A RAPID AND SENSITIVE TECHNIQUE FOR THE DETERMINATION OF THE 2'-O-METHYLNUCLEOSIDE RATIO OF AN RNA MOLECULE

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ABSTRACT

A RAPID AND SENSITIVE TECHNIQUE FOR THE DETERMINATION OF THE 2'-O-METHYLNUCLEOSIDE RATIO OF AN RNA MOLECULE

By

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A method is presented for the determination of the 2'-O-methylnucleoside ratio in an RNA molecule. The RNA to be analyzed is enzymatically digested to the nucleoside level with a combination of pancreatic ribonuclease, snake venom phosphodiesterase, and bacterial alkaline phosphatase.

Isolation of the 2'-O-methylnucleosides is achieved on an AG 1x4(borate) column. The ribonucleosides are retained on the column since a negatively charged borate complex forms across the cis-2', 3'-hydroxyl groups. Recovery of the 2'-O-methylnucleosides is good but deoxyribonucleoside contamination is possible if the RNA sample is not free of DNA.

The isolated 2'-O-methylnucleosides are trimethylsilylated with BSA to form volatile derivatives for a gas-liquid chromatographic separation. The separation is achieved on the slightly polar liquid phase OV-17 under isothermal conditions. Peak areas are determined by weighing. The derivatives are stable at room temperature for a minimum of three hours and their response to the hydrogen flame detector of the GLC is reproducible.

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Lee M. Pike

A THESIS

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Dedicated to my wife, Mary and to our son, Lee, Jr.

I thank Dr. Fritz Rottman for his guidance in my research and graduate training. My thanks also go to Joseph Abbate, Brian Dunlap, and the other members of the Rottman group for their assistance and beneficial discussions in the course of this work.

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LIST OF ABBREVIATIONS

A	Adenosine
Am	2'-O-methyladenosine
A_{260}	Absorbance at 260 nanometers
BSA	N, O-bis (trimethylsilyl) acetamide
BSTFA	N, O-bis (trimethylsilyl) trifluoroacetamide
C	Cytidine
Cm	2'-O-methylcytidine
DEAE-cellulose	Diethylaminoethylcellulose
eV	Electron volt
G	Guanosine
Gm	2'-O-methylguanosine
GLC	Gas-liquid chromatography
HMDS	Hexamethyldisilizane
mRNA	Messenger RNA
OV-1	Methyl silicon gum
OV-17	Methyl-phenyl silicon gum
RNA	Ribonucleic acid
rRNA	Ribosomal RNA

sRNA	RNA soluble in 2M NaCl
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
tRNA	Transfer RNA
TSIM	Trimethylsilylimidazole
U	Uridine
Um	2'-O-methyluridine

LITERATURE REVIEW

Ribonucleic acid is a polymer containing principally the purine and pyrimidine nucleosides adenosine, guanosine, cytidine, and uridine. Methylated modifications of these four nucleosides occurring naturally are known as "trace" or "minor" nucleosides, since they represent only a small fraction of the total nucleosides present. Thirteen different ring-methylated nucleosides and five nucleosides methylated on the 2'-hydroxyl group of the ribose moiety have been identified (Starr and Sells, 1969). The ribose methylated nucleosides are of interest to us with respect to the influence of a blocked 2'-hydroxyl group on the structure, function, and metabolism of RNA molecules (Bobst et al, 1969; Price and Rottman, 1970; Dunlap et al, 1971).

Distribution of 2'-O-methylnucleosides

The occurrence of 2'-O-methylnucleosides in RNA was first demonstrated by Smith and Dunn (1959). They were isolated in low yield from the alkaline stable oligonucleotides in RNA from such varied sources as wheat germ, tobacco and beet leaves, and rat liver. Biswas and Meyers (1960) reported the presence of Cm in a preparation of cytidine obtained from acid hydrolysis of RNA in the blue-green algae *Anacystis nidulans*. Bacterial, yeast, and several mammalian RNA's were shown by Hall (1964) to contain significant levels of the 2'-O-methylnucleosides.

Lane and his co-workers have developed techniques (described below) for detecting the presence and distribution of the 2'-O-methylnulceosides. Their studies have included characterization of the 2'-O-methylation patterns of RNA from wheat germ (Singh and Lane, 1964a), yeast (Gray and Lane, 1967), and Esherishia coli (Lane and Tamaoki, 1969).

The 2'-O-methylnucleosides are ubiquitous; they have been found in all organisms assayed to date, and in nearly all classes of RNA. The exceptions are viral and bacterial mRNA (Moore, 1966), and 5S rRNA from bacteria (Brownlee et al, 1967) and mammals (Forget and Weissman, 1968). Eukaryotic mRNA has not been analyzed for the presence of 2'-O-methylnucleosides.

