were tightly capped with a teflon lined cap. Acetonitrile was added to give a 3:1 v/v acetonitrile to BSA ratio. The different amounts of BSA added corresponded to 5, 25, 50, and 100 molar excess, respectively. The samples were heated in the closed tubes for 30 min at 150° in an oil bath with magnetic stirring, then cooled to room temperature and aliquots of 3 to 5 μl were injected into the gas chromatograph. The molar response of the TMS base relative to phenanthrene, $RMR_{b./phen.}$, was calculated from the measured areas of the two experimental chromatographic peaks as follows:

$$RMR_{B./Phen.} = \frac{\frac{A_{B.}}{W_{B.}/GFW_{B.}}}{\frac{A_{Phen.}}{W_{Phen.}/GFW_{Phen.}}}$$

$$RMR_{B./Phen.} = \frac{A_{B.}/moles_{B.}}{A_{Phen.}/moles_{Phen.}}$$

$RMR_{B./Phen.}$ = Molar response of base relative to phenanthrene
$A_{B.}$ = Area of chromatographic peak for base
$A_{Phen.}$ = Area of chromatographic peak for phenanthrene (I.S.)
GFW = Gram formula weight

The data presented in Fig. 1 show that a minimum of a 50 molar excess of BSA was necessary to obtain a maximum yield of the TMS derivative of guanine.

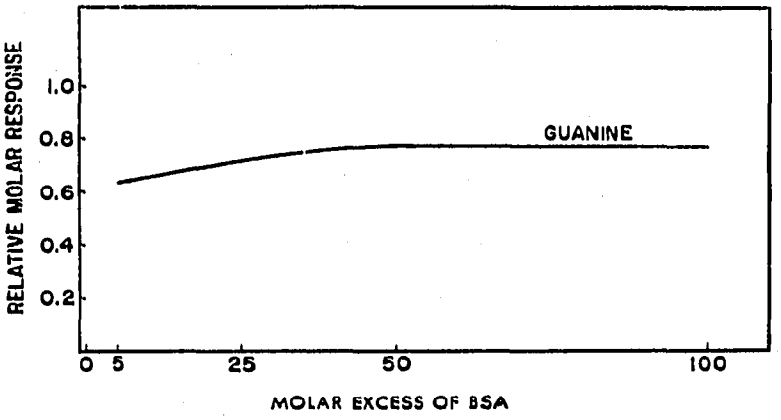

Fig. 1. Effect of reagent concentration on silylation in a closed tube at 150° for 30 min.

Time and temperature required for maximum derivatization. Samples containing *ca.* 1.0 mg of each of two bases (A and T, and, G and U) and phenanthrene were accurately weighed into sample vials (described in previous section) and 0.80 ml of BSA (*ca.* 100 molar excess) and 2.4 ml of acetonitrile were added. Duplicate samples were then stirred for 5, 15, 30, and 60 min at temperatures of 125, 150, and 170°. The samples were then immediately cooled to room temperature and 3–5 μl aliquots were analyzed by GLC. The relative molar response, $RMR_{B./Phen.}$, for each base was calculated according to the previously described formula and plotted as a function of reaction time (Fig. 2). Identical curves were obtained at temperatures of 125° and 170° for uracil, thymine, and adenine. Guanine gave a significantly lower molar response at temperatures of 100° and 125° at all reaction times studied. Also, silylation at 170° severely increased the number of tube cap failures and sample leakages, and did not significantly reduce the time required to obtain maximum conversion of guanine to its TMS derivative.

From these experiments, the optimum silylation conditions for the purine and pyrimidine bases were found to be heating at 150° (in a closed tube) for 45 min with a 50–100 molar excess of BSA.

As a result of using the large molar excess of BSA, a problem was encountered with the base, cytosine. This increase in amount of BSA resulted in the formation of two chromatographic peaks for cytosine, and the peak area of either of the two cytosine peaks was a function of silylation time and temperature. It was also observed that a single peak (the first) with a relatively high response was obtained for cytosine on silylation at room temperature for 30 min with a 100 molar excess of BSA. However, as previously demonstrated, silylation of the other bases at room temperature was not complete, but the area for the first eluted cytosine peak was constant and re-

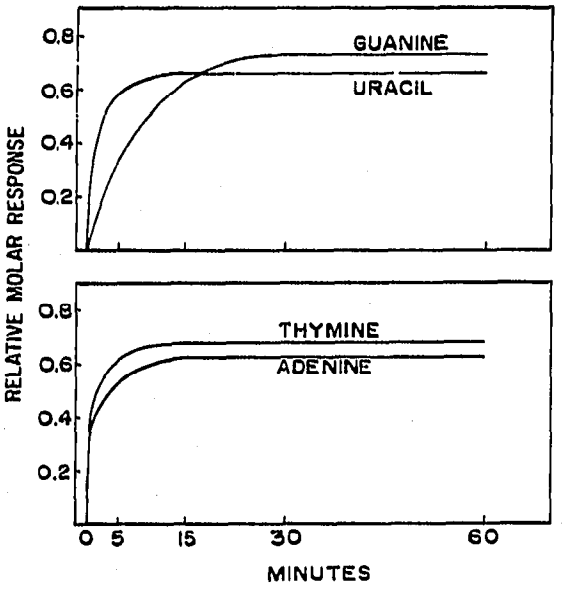

Fig. 2. Effect of silylation time at 150° on relative molar response.