Correlation of 2'-O-methylnucleosides with rRNA processing

Studies on high molecular weight rRNA isolated from eukaryotic cells indicate that the major site of methylation in these molecules is the 2'-hydroxyl group of ribose (Wagner et al, 1967; Iwanami and Brown, 1968; Lane and Tamaoki, 1969). The kinetic studies of Greenberg and Penman (1966) and Zimmerman and Holler (1967) showed that preribosomal 45S RNA is methylated as it is synthesized in the nucleolus. This methylation has been reported to be a prerequisite for maturation and processing of 45S RNA into 18S and 28S RNA (Vaughan et al, 1967). In HeLa cells, the processing of the 45S RNA into 18S and 28S RNA has been demonstrated to involve no significant loss of 2'-O-methyl groups (Wagner et al, 1967;

Weinberg et al, 1967). Conservation of 2'-O-methyl groups is also high in the maturation process of 45S RNA in Novikoff hepatoma ascites cells with a loss of only 15-17% (Choi and Busch, 1970). These results are consistent with a processing mechanism for 45S RNA involving specific loss of unmethylated or undermethylated regions of the molecule.

Correlation of 2'-O-methylnucleosides with nuclear RNA metabolism

Another group of RNA molecules displays a relationship between its 2'-O-methylation patterns and metabolism procedures. The nucleus contains a group of six to nine RNA molecules of discrete sizes ranging from 4-7S which were reported independently in rat liver by Dingman and Peacock (1968) and in HeLa cells by Weinberg and Penman (1968). They were subsequently shown to be present in cells of several organisms and many vertebrate tissue culture lines (Rein and Penman, 1969). Many of these RNA molecules were shown to be highly methylated since they were heavily labeled in ^{14}C-methylmethionine labeling experiments (Weinberg and Penman, 1968; Zapisek et al, 1969; Moriyama et al, 1969).

The distribution of these methyl groups has been examined in the nuclear RNA from two sources. Three fractions of Chinese hamster cell nuclear RNA have been shown by Zapisek et al, (1969) to have a large fraction of their methyl label in 2'-O-methyl groups. Ro-Choi et al (1970) have isolated the nuclear 4.5S RNA$_{III}$ from Novikoff hepatoma ascites cells and shown that five of the eighty-

five nucleotides it contains are 2'-O-methylated, including the 3'-terminal nucleoside (El-Khatib et al, 1970).

Metabolically these nuclear RNA molecules are very stable (Weinberg and Penman, 1969; Moriyama et al, 1969). Several of the molecules are as stable as rRNA, while others have half lives of one day or more. This stability distinguishes these small molecular weight nuclear RNA molecules from the bulk of nuclear RNA which has a very rapid turnover rate (Soeiro et al, 1968; Aronson and Wilt, 1969). Whether or not there exists some relationship between metabolic stability and the degree of 2'-O-methylation is unknown, but Novikoff hepatoma ascites cell nuclear 4.5S RNA_1, one of the least stable of these nuclear RNA molecules (Ro-Choi, 1971), has been shown to contain no 2'-O-methyl groups among its 102 nucleotides (Ro-Choi et al, 1971).

Quantitation of 2'-O-methylnucleoside content of an RNA molecule

Two techniques arose independently for the early quantitative analysis of the 2'-O-methylnucleoside levels in RNA. Singh and Lane (1964 a and b) utilized the property of alkaline stability of oligonucleotides containing blocked 2'-hydroxyls. The stable oligonucleotides were separated from the mononucleotides by salt fractionation on a DEAE-cellulose column. Two dimensional paper chromatographic separation of the sixteen dinucleoside monophosphates allowed estimation of the abundance of 2'-O-methylnucleosides based on the percentage of the initial ultraviolet absorbing material recovered

from each spot on the chromatogram. Alternately, the di- and tri-nucleotide fraction could be enzymatically digested to the nucleoside level. Paper chromatography in several steps could then be used to separate the eight resulting nucleosides; they could be quantitated by recovery of ultraviolet absorbing material as described above.

If an attempt is made to increase the sensitivity of the above assay by labeling with ^{14}C-methyl groups, an exaggerated estimate of sugar methylation may be obtained. RNA may contain other dinucleotide sequences which display alkaline stable properties although they are methylated only on their bases (eg. N^6,N^6-dimethyladenylyl-(3', 5')-N^6,N^6-dimethyladenosine-2'(3')-phosphate; Nichols and Lane, 1966a). There is the additional problem of coincident base methylation of some of the 2'-O-methylnucleosides (eg. N^4-methyl-2'-O-methyl-cytidylyl-(3', 5')-cytidine; Nichols and Lane, 1966b).

In the second technique, Hall (1964) made use of the inability of 2'-O-methylnucleosides to form complexes with borate; ribonucleosides will form such complexes across the (cis)2',3'-hydroxyl groups. RNA was hydrolyzed to its constituent nucleosides enzymatically and the nucleosides were fractionated on a partition column of diatomaceous earth with a solvent system of 1-butanol, ammonium hydroxide and sodium borate. Paper chromatographic separation of the 2'-O-methylnucleosides allowed estimation of their abundance based on the percentage of the intial ultraviolet absorbing material recovered

for each 2'-O-methylnucleoside.

These two techniques have been the only available methods for measuring the amount of each 2'-O-methylnucleoside in an RNA molecule. The enrichment or isolation step is necessary since the 2'-O-methylnucleosides represent a small fraction of the total nucleosides in RNA and would otherwise be masked. However, the paper chromatographic separations used in both of the above techniques are time consuming (approximately one day for each dimension or system used). In addition, they require sufficient quantities of the nucleosides for complete resolution and exact measurement of ultraviolet absorbance after elution from the chromatogram (ca 20 nmole or 0.25 A_{260} of each nucleoside per determination).

Abbate and Rottman (1971) recently developed a more rapid and sensitive technique for quantitation of the total percentage of nucleosides which are 2'-O-methylated. It is based on a report (Baskin and Dekker, 1967) that perchloric acid hydrolysis of ^{14}C-methyl labeled RNA released only sugar O-methyls as ^{14}C-methanol; labeled base methyls were not released. The ^{14}C-methanol was isolated by repeated co-distillation with 50% methanol to determine the amount of 2'-O-methylation.

The method of Abbate and Rottman (1971) has several advantages. The perchloric acid hydrolyzate is directly analyzed for the released methanol by gas chromatography, reducing the risk of loss by handling. Since the methanol originating from the 2'-O-methyl group is not

diluted with added methanol, the ^{14}C-label is not necessary. Virtually any RNA sample or mixture of nucleosides can be analyzed. However, the data obtained reflects only the total abundance of sugar hydroxy-methylation, regardless of which nucleosides are methylated.

GLC of nucleosides

The use of gas-liquid chromatography (GLC) for analysis of nucleic acids is a relatively new technique. Base ratios can be determined using a mixture of bases obtained by perchloric acid hydrolysis of RNA (Hashizume and Sasaki, 1968), but quantitation of 2'-O-methylnucleosides in a RNA molecule would require analysis of a mixture of intact nucleosides. Since nucleosides have a low volatilities, it is necessary to render them less polar and more volatile by blocking the amino or hydroxyl groups on the base in order to elute them from a GLC column at temperatures which will not result in their decomposition.

Miles and Fales (1962) used various combinations of the acetyl, methyl, or isopropylidene derivative for this purpose, but they experienced difficulties with multiple derivatives and trailing of cytidine and guanosine. Hancock and Coleman (1965) used trimethyl-silyl (TMS)derivatives with more success. Subsequent evaluation of the TMS and other silyl derivatives of the nucleosides by Sasaki and Hashizume (1966), Gehrke et al (1967), and Hancock (1968) has led to the conclusion that the TMS derivatives are most suitable since the problem of multiple derivatives is minimal.

Hancock and Coleman (1965) and Sasaki and Hashizume (1966) used the procedure developed by Sweeley et al (1963) for silylation of sugars to form TMS derivatives of nucleosides. The dry standard nucleosides were dissolved in a mixture of pyridine, hexamethyldisilizane (HMDS) and trimethylchlorosilane (TMCS); silylation time ranged from 5 to 120 minutes with or without heating to enhance the reaction. The resulting mixture of derivatives was injected onto a GLC column consisting of a diatomaceous earth support coated with a liquid phase of 1 to 5% methyl silicone gum (OV-1 or S E-30). Peaks were obtained for all four nucleosides, but they were asymmetrical with broad shoulders leading adenosine and guanosine and all four trailed badly.

Gehrke et al (1967) achieved a measure of success when they added N, O-bis (trimethylsilyl) acetamide (BSA) to some nucleosides dissolved in acetonitril for silylation; they did not include cytidine, which has been difficult to derivatize. Jacobson et al (1968) used only BSA for a very successful derivatization of both standard nucleoside mixtures and nucleosides obtained by enzymatic hydrolysis of RNA. BSA was added directly to the lyophilized incubation mixture and heated at 120° for 120 minutes. The TMS derivatives were resolved on a GLC column packed with a methyl-phenyl silicone gum (4% OV-17 on Gas Chrom Q) with a programmed temperature increase of two degrees per minute from 160° to 240°. Single peaks were obtained for all derivatives with the exception of TMS-

guanosine which had two peaks. Theirs was the first successful attempt at a gas chromatographic quantitation of the nucleoside ratios in RNA.

INTRODUCTION

The purpose of this research was the development of a more rapid and sensitive assay for quantitation of 2'-O-methylnucleoside ratios than the elaborate and involved paper chromatographic systems currently in use. Coupled with knowledge of the total abundance of 2'-O-methylnucleosides in an RNA molecule obtained by the method of Abbate and Rottman (1971), the 2'-O-methylnucleoside ratio would permit calculation of the abundance of each 2'-O-methylnucleoside in that RNA molecule. The two techniques would thus fingerprint the 2'-O-methylation patterns in RNA molecules. These fingerprints could be used to compare a variety of RNA molecules for inherent differences or changes in the 2'-O-methylation pattern.

MATERIALS

Adenosine, guanosine, cytidine, and uridine were purchased from Sigma Chemical Company. The 2'-O-methyladenosine (Am) was prepared from adenosine treated with diazomethane in an aqueous solution of 1, 2-dimethoxymethane by the method of Broom and Robins (1965) as modified by Rottman and Heinlein (1968). The 2'-O-methylcytidine (Cm) was prepared by a similar chemical methylation of cytidine (Rottman and Johnson, 1969). The 2'-O-methyluridine (Um) was prepared by deamination of Cm with potassium nitrite in acetic acid as described by Martin et al (1968). The alkaline stable dinucleosides monophosphates and 2'-O-methylguanosine (Gm) were isolated from wheat germ rRNA using the techniques of Singh and Lane (1964b). Commercial wheat germ was purchased locally.