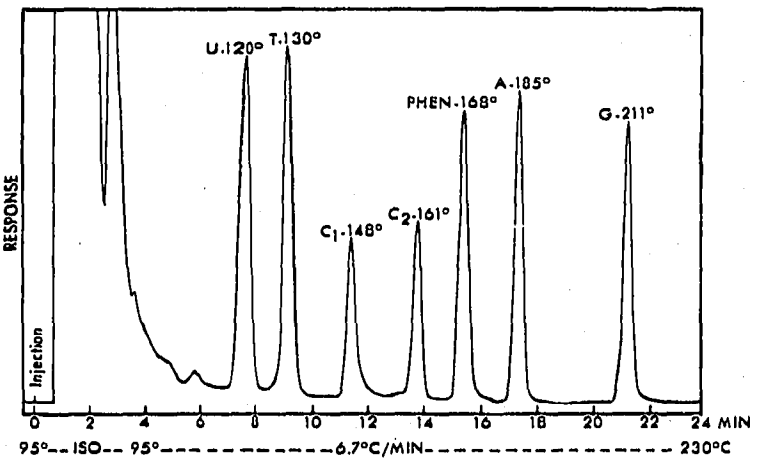

Fig. 3. Chromatographic separation of purine and pyrimidine TMS derivatives. Column: 4 w/w% SE-30 on 80–100 mesh, High Performance Chromosorb G, 1 m × 4 mm I.D. Each peak represents *ca.* 1 μg of each base.

producible at 150° for 45 min. Therefore, these reaction conditions can be used for the quantitative analysis of the five main bases.

Relative molar response of the purine and pyrimidine bases (hydrogen flame ionization detector). Optimum conditions for silylation of the five main bases (U, T, C, A, and G) using BSA were chosen from the results of the previous experiments. These conditions were then used for the determination of $RMR_{B./Phen.}$ for each of the following purine and pyrimidine bases: uracil, thymine, purine, cytosine, 5-methyl cytosine, hypoxanthine, adenine, xanthine, and guanine. The results of these determinations are given in Table I including standard deviations for the $RMR_{B./Phen.}$ of four of the bases, U, T, A, and G. Two peaks were observed for 5-methyl cytosine similar to those obtained for cytosine. The $RMR_{C.}$ shown for cytosine was determined by stirring the samples at room temperature for 30 min (until the second peak just began to appear). All of the chromatographic peaks exhibited desirable peak shapes with very little tailing. A typical chromatogram of the five main bases is shown in Fig. 3. This chromatogram represents 1 μg of each base injected.

(5) *Quantitative analysis of the five main purine and pyrimidine bases*

Calibration curves were prepared to demonstrate the quantitative aspects and reproducibility of the linear range of response for the formation of the TMS-base derivatives with BSA. A sample weight range of 25–2000 μg of each base was used. Phenanthrene was used as the internal standard.

Preparation of stock solution of bases. A stock solution containing the five main bases was prepared to facilitate the handling of samples containing amounts of material less than could be accurately weighed. No single organic solvent was suitable to dissolve all five bases, therefore, ammonium hydroxide, which could be removed by

TABLE I

RELATIVE MOLAR RESPONSE OF THE TMS DERIVATIVE OF PURINE AND PYRIMIDINE BASES USING A F.I.D.[a]

Compound	*Retention temperature*[b] (°C)	*RMR*[c]	*S.D.*[d]
Uracil	120	0.67	0.010
Thymine	130	0.68	0.012
Purine	140	0.38	
Cytosine	148; 161	0.54[e]	
5-Methyl cytosine	152; 165	0.45[f]	
Hypoxanthine	176	0.58	
Adenine	185	0.60	0.004
Xanthine	202	0.71	
Guanine	211	0.72	0.006
Phenanthrene (I.S.)	172	1.00	

[a] F.I.D. = flame ionization detector.
[b] Chromatographic conditions are described in experimental, section 3.
[c] RMR determined from at least two independent determinations. RMR = relative molar response to phenanthrene as I.S.

$$\text{RMR} = \frac{\text{molar response of bases}}{\text{molar response of phenanthrene}}$$

[d] Standard deviation calculated from more than 4 independent determinations.
[e] Silylation for 30 min at room temperature.
[f] Response based on first eluted peak.

evaporation, was used. The stock solution was prepared by weighing 25.0 mg of each of the five bases (U, T, C, A, and G) into a 100 ml volumetric flask and dissolving the bases with 7.5 *N* ammonium hydroxide, then diluting to volume. Complete solution of the bases was achieved.

Preparation of known samples. Exact aliquots of the stock solution (8.00, 4.00, 1.00, 0.50, and 0.10 ml) containing 2.000, 1.000, 0.250, 0.125, and 0.025 mg, respectively of each base were placed in screw necked culture tubes. The samples were dried by directing a stream of dry filtered air into the tubes while heating on a steam bath, then vacuum dried over P_2O_5 at 50° for 24 h.

Silylation and analysis of samples. An internal standard solution of phenanthrene in acetonitrile (50 ml of 0.177 mg/ml) was prepared. Exactly 10.00, 6.00, 1.50, 0.75, and 0.45 ml of this solution were added to the known samples prepared from aliquots of the stock solution of the bases, respectively. BSA (volumes of 4.0, 2.0, 0.50, 0.25, and 0.15 ml, respectively) was added to each sample. A magnetic stirring bar was placed in each tube; the tube was then tightly capped and heated for 45 min at 150° with magnetic stirring.

After cooling to room temperature, the samples were analyzed by GLC and the experimental peak area of each base relative to the phenanthrene peak area was calculated. Each of the samples contained approximately a one hundred molar excess of BSA and had a 3:1 v/v ratio of acetonitrile to BSA. The actual concentrations of the bases in each sample were in the range of 0.04–0.143 mg/ml.

The calibration curves shown in Fig. 4 demonstrate that the derivatization procedure gave linear calibration curves over an 80 fold increase in sample size. Excellent linearity and reproducibility was obtained over this sample weight range with

a relative standard deviation ranging from 1.1 % for uracil to 3.1 % for cytosine. The calibration curve for cytosine was prepared using only the first eluted peak area. These calibration curves demonstrate that precise and accurate quantitative analysis of the purine and pyrimidine bases can be achieved from an aqueous sample.

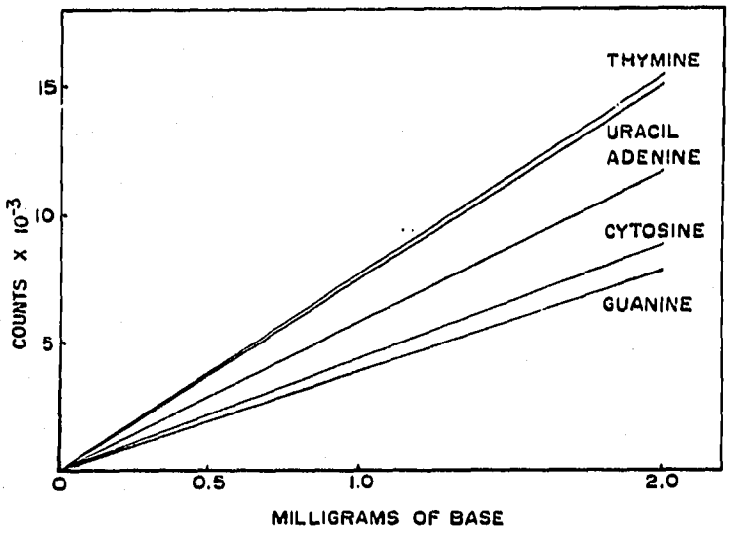

Fig. 4. Calibration curves for the purine and pyrimidine bases.

Minimum detectable amount (MDA). The second largest calibration sample was used for the determination of the minimum detectable amount (MDA). This sample contained 1.0 mg of each of the five main bases in a total volume of 8.0 ml (6.0 ml of acetonitrile and 2.0 ml of BSA). An aliquot of this solution was diluted 1:5 with acetonitrile thus containing 1 mg/40 ml (solution A). An aliquot, 1.00 ml, of solution A was then diluted 1:5 with acetonitrile (solution B) containing 1 mg of each base/200 ml. These solutions were then analyzed by GLC using various size injections and adjusting the instrument attenuation (*i.e.*, sensitivity) until a signal to noise ratio of 3:1 was obtained. This was considered the MDA. The MDA for the bases injected was 3–5 ng, or *ca.* 3×10^{-11} moles of each base.

(6) *Determination of the relative molar response of selected nucleosides and nucleotides*

Selected nucleosides and nucleotides were subjected to silylation under the conditions previously chosen for the purine and pyrimidine bases. The samples were subsequently analyzed by GLC to determine their retention temperatures and molar response values relative to phenanthrene. Even though these conditions were not necessarily optimal for the nucleosides and/or nucleotides, the response values could be useful in the analysis of biological samples containing mixtures of all of the nucleic acid components.

Individual samples containing *ca.* 1.0 mg of the following nucleosides or nucleotides with *ca.* 1.0 mg of phenanthrene (I.S.) were prepared: uridine, thymidine, adenosine, guanosine, uridylic acid, and adenylic acid. BSA (0.5 ml, *ca.* 100 molar excess) and 1.5 ml of acetonitrile were added to each nucleoside, and 0.8 ml of BSA (*ca.* 100 molar excess) and 2.4 ml of acetonitrile were added to each of the nucleotides. The samples were reacted at 150° for 45 min, then cooled to room temperature and analyzed by GLC.

TABLE II

RELATIVE MOLAR RESPONSE OF SELECTED NUCLEOSIDES AND NUCLEOTIDES AS TMS DERIVATIVES

Compound	*Retention temperature* (°C)	*RMR*[a]	*RMR range*
Thymidine[b]	230		
Uridine	234	1.19	± 0.10
Adenosine	260	1.60	± 0.10
Guanosine	265	1.37	± 0.09
Uridylic acid	270	0.61	± 0.05
Adenylic acid	276	0.98	± 0.03

[a] Calculated from at least two independent observations; samples heated for 45 min at 150° with a 100 molar excess of BSA.

[b] RMR not calculated due to two unresolved peaks for thymidine. RMR = relative molar response to phenanthrene as I.S.

Single chromatographic peaks were obtained for the compounds investigated which contained ribose; however, when the same procedure was used for thymidine, a deoxyribose containing compound, two unresolved peaks resulted. A chromatographic peak for cytidylic acid was obtained on room temperature silylation for extended periods of time (*e.g.* 24 h). HASHIZUME AND SASAKI[10] using HMDS and TMCS reported unsuccessful silylation of cytidylic acid. The relative molar responses of some of the nucleosides and nucleotides have been determined and are given in Table II. A mixture of ribonucleosides was chromatographed under the conditions used for the purine and pyrimidine bases, but complete separation was not achieved at these conditions as shown in Fig. 5. In particular, cytidine and guanosine were not separated at all. Further investigations are necessary to delineate the optimum silylating, chromatographic, and instrumental conditions for the successful analysis of the nucleosides and nucleotides.

(7) *Silylation using hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS), and BSA*

The results of SASAKI AND HASHIZUME[11] showed that a single peak for cytosine was obtained using the HMDS–TMCS reagents. This was not the case, however, using the BSA silylation procedure, since cytosine under these conditions yielded two peaks. These differences necessitated a comparison of these two derivatization procedures under similar experimental and chromatographic conditions. The HMDS–TMCS and BSA procedures are described as follows:

HMDS–TMCS method. A mixture of *ca.* 2.0 mg of each of the five main bases (uracil, thymine, cytosine, adenine, and guanine) and phenanthrene (I.S.) was weighed into a 25 ml Claisen flask. HMDS (0.2 ml), TMCS (0.1 ml), and 0.7 ml of pyridine as solvent were added and the mixture was refluxed for 1 h under anhydrous conditions, then cooled to room temperature.

BSA method. A mixture of *ca.* 1.0 mg of adenine and *ca.* 1.0 mg of phenanthrene was silylated with 0.4 ml of BSA and 1.2 ml of acetonitrile at 150° for 45 min in a closed tube.