Snake venom phosphodiesterases (Russell's viper and Crotalus adamenteus) and bacterial alkaline phosphatase (BAPSF) were purchased from Worthington Biochemical Corporation. Bovine pancreatic ribonuclease A was purchased from Sigma Chemical Company. Bio-Rad AG 1x4 (Cl^-), 100/200 mesh and Dowex-50, W-X8, 100/200 mesh were purchased from Bio-Rad Laboratories. N, O-Bis (trimethylsilyl) acetamide (BSA), N, O-bis (trimethylsilyl) trifluoracetamide (BSTFA) and trimethylsilylimadazole (TSIM) were purchased from Regis Chemical Company. The 2% OV-17 (methyl, phenyl silicon gum) on 80/100 mesh Gas Chrom Z was

the generous gift of W. W. Wells. The 1% OV-1 (methyl silicon gum) on 100/200 mesh Gas Chrom Z was purchased from Applied Science Laboratories. All other chemicals were reagent grade.

METHODS

Purification of Russell's viper venom phosphodiesterase

Russell's viper venom phosphodiesterase had been purified to remove contaminating phosphatase activity. This was achieved on a cation exchange resin (Dowex-50, W-X8, 100/200 mesh) column washed with sodium hydroxide and equilibrated with sodium acetate (Keller, 1964).

Hydrolysis of RNA

Digestion of RNA was effected with the following reaction mixture per milligram of RNA: alkaline phosphatase, 15 ug; ribonuclease A, 20 ug; purified Russell's viper venom phosphodiesterase, 45 ug of crude Crotalus adamenteus venom phosphodiesterase, 45 ug; in a total volume of 0.3 ml which was 0.1M in ammonium carbonate buffer, pH 8.8 and 2mM in magnesium acetate. A 24 hour digestion at 37°C achieved hydrolysis to the nucleoside level. Completeness of the reaction was checked by electrophoresis on Whatman #1 for 90 minutes at 400v in 0.05M Tris-acetate, pH 7.5.

Incubation of a pyridine and water mixture (1:1, v:v) with RNA, nucleotides, or adenosine was done in sealed glass tubes. The tubes were heated for various times a 108° and the contents were analyzed by paper chromatography after removal of pyridine.

Isolation of 2'-O-methylnucleosides

Bio-Rad AG1x4 (Cl^-) was converted to the borate form by washing with 1.0N sodium hydroxide until the silver nitrate test

was negative for Cl^-, washing with H_2O, and finally washing with 0.7M boric acid. An 8.0 x 1.6 cm column was used for an incubation mixture of 10 mg of RNA. Prior to use the column was washed with 500 ml 0.7M boric acid and finally equilibrated with 500 ml 0.3 M ammonium borate, pH 6.0.

The pH of the nucleoside sample was adjusted to 6 with 1.0N HCl prior to addition to the column. The 2'-O-methylnucleoside fraction was eluted in 450 ml 0.3M ammonium borate, pH 6.0 at a flow rate of 1.0 ml per minute. The sample was lyophilized and the boric acid was removed as the methyl ester by repeated evaporation from methanol (Balle et al, 1966).

Nucleoside derivatization

Derivatizations of the nucleosides for GLC were performed in ½ dram vials sealed with a teflon-lined screw cap. Trimethylsilylation was done with BSA, BSTFA, or TSIM. BSA was added in a 100 mole excess and the solution was heated for 120 minutes at 125° (Jacobson et al, 1968). BSTFA was added in 100 mole excess and the solution was heated for 90 minutes at 90°. TSIM was added in 100 mole excess and the solution remained at room temperature for 1-2 hours before injection.

Occasionally, the nucleosides were treated with methyoxyamine-hydrochloride or methanolic acetic anhydride as described below prior to silylation. Methoxyamine-hydrochloride was added in 40 mole excess with dry pyridine as a solvent. The solution was heated

for 3 hours at 75°, evaporated by a stream of dry nitrogen, and the nucleosides were silylated with BSA as above. Acetylation was done in a solution of 0.1 ml acetic anhydride and 0.4 ml absolute methanol. The solution was treated for 2 hours at 80° or remained at room temperature from 5 to 96 hours. Following evaporation of the solvent by a stream of dry nitrogen, the nucleosides were silylated with BSTFA as above.

GLC analysis

GLC analyses were conducted on a Hewlett-Packard FandM 402 High Efficiency Gas Chromatograph equipped with a flame ionization detector. The instrument was connected to a 1 mV Sargent Model SRG recorder. The column consisted of U-shaped glass tubing (6 ft x in i.d.) packed with 1% OV-1 on silanized Gas Chrom Q or 2% OV-17 on silanized Gas Chrom Z. The columns were conditioned prior to use as follows: a) nitrogen flow for 5 minutes at room temperature; b) heated to 350° for 1 hour with no carrier flow; c) cooled to room temperature; and d) column heated to 250° for 24-48 hours with flowing nitrogen. The oven temperature was kept 40-50° higher than normal operating temperature when the column was not in use.

Chromatographic conditions for OV-17 columns were: isothermal column temperature of 195-205° C; injection heater, 200°; detector heater, 210°; and carrier gas, nitrogen, 45 cc per minute. Conditions for OV-1 columns were: isothermal column temperature of 160-

180°; injection heater, 170°; detector heater, 180°; and carrier gas, nitrogen, 45 cc per minute.