The derivatized samples for both methods were chromatographed under the same instrumental conditions on the 4 w/w % SE-30 column.

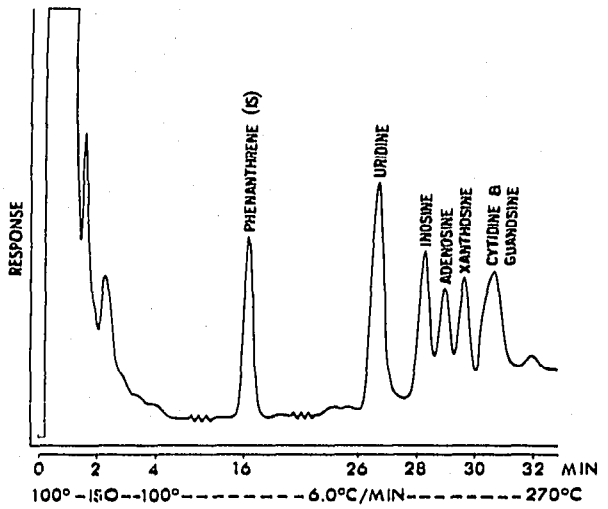

Fig. 5. GLC chromatogram of ribonucleosides.

Chromatographic separation of the derivatized bases using the HMDS–TMCS method was excellent and was equivalent to the separation achieved with the derivatives prepared with BSA. Only one peak for cytosine was observed for the HMDS–TMCS method as had been previously reported[11]. However, the molar response values relative to phenanthrene were considerably lower when using HMDS–TMCS, except for adenine, than those obtained using BSA. The results of this comparison are given in Table III. It is apparent from the differences in relative molar response,

TABLE III

COMPARISON OF HMDS–TMCS AND BSA SILYLATION METHODS

Base	*Relative molar response*[a] *HMDS–TMCS*[b]	*BSA*[c]	*% Increase with BSA*
Uracil	0.60 ± 0.03	0.67	11.7
Thymine	0.55 ± 0.02	0.68	23.6
Cytosine	0.43 ± 0.02	0.54[e]	25.6
Adenine	0.61 ± 0.03	0,60, 0.62[d]	not significant
Guanine	0.56 ± 0.03	0.72	28.6

[a] Relative molar response to phenanthrene as I.S.
[b] Average of two independent runs.
[c] Values obtained from Table I.
[d] RMR for adenine reconfirmed three months later.
[e] Silylation at room temperature.

that in general, the percent silylation or yield of derivative is greater using BSA than with HMDS–TMCS. Yield of derivative is one of the most important single factors affecting the precision and accuracy of a gas chromatographic method of quantitative analysis. A low yield could result in poor precision and severely limit the usefulness of the method. Even though the BSA method results in two peaks for cytosine, the relative area of the first of the two peaks was reproducible under the selected conditions and a linear calibration curve was obtained. This point of a single peak

for cytosine is the only discernible advantage of the HMDS–TMCS method. The method using BSA has the advantages of speed, simplicity, and higher yield of derivative. Therefore, it is a superior method of derivatization.

(8) Effect of ammonium chloride on base silylation with BSA

HASHIZUME AND SASAKI[15] reported a method for the GLC determination of orthophosphoric acid as the TMS derivative. They used this method for the analysis of orthophosphate obtained from perchloric acid hydrolysates of nucleotides. In their procedure the perchloric acid hydrolysate was first made alkaline with potassium hydroxide and then neutralized with hydrochloric acid. In the next step, concentrated ammonium hydroxide was added and the resulting mixture was evaporated to dryness at 100°. The purpose was to precipitate the perchlorate anion as the potassium salt, and to convert the tripotassium phosphate to the respective ammonium salt. The ammonium salt was then directly trimethylsilylated using hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) in pyridine, without the removal of potassium perchlorate, ammonium and potassium chlorides, etc.

The described procedure[15] was investigated in our laboratory for the determination of base ratios in yeast RNA, except that BSA was substituted as the silylation reagent. BSA was previously demonstrated to be a superior silylating reagent. Initial studies showed that this method was not satisfactory due to the presence of the TMS-orthophosphate, which partially overlapped the TMS uracil peak. Also additional interfering peaks for the TMS derivatives of ribose and its degradation products were present. However, one very interesting observation was made. Silylation with BSA normally yields two derivatives for cytosine, but in this case, only a single peak for cytosine was observed. Ammonium chloride is produced in the previously described reaction sequence when ammonium hydroxide is added to the sample containing chloride ions and also in the reaction when HMDS–TMCS is used. Therefore, this observation suggested an investigation of the effect of NH_4Cl on trimethylsilylation with BSA.

Seven mixtures containing *ca.* 1 mg each of cytosine and phenanthrene (I.S.) were weighed into 16 × 75 mm screw top culture tubes. Approximately 0, 10, 25, 50, 75, 100, and 200 mg amounts of NH_4Cl were added. Anhydrous acetonitrile (1.2 ml), 0.4 ml BSA, and an 8 × 15 mm teflon covered magnetic stirring bar were added to each tube. The tubes were tightly capped with teflon lined caps, heated at 150° for 45 min, and then cooled to room temperature. These samples were analyzed by GLC.

The same experiment was repeated for the bases, uracil, thymine, adenine, and guanine, except only four samples of the bases, containing *ca.* 0, 10, 50, and 100 mg of NH_4Cl were analyzed.