A 1-7 ul aliquot containing 0.03-1.0 ug (0.12-3.8 nmole) of each nucleoside was injected into the gas chromatograph. Peaks were compared by cutting out the peaks, weighing them, and correcting for differences in attenuation and chart speed of the recorder.

Mass Spectral Analysis

Low resolution mass spectra were recorded on an LKB-9000 combined gas-liquid chromatograph-mass spectrometer using an ionizing energy of 70 eV. The ion source was a 290° and the molecular separator was at 230°. The trap current was 60 uamps. The GLC column consisted of coiled glass tubing (4 ft x 3 mm i.d.) packed with 3% SE-30 on silanized Supelcoport (100 120 mesh). Helium carrier gas was maintained at 35 cc per minute. The column had a linear program rate of 2° per minute from an initial temperature of 220° to a final temperature of 250°. The data was processed by an on-line digital computer system (Sweeley et al, 1970).

EXPERIMENTAL

I. RNA hydrolysis and isolation of 2'-O-methylnucleosides.

Enzymatic RNA hydrolysis

Enzymatic digestions were attempted on several types of RNA by the procedures described in the methods section. Pancreatic ribonuclease was added to endonucleolytically cleave the RNA into short pieces to create more sites for the exonucleolytic action of snake venom phosphodiesterase. Bacterial alkaline phosphatase was added to hydrolyze the phosphate from the resulting nucleotides producing nucleosides for subsequent analysis. The digestions were done at alkaline pH to create optimal conditions for the alkaline phosphatase and the snake venom phosphodiesterase.

The products of the enzymatic digestions were assayed by a 90 minute electrophoresis at pH 7.5 in 0.05M Tris-acetate buffer at 400V. Standards used to check for completeness of reaction were: a) a mixture of the four normal ribonucleosides; b) a mixture of the four normal 5'-ribonucleotides; and c) a mixture of wheat germ alkaline stable dinucleoside monophosphates. The uncharged nucleosides remain at the origin, while 5'-mononudeotides and dinucleoside monophosphates migrate towards the positive pole.

The reaction was judged incomplete if ultraviolet absorbing material was present in any region of the electrophoreogram other than the nucleoside region; a 3% contamination was detectable.

By this criterion, Crotalus adamenteus venom phosphodiesterase and purified Russell's viper venom phosphodiesterase were equally successful in accomplishing the digestion. However, Russell's viper venom phosphodiesterase may be preferred since it has been purified to eliminate nucleoside deaminase activity.

Behavior of nucleosides on AG 1x4 (borate) column

Isolation of the 2'-O-methylnucleoside fraction is necessary to avoid its being masked by the ribonucleosides as discussed in the Literature Review. The technique developed in this research utilizes the formation of a borate-ribonucleoside complex, but separation is effected on a simple AG 1x4 (borate) column rather than the elaborate partition column described by Hall (1964). The separation depends on a borate complex forming with the ribonucleosides and their consequent retention on the resin. The 2'-O-methylnucleosides pass through the column with the purines being held up slightly on the basis of a partial charge or polarity of the base. Furukawa et al (1965) used a similar Dowex-1 column to isolate 2'-O-methylated intermediates in a chemical synthesis of 2'-O-methyluridine. There has not been a report of the use of this column with RNA hydrolyzates.

Deoxyribonucleosides represent a potential source of contamination in this technique. Since they lack the 2'-hydroxyl, no borate complex is formed and they chromatograph similarly to the 2'-O-methylnucleosides on the AG 1x4 (borate) column. All RNA molecules investigated must therefore be ascertained to be free of DNA or be treated extensively

with deoxyribonuclease during their purification.

Recovery of standard 2'-O-methylnucleosides from the AG 1x4 (borate) column was determined to insure that no loss occurred during this isolation. In addition, the ability of the column to retain ribonucleosides was checked by applying a mixture of U, A, C, and G (in an undetermined ratio) to the column and monitering the effluent for the appearance of material which absorbs at 260 nm.

A profile of the first 200 ml of the elution of a mixture of Um, Am, and Cm from the column as described in Methods is shown in Figure 1. The first peak contains Um and Cm and their elution is complete in 200 ml. The broad second peak contains Am, but its recovery is not complete until about 400 ml have passed through the column. Elution of Gm would begin under the Am peak and would continue through another 50 ml volume after the Am elution is complete.

The recovery of each 2'-O-methylnucleoside was tested in duplicate and the results are reported in Table 1. Amounts were determined by absorbance at 260 nm at pH 6 (neutral). The results were good for Um, Am, and Cm with recovery of over 95% of the approximately 20 A_{260} units used. Further experiments were limited by the minimal amounts of Gm available. A slight error in determining the absorbance in this low range would have a larger effect than in the previous cases, however. Larger amounts of Gm will be available in the future since it is being chemically