The data from this investigation on the effect of NH_4Cl showed that a level of 10 mg of NH_4Cl per 1 mg of cytosine caused the second peak for cytosine to be decreased to 5 % of the first peak. If more than 10 mg of NH_4Cl were added the second peak for cytosine was absent. The NH_4Cl effect on the silylation of the five main purine and pyrimidine bases is graphically shown in Fig. 6. An important observation is that a significant increase in the relative response of cytosine, adenine, and guanine resulted on the addition of 10 mg of NH_4Cl. However, the amount of NH_4Cl present in the reaction for each mg of base is very important, as the $RMR_{B./Phen.}$ for adenine

and guanine were decreased by *ca.* 50 and 80%, respectively, in the presence of 50 mg of NH_4Cl.

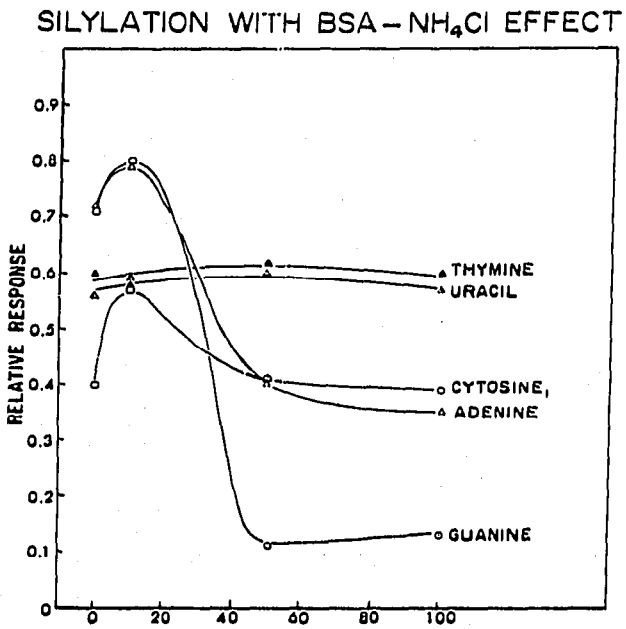

Fig. 6. mg of NH_4Cl/mg of Base.

Several other salts were also investigated to further define this effect. These included ammonium phosphate ($NH_4H_2PO_4$), potassium phosphate (KH_2PO_4), and sodium chloride. The phosphate salts were selected because of their presence in the hydrolysates of RNA and DNA prepared by the procedure of HASHIZUME AND SASAKI[15]. Sodium chloride was selected because of its assumed non-reactivity since both of the phosphate salts would enter into the silylation reaction. Similar results were obtained for potassium and ammonium phosphates as for NH_4Cl. At K and NH_4 phosphate salt levels of 10 mg/mg of cytosine, an enhanced response for cytosine was observed. At the 50 mg level only a very small cytosine peak and a very large TMS phosphate peak were present in the sample with $NH_4H_2PO_4$. This is explained by the large excess of $NH_4H_2PO_4$ reacting with the BSA, thus leaving only a small amount of BSA reagent for derivatization of cytosine. Also, a decrease in the RMR of cytosine was observed in the presence of 50 mg of KH_2PO_4 but not of the magnitude as for $NH_4H_2PO_4$. This is presumably due to the less reactivity of KH_2PO_4 with BSA as compared to $NH_4H_2PO_4$. Both the ammonium and potassium phosphate salts resulted in the loss of the second peak for cytosine at the 50 mg level. The effect of NaCl on the relative response of cytosine at the 50 mg/mg level was negligible and caused no loss of the second cytosine peak.

The second peak normally present on silylation of cytosine with BSA can be eliminated by adding 8–15 mg of NH_4Cl per mg of base. Further, the RMR of adenine and guanine is significantly enhanced on using an amount of NH_4Cl up to 15 mg. The effect of NH_4Cl on the silylation of uracil and thymine was negligible and has no apparent advantage or disadvantage in their derivatization. However, the fact that the amount of NH_4Cl present in the reaction is very critical with cytosine, adenine, and especially guanine, makes its use questionable since the successful quantitation

of the bases has previously been demonstrated, even though cytosine yields two derivatives under those conditions.

(9) *Evaluation of GLC column support phases for the TMS bases*

Chromatographic difficulties were encountered in reproducibly preparing the SE-30 column on High Performance Chromosorb G and necessitated the treatment of the prepared columns with a silylating reagent (Silyl 8) to obtain satisfactory response for the bases. Thus, to improve chromatographic precision and separation, a GLC column evaluation study was made on the following columns:

(1) 4 w/w % SE-30 on 80–100 mesh, High Performance Chromosorb G (previously used column for the TMS bases);

(2) 4 w/w % SE-30 on 80–100 mesh acid-washed Chromosorb G, heat treated for 15 h at 550°;

(3) 0.2 w/w % SE-30 on 100–120 mesh Corning Glass Beads (Type 0201);

(4) 8 w/w % SE-30 on 100–120 mesh Supelcoport (Chromosorb W type).

All columns were 1 m × 4 mm I.D. pyrex glass U-tubes. Columns Nos. 1 and 2 were preconditioned for 16 h at 275° with a carrier gas (N_2) flow rate of *ca.* 75 ml/min. Column No. 3 was preconditioned similarly except at 230°, because of the low loading percentage necessary for the glass bead column. Column No. 4 was also conditioned at 230° because both columns (3 and 4) were in the same chromatographic oven. The columns were then checked with a standard TMS base mixture.

The Supelcoport column required no silylation pretreatment and was found to be superior to the 4 w/w % SE-30 on 80–100 mesh, High Performance Chromosorb G column that had been used routinely for the preceding investigations. The columns of 4 w/w % SE-30 on acid-washed, heat treated Chromosorb G and the 0.2 w/w % SE-30 glass bead column were found to be totally unacceptable. The need for the higher percentage (8 w/w %) loading of the liquid phase on the Supelcoport column was to provide an approximately equal liquid phase film thickness compared to the 4 w/w % loading on the Chromosorb G columns. Supelcoport has approximately twice as much surface area per unit weight. The Supelcoport column was used for all subsequent investigations.