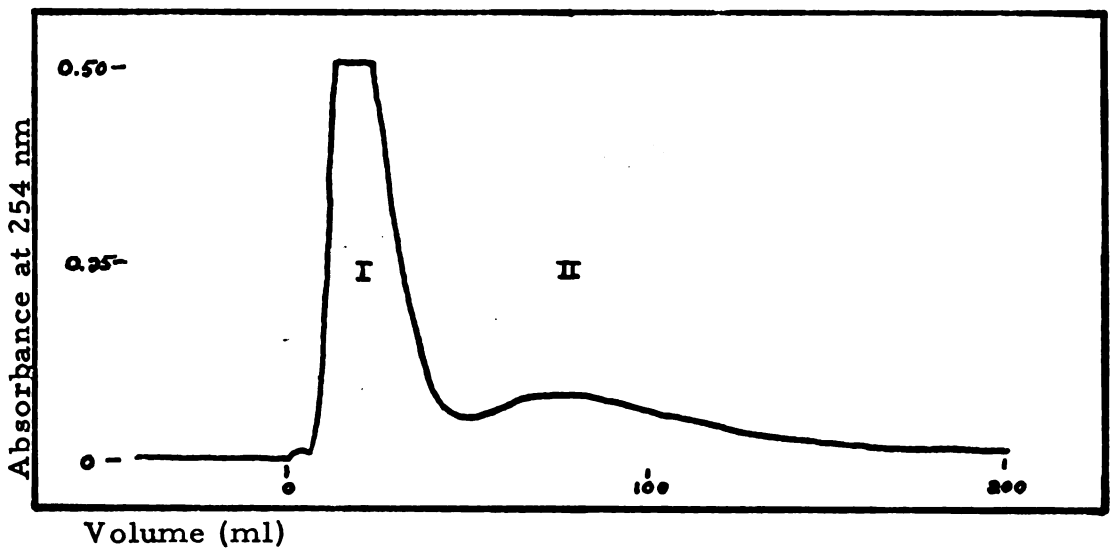

Figure 1. An elution profile of the 2'-O-methylnucleosides from an AG 1x4 (borate) column.

A mixture of Um (0.97 umole), Am (0.50 umole), and Cm (1.61 umole) were applied in 0.5 ml H_2O at pH 6 to an AG 1x4 (borate) column which has been prepared as described in Methods. Elution was effected with 400 ml 0.3M ammonium borate, pH 6.0; a profile of the first 200 ml of that elution is shown above. Peak I contains Um and Cm; peak II contains Am.

Nucleoside	Amount Applied (umole)	Amount Recovered (umole)	Percent Recovered
Um-1	2.06	2.06	100
Um-2	2.02	2.04	101
Am-1	1.31	1.26	96.2
Am-2	1.31	1.29	98.4
Cm-1	2.85	2.78	97.5
Cm-2	3.12	3.02	96.7
Gm-1	0.56	0.49	88
Gm-2	0.55	0.45	82

Table 1. The recovery of 2'-0-methylnucleosides from the AG 1x4 (borate) column.

Two samples of each 2'-0-methylnucleosides were applied to separate AG 1x4 (borate) columns in 1.0 ml H_2O at pH 6, followed by a rinse of 0.5 ml H_2O. The nucleosides were eluted with 0.3M ammonium borate, pH 6.0, at a flow rate of 1 ml per minute. Fractions were analyzed with a Gilford UV Spectrophotometer for the recovery of 2'-0-methylnucleosides. All recoveries were complex within the first 450 ml of column effluent as discussed in the text.

synthesized in our laboratory. If the recovery cannot be increased, data will be obtained to calculate a recovery factor to normalize the Gm content of the column effluent of an RNA hydrolyzate prior to further analysis.

The retention of a mixture of ribonucleosides was tested in triplicate under the same conditions by which the 2'-O-methylnucleosides were completely eluted. As shown in Table 2, 210 A_{260} units of an approximately equimolar solution of ribonucleosides were still retained after 500 ml of the buffer had passed through the column. The capacity of the column was seen to be closely related to its size since the application of a larger amount of ribonucleosides resulted in an earlier elution.

Isolation and characterization of 2'-O-methylnucleoside fraction from wheat germ rRNA

The 2'-O-methylnucleosides were recovered from the Crotalus adamenteus venom phosphodiesterase digestion of wheat germ rRNA using the AG 1x4 (borate) column. The digestion mixture was separated into three portions of approximately 200 A_{260} units each. The fractions were applied to separate columns and the 2'-O-methylnucleosides were eluted under the previously described conditions to effect their separation from ribonucleosides. The amount recovered is reported in Table 3, both as A_{260} units and as percentage of 260 nm absorbing material applied to the column. There is some variation, but the amount recovered is in the range of the 1.7 percent

Nucleoside	Amount Applied (A_{260})	Amount Recovered (A_{260})	Percent Recovered	Recovery Volume (ml)
UACG-1	210	0	0	480
UACG-2	250	0	0	420
UACG-3	210	0	0	510

Table 2. The retention of ribonucleosides on the AG 1x4 (borate) column.

Three aliquots of an approximately equimolar mixture of U, A, C, and G were applied to separate AG 1x4 (borate) columns in 1.0 ml H_2O at pH 6. The column was washed with 0.3M ammonium borate, pH 6.0, at a flow rate of 1 ml per minute. Fractions were analyzed with a Gilford UV Spectrophotometer for the appearance of eluted material. The indicated recovery volumes were collected prior to the elution of any detectable ribonucleoside.

Crotalus adamenteus Venom Phosphodiesterase Digestion	Amount Applied (A_{260})	Amount Recovered (A_{260})	Percent Recovered
1	210	5.2	2.5
2	210	3.5	1.7
3	200	5.5	2.8

Table 3. The isolation of 2'-*O*-methylnucleosides from wheat germ rRNA.

A single incubation mixture containing 28 mg of wheat germ rRNA was digested with *Crotalus adamenteus* venom phosphodiesterase as described in Methods. Completeness of the reaction was shown by electrophoresis as discussed above. The mixture was adjusted to pH 6 by addition of 1.0N HCl and the A_{260} was recorded. The mixture was divided into three portions and each portion was applied to a separate AG 1x4 (borate) column. The 2'-*O*-methylnucleoside fraction was eluted with 0.3M ammonium borate, pH 6.0. Recoveries are given as amounts of abosrbing material recovered and as percent of the amount applied.

value reported for the 2'-O-methylnucleoside content of wheat germ ribosomal RNA (Abbate and Rottman, 1971).

The 2'-O-methylnucleoside fraction was eluted from the AG 1x4 (borate) column in 450 ml 0.3 M ammonium borate; this is equivalent to 8.3 grams of boric acid. Removal of the borate was accomplished by lyophilizing the column effluent and evaporating several times from methanol. Ammonia is volatile and the boric acid was removed as the methyl ester (Balle et al, 1966).

Following removal of the boric acid by evaporation from methanol as the methyl ester, the 2'-O-methylnucleoside fraction designated 1 was checked by paper chromatography in 4-1-2 solvent (ethyl acetate, n-propanol, water; 4:1:2; v:v:v; upper phase). This solvent resolves normal nucleosides into two spots--one containing U and A and the other containing C and G. The 2'-O-methylnucleosides and deoxyribonucleosides are similarly resolved. Since the six spots are cleanly separated, contamination of the 2'-O-methylnucleoside fraction with ribonucleosides or deoxyribonucleosides would be indicated by the presence of these spots on the chromatogram.

After spotting the sample and appropriate standards, the chromatogram was developed for six hours. There was some difficulty with salt, but the chromatogram showed that while there was no ribonucleoside contamination, small amounts of deoxyribonucleo-

sides were present. These results indicate that while there is no contamination with ribonucleosides, extra care must be taken to remove DNA during the RNA preparations to avoid contamination with deoxyribonucleosides.

Pyridine and water hydrolysis of RNA

Enzymatic hydrolysis of RNA is effective, but the nucleoside fraction is contaminated with protein at the conclusion of the hydrolysis. The possibility of using a chemical hydrolysis of RNA was investigated with the objective of eliminating any potential difficulty imposed by the presence of protein in the isolation or subsequent resolution of the 2'-O-methylnucleoside fraction.

Alkaline hydrolysis of RNA is not satisfactory in isolation of 2'-O-methylnucleosides since the phosphate forms a cyclic intermediate across the cis-2',3'-hydroxyls as part of the initial cleavage. Hydrolysis of RNA by ammonia under pressure, first reported by Levene and Jacobs (1910) completely deaminates cytidine (Gullard and Hobday, 1940) and probably proceded by an alkaline hydrolysis mechanism in any case. Mineral acid hydrolysis of RNA results in cleavage of the glycosidic bond with purines while perchloric acid hydrolysis results in complete destruction of all nucleosides

to the free base level (Hashizume and Sasaki, 1968). Catalytic hydrolysis of RNA with lanthanum was reported by Allen and Bacher (1951) but they report a 2-3% loss of 260 mu absorbing material per hour.

Bredereck et al (1941) refluxed yeast RNA in a pyridine-water mixture for 108 hours and reported hydrolysis of the RNA with recovery of the four ribonucleosides. They used the procedure as a source of nucleosides rather than as a method of RNA analysis. If no undesirable side reactions occur, hydrolysis of RNA by incubation in pyridine and water would be attractive since pyridine could be easily removed when the hydrolysis was complete. After several evaporations, an incubation mixture would contain only nucleosides and trace amounts of phosphate. Since total recovery of the nucleosides from a hydrolyzed RNA sample is essential if they are to be used to determine nucleoside ratios, this research included a study of the effect of the pyridine and water incubation on nucleosides and nucleotides.

The pyridine-water hydrolysis of RNA was investigated with respect to recovery of nucleosides and formation of by-products. The initial study was a time analysis of the hydrolysis of yeast sRNA in order to establish the length of time necessary to achieve degradation to the mononucleoside level. Sealed vials containing 20 mg of yeast sRNA in a total volume of 1.0 ml (pyridine, water; 1:1; v:v) were heated at 108^{o} and samples were taken after one, three, four, and six days. After several evaporations with water

to remove pyridine, the samples were spotted on Whatman #1. A mixture of the four ribonucleosides, a mixture of the four ribonucleotides, a mixture of alkaline stable wheat germ dinucleoside monophosphates, and a sample of unhydrolyzed yeast sRNA were included as standards. The chromatogram was developed for 18 hours by descending chromatography with Heppel's solvent (n-propanol, ammonia, water; 55:10:35; v:v:v). Heppel's solvent leaves long oligonucleotides or unhydrolyzed RNA at the origin. Short oligonucleotides (< 10) and mononucleotides are resolved by size with the shorter chains having the larger R_f values. Nucleosides move even faster than the mononucleotides.

After one day of incubation the yeast sRNA had been hydrolyzed into a mixture of nucleosides, nucleotides, and short oligonucleotides. Some material still chromatographed in the dinucleoside monophosphate region after three days, but hydrolysis to the nucleoside level was complete after four days. Comparison of the three, four, and six day samples indicated an increasing amount of fluorescent material with time. This fluorescent material chromatographed in the region of the nucleosides, but it has an unique R_f.

Since it was known to take at least four days to hydrolyze yeast sRNA by this approach, the fate of several nucleotides during an incubation under similar conditions was studied. Recovery of nucleoside as measured by absorbance at 260 nm initially seemed very good; the values were in the range of 95%. However, analysis

of the products of the incubation indicated deamination and cleavage of the glycosidic bond were occurring after only 40 hours of incubation. In addition, the fluorescent material seen in the yeast sRNA hydrolyzate began to appear upon further incubation.

The fate of adenosine during a pyridine-water incubation was followed in a time course analysis. As shown in Table 4, adenosine was either hydrolyzed to the free base or deaminated (or both) to a significant extent in the period of time necessary to hydrolyze the yeast sRNA sample. Values for adenosine are maximum values since they may contain unresolved hypoxanthine. At least 16% of the adenosine has been destroyed in the first 38 hours of incubation. This value increases to a minimum of 19% in the four days which were required to hydrolyze yeast sRNA. The data is not sufficient to determine a rate of destruction but a definite decrease in amount of adenosine with time is seen.

It is of interest that no fluorescent material was produced in the adenosine incubation, even at the longest incubation period. The fluorescent compounds may occur as a result of some reaction of phosphate ion with the nucleosides or with pyridine since the fluorescent material appeared following hydrolysis of the monophosphates (eg. AMP) as well as the hydrolysis of RNA.

Extensive destruction of the nucleoside is occurring in the time periods necessary for complete hydrolysis of yeast sRNA by pyridine-water reflux. The appearance of cleavage and demination

Time of Incubation (hours)	Adenosine (Percent)	Adenine (Percent)	Inosine (Percent)
38	83.7	8.1	8.1
67	81.8	9.1	9.1
84	83.3	8.6	8.0
96	80.7	7.8	11.4
108	76.0	11.5	12.5
120	77.8	9.7	12.4
133	77.4	8.4	14.1
144	75.1	9.3	15.6

Table 4. The progress of destruction of adenosine by incubation with pyridine and water.

Vials containing 4.1 umoles of adenosine were incubated as described in Methods and samples were taken at various times. After several evaporations from water on a flash evaporator to remove pyridine, the samples were spotted on Whatman #40 (acid washed). Adenosine and its deamination product, inosine, were included as standards, as well as adenine and hypoxanthine, the respective products of hydrolysis of their glycosidic bonds. The chromatogram was developed for 18 hours by descending chromatography with 86% n-butanol as solvent. After chromatography, the standards were arrayed in the following order by increasing R_f value: inosine, adenosine, hypoxanthine and adenine. The spots corresponding to adenosine, adenine, and inosine were cut out and eluted in 1.0 ml of 0.1N HCl. Since hypoxanthine nearly cochromatographed with adenosine, it was not possible to quantitate this base. The A_{260} for each spot was measured and the values were converted to moles using the respective extinction coefficients.

products of adenosine when the nucleoside alone is incubated is enough to invalidate the approach. Production of a fluorescent by-product when the nucleotide or intact RNA is incubated renders the procedure completely unacceptable for quantitation of nucleoside ratios.

II. Derivatization and GLC of nucleosides.

The mixture of 2'-O-methylnucleosides obtained from the AG 1x4 (borate) column must be resolved and quantitated for a ratio determination. For my purposes, GLC was investigated as a potential tool for this resolution due to its sensitivity and convenience. Jacobson et al (1968) reported a lower limit of 0.1 ug (ca 0.4 nmole) of each nucleoside in their GLC determination of nucleoside ratios, which is much less than the amount required for paper chromatographic determinations (ca 20 nmole). Several analyses may be performed in a relatively short time. A single sample may be injected several times, giving data which is statistically more significant than the single A_{260} reading available after paper chromatography.

BSA derivatives

The 2'-O-methylnucleosides must be derivatized to increase their volatility prior to a GLC analysis. The BSA silylation procedure of Jacobson et al (1968) was used. The TMS-derivatives formed from the 2'-O-methylnucleosides must have well resolved

peaks for the determination of a ratio. Two column liquid phases were investigated for this separation. They were OV-1, the non-polar methyl silicon gum, and the slightly polar methyl-phenyl silicon gum OV-17 used by Jacobson et al (1968). The resolution of nucleosides achieved by Jacobson et al depended on increasing the column temperature from 160° to 240° by temperature programming to elute the derivatives from a 4% liquid phase. Lower percentages of liquid phases were used in this work in an attempt to elute the peaks isothermally, thus decreasing the time of analysis and creating more reproducible conditions.

GLC behavior and retention times of nucleoside derivatives.

Treating 2'-O-methylnucleosides with BSA as described in the methods section results in a single derivative for Um and Am