(10) *Comparative study of silylation reagents.*

The increased acceptance of the trimethylsilyl derivative in analytical GLC has brought about the development and introduction of new silylation reagents and also modifications of existing silylation mixtures. One of these new reagents, bis(trimethylsilyl)trifluoroacetamide (BSTFA), was synthesized in our laboratories[13] and has proven especially useful for derivatization of amino acids. Also, a reagent mixture of catalytic amounts of TMCS in BSA has been reported to have advantages for certain classes of compounds. This is explainable by the fact that as TMCS reacts with a replaceable hydrogen, HCl is formed which could have a catalytic effect on the silylation reaction. Due to the demonstrated usefulness of these reagents, it was necessary to evaluate and compare them with the presently used BSA reagent for the silylation of purine and pyrimidine bases.

Eight mixtures containing *ca.* 1.0 mg each of uracil, cytosine, adenine, guanine, and phenanthrene (I.S.) were accurately weighed into the previously described pyrex culture tubes. Four different silylation reagent solutions were used each at a 100

molar excess to total bases, and two independent samples were analyzed with each reagent. The solutions were:

(1) BSA–acetonitrile, 1.5:4.5 ml;
(2) TMCS (1 v/v %) in BSA–acetonitrile, 1.5:4.5 ml;
(3) TMCS (0.1 v/v %) in BSA–acetonitrile, 1.5:4.5 ml;
(4) BSTFA–acetonitrile (1:2.15 v/v) 1.9/4.1 ml*.

After the reagents and a stirring bar were added (previously described), the reaction tubes were tightly capped and heated at 150° for 45 min, then cooled to room temperature and the reaction mixtures analyzed by GLC using the normal operating conditions.

In regard to analytical derivatization yield there was no particular advantage or disadvantage in using BSTFA, or TMCS in BSA, in place of the previously used BSA as the silylating reagent since the $RMR_{B./Phen.}$ values obtained for the four different silylating reagent solutions were in good agreement (Table IV). This demonstrated that these silylating reagents were essentially equivalent under the reaction conditions used. The only discernible advantage of any of the reagents compared to BSA was that the chromatograms obtained with BSTFA showed a better base line with fewer small extraneous peaks. Also the injection peak was smaller due to the greater volatility and lower detector response of BSTFA and its by-product, N-trimethylsilyltrifluoroacetamide (MSTFA).

The major advantage of using BSTFA over BSA was not shown by the chromatograms but results from the complete miscibility of BSTFA with acetonitrile. With BSA, the required ratio of solvent for miscibility is 3:1 v/v, acetonitrile–BSA. Also it has been previously demonstrated that a 100 molar excess of BSA to total bases is required for optimum derivatization. Thus, the concentration of the bases in solution is limited. BSTFA does not present these limitations and therefore different sample weights of bases and volumes of solvent can be used. This permits one to analyze on a micro scale as the sample solution can be concentrated to a smaller volume.

(11) Application of BSA silylation to the analysis of yeast RNA

A major objective of this research was the application of the developed method of analysis of nucleic acid components to biological materials. Originally there were a number of possible approaches to the problem. One such approach involved the hydrolysis of the nucleic acid material with 1 *N* HCl to obtain the purine bases and pyrimidine nucleotides. Our investigations to this time indicated that the purine and pyrimidine bases could be more successfully analyzed by GLC than the nucleosides or nucleotides. The analysis of the larger, more complex nucleoside and nucleotide molecules would present chromatographic and quantitative derivatization problems. Thus, our research was oriented toward complete hydrolysis of the nucleic acids to the bases, followed by derivatization and chromatography.

As early as 1951 it was shown by MARSHAK AND VOGEL[16] that a concentrated perchloric acid hydrolysis of RNA or DNA would quantitatively yield the free purine

* With BSA–acetonitrile, the 1:3 v/v ratio is necessary for complete miscibility. However, this is not the case with BSTFA as it is miscible in all proportions with CH_3CN. Therefore, the volume ratio was changed for BSTFA so that the total final volume (6.0 ml) would be constant and the concentration of bases would be the same in all samples and experiments, and 100 molar excess of BSTFA.

TABLE IV

A COMPARATIVE STUDY OF SILYLATION REAGENTS

Silylation reagent	$RMR_{B./Phen.}$[a]					
	Uracil		*Average*	*Cytosine*$_1$		*Average*
BSA	0.60	0.60	0.60	0.41	0.43	0.42
1 v/v % TMCS in BSA	0.61	0.62	0.61	0.47	0.43	0.45
0.1 v/v % TMCS in BSA	0.63	0.62	0.62	0.43	0.42	0.42
BSTFA	0.63	0.63	0.63	0.42	0.36	0.39
	Adenine		*Average*	*Guanine*		*Average*
BSA	0.57	0.57	0.57	0.69	0.69	0.69
1 v/v % TMCS in BSA	0.56	0.59	0.58	0.72	0.71	0.72
0.1 v/v % TMCS in BSA	0.58	0.59	0.59	0.73	0.71	0.72
BSTFA	0.57	0.56	0.57	0.72	0.69	0.70

[a] All samples silylated at 150° for 45 min; 100 molar excess of silylating reagent. Each value is a single determination on an independent sample. Molar responses relative to phenanthrene (I.S.).

and pyrimidine bases. Much later, 1966, MACGEE[7] used this hydrolysis procedure in conjunction with a short ion-exchange clean-up process, for removal of the released phosphoric acid, ribose, and the ribose degradation products, to give purified purine and pyrimidine bases. Subsequently the N-methyl derivatives were prepared with TMAH and the base ratios determined by GLC. This procedure, however, was not without complications. The method of derivatization employed was not satisfactory as it resulted in multiple derivatives of two bases, adenine and cytosine, and necessitated the selection of a single peak for each compound for calculations. For these reasons a better derivatization and GLC analysis method was needed.

Our previously described trimethylsilyl derivatization procedure offered the possibility of quantitative analysis, once the problems of hydrolysis and purification were solved.

The following analytical–chromatographic method represents a combination of our silylation derivatization reactions with a modification of MACGEE's perchloric acid hydrolysis of the nucleic acids and ion-exchange clean-up procedure.

Sample hydrolysis and preparation. A 5.0 mg sample of yeast RNA was weighed into a 16 × 75 mm culture tube and 0.5 ml of 70 % perchloric acid was added. The tube was capped and the sample heated at 100° for 40 min with occasional shaking. The sample was then cooled in an ice-bath and made alkaline by the dropwise addition of 1.0 ml of ice-cold 8 *N* KOH with agitation. After the alkaline sample was held in an ice-bath for ½ h, the insoluble potassium perchlorate was centrifuged and the supernatant removed. The precipitate was then washed three times with 0.5 ml volumes of ice-cold 1 *N* KOH. The original supernatant was combined with the three washes and held in an ice-bath until placed on the anion-exchange column.

Ion-exchange clean-up. An anion-exchange column of Dowex 1-X2 formate, 100–200 mesh, was prepared with a resin bed of 9 × 105 mm. The alkaline sample (pH ⩾ 13) was then placed on the anion-exchange column and the liquid level was allowed to drop to the resin level. Double distilled water (50 ml) was then passed through the column and discarded. Twenty (20) ml of 1.0 *M* formic acid eluent were then passed through the column. The first 7 ml were discarded and the next 13 ml

were collected in a 16 × 150 mm culture tube. The collected fraction was evaporated to approximately 2 ml on a 100° sand bath while being flushed with a stream of filtered N_2 gas. The sample was then taken to dryness by lyophilization.

Derivatization and GLC analysis. The dried sample containing the purified purine and pyrimidine bases was silylated at 150° for 45 min using a 100 molar excess of BSA and a 3:1 v/v ratio of acetonitrile–BSA. The sample was then cooled to room temperature and analyzed by GLC. The GLC column used was 8 w/w% SE-30 on Supelcoport.

A series of standard mixtures of the bases was carried through this total procedure (*i.e.*, hydrolysis, ion-exchange, derivatization, and GLC analysis). Phenanthrene (I.S.) was added after lyophilization just prior to derivatization. At the same time a series of standard mixtures containing phenanthrene were carried through the derivatization and chromatography steps only, and by comparison of the RMR data, the percent recovery was determined (Table V). Although the percent recovery was approximately 82%, the recovery of the four bases was equivalent (± 1.5%). This demonstrates a non-selective loss through the total procedure. The losses were probably incurred in the transfer step (centrifugation), and refinement of the manipulative techniques should improve the recovery.

Table VI gives the results obtained for the base ratio determination of purified yeast RNA. The values are for duplicate analyses on three independent samples and demonstrate excellent precision for base ratio analysis. This experiment was undertaken for precision studies only. Yeast RNA can vary considerably in its base ratios depending on its source[17], and therefore, the values cannot be checked against the literature. A typical chromatogram of the TMS bases from yeast RNA is shown in Fig. 7. Although phenanthrene was added to this sample, it is not necessary for base ratio determination. However, if the absolute amount of bases is desired, it is necessary to add an internal standard such as phenanthrene. Also, it is essential for base ratio analysis that standard mixtures be prepared and analyzed concurrently with the biological samples to normalize for response variations due to changing gas chromatographic column characteristics and/or instrumental changes.

TABLE V

RECOVERY OF BASES TAKEN THROUGH COMPLETE METHOD

Base		$RMR_{B./Phen.}$[a, b]	*Average*	*Recovery* (%)
Uracil	Procedure[c]	0.49 0.50 0.49	0.49	80.3
	Standard	0.61 0.60	0.61	
Cytosine$_1$	Procedure[c]	0.36 0.35 0.35	0.35	83.3
	Standard	0.42 0.42	0.42	
Adenine	Procedure[c]	0.50 0.51 0.51	0.51	80.9
	Standard	0.62 0.64	0.63	
Guanine	Procedure[c]	0.64 0.63 0.63	0.63	82.9
	Standard	0.76 0.77	0.76	

[a] Each value represents duplicate analyses of an independent sample.

[b] $RMR_{B./Phen.} = \frac{A_{B.}/moles_{B.}}{A_{I.S.}/moles_{I.S}}$

[c] Mixtures carried through complete method, hydrolysis, ion-exchange, derivatization, and chromatography.

TABLE VI

GLC DETERMINATION OF BASE RATIOS IN YEAST RNA

Base	*Mole ratio*[a]			*Average*
Uracil	0.95	0.94	1.00	0.96
Cytosine	1.06	1.04	1.05	1.05
Adenine	1.00	1.00	1.00	1.00
Guanine	1.20	1.19	1.21	1.20

Sigma range of 0.029–0.037 with an average rel. S.D. of 3.0%.

[a] Base ratios relative to adenine assigned 1.00. Each value represents duplicate analyses of an independent sample.

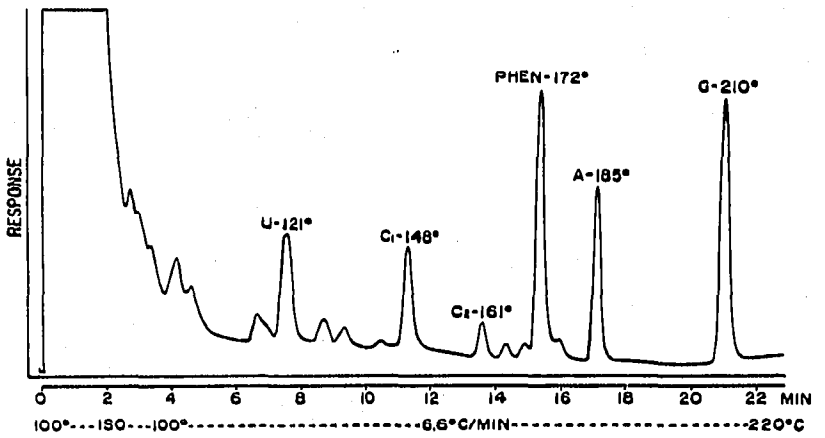

Fig. 7. Chromatogram of bases from yeast RNA. Column: 8 w/w% SE-30 on 100–120 mesh Supelcoport, 1 m × 4 mm I.D. pyrex glass. Each peak represents *ca.* 0.5 μg of base.

CONCLUSIONS

The major purpose of this investigation was to develop a quantitative gas–liquid chromatographic method of analysis for the components of nucleic acids (*i.e.*, purine and pyrimidine bases, nucleosides, or nucleotides), and further to apply this method to the analysis of biological material. Gas–liquid chromatography has the inherent advantages of speed, accuracy, and simplicity over other methods of analysis. However, before successful GLC of these compounds can be accomplished, they must first be converted to suitable volatile derivatives, as the compounds themselves are not sufficiently volatile.

The N-methyl derivatives of the purine and pyrimidine bases, prepared by thermal dissociation of their tetramethylammonium salts, as reported by MACGEE[6] were investigated and found to be unsuited for quantitative analysis due to the formation of multiple chromatographic peaks for cytosine, adenine, and guanine. However, investigation of the trimethylsilyl (TMS) derivatives of the bases demonstrated these derivatives to be far superior to the N-methyl compounds and well suited for the analysis of nucleic acid components, thus the TMS derivative was the derivative of choice.

A new silylating reagent, bis(trimethylsilyl)acetamide (BSA), was evaluated and found to be an excellent reagent for silylation of the bases. A procedure for the

quantitative analysis of the bases was developed. The optimum derivatization conditions were heating the bases in a closed tube at 150° for 45 min, with a 100 molar excess of BSA to total bases, and a 3:1 v/v acetonitrile–BSA ratio. Calibration curves for the five main bases (U, T, C, A, and G) were prepared and found to be linear over a sample weight range of 25–2000 μg of base. The relative standard deviations ranged from 1.1 % for uracil to 3.1 % for cytosine.

The minimum detectable amount (MDA) using the hydrogen flame ionization detector, was determined to be 3–5 $\times$ 10^{-9} g or *ca.* 3 $\times$ 10^{-11} moles of each base injected. The relative molar responses of the five main purine and pyrimidine bases and other bases, nucleosides, and nucleotides were determined relative to phenanthrene as internal standard. Both cytosine and 5-methyl cytosine exhibited two chromatographic peaks using the optimum derivatization conditions; however, these conditions were found to be necessary for the reproducible formation of the guanine TMS derivative, and the relative peak area remained constant under these conditions.

When the BSA method of derivatization was compared to the HMDS–TMCS method of SASAKI AND HASHIZUME[11], which was published during the course of this investigation, BSA was found to have the advantages of speed, simplicity, and most important, a higher yield of derivative. Therefore, it was shown to be a superior method of derivatization.

Ammonium chloride, when added to the BSA derivatization reaction, was found to have an effect on the silylation of the bases. At an ammonium chloride level of slightly greater than 10 mg per mg of base, the second peak for cytosine was not present and the relative response of the first peak was greater. Also, at a level of 10 mg of ammonium chloride per mg of base, the relative response of adenine and guanine were greater. However, a further small increase in the ammonium chloride concentration (*i.e.*, to 25 mg of NH_4Cl/mg of base) caused a very significant decrease in the response of adenine and guanine. The fact that the amount of NH_4Cl present was very critical with cytosine, adenine, and guanine, even though it showed no effect on uracil and thymine, makes its use questionable, since the successful quantitation of the bases in the absence of NH_4Cl has previously been demonstrated.

An evaluation of four GLC columns for the TMS bases showed that a column of 8 w/w % SE-30 on 100–120 mesh Supelcoport provided good resolution and stability, and was superior to the previously used column of 4 w/w % SE-30 on High Performance Chromosorb G.

A comparison was made of BSA as a silylation reagent with the new reagent, bis(trimethylsilyl)trifluoroacetamide (BSTFA), which was synthesized in our laboratory, and reagent solutions of 0.1 v/v % TMCS in BSA, and 1.0 v/v % TMCS in BSA. There were no significant differences in regard to analytical derivatization yield with any of these reagents. However, advantages of BSTFA include "cleaner" chromatograms with fewer extraneous peaks, and an important advantage arising from the complete miscibility of BSTFA in the solvent, acetonitrile. This latter advantage stems from the fact that silylation of the bases requires a 100 molar excess of BSA, and a required solvent–reagent ratio of 3:1 v/v acetonitrile–BSA for miscibility. Since BSTFA does not present these limitations, different sample weights of bases and volumes of solvents can be used. Also, recent research in our laboratory shows that the silylation time with BSTFA can be reduced to 15 min. This permits one to make analyses on a micro scale as the sample solution can be concentrated to a small

volume with sample integrity being maintained. From these studies it was concluded that BSTFA is the silylating reagent of choice.

The application of the developed method for purine and pyrimidine base analysis to the analysis of biological materials was accomplished using perchloric acid hydrolysis and anion-exchange removal of biological interferences prior to derivatization and GLC analysis. The combined hydrolysis and purification procedures were shown to give a recovery of the bases of *ca.* 82 % with the recovery of the four bases being equivalent (± 1.5 %), thus demonstrating a non-selective loss of individual bases. When the method was used for the analysis of purified yeast RNA, the base ratio determination of three independent samples demonstrated the excellent precision of the method with an average relative standard deviation of 3.0 %. Although the method has not been applied to analysis of microgram amounts of RNA and DNA, it should not prove difficult to accomplish with minor refinements of technique.

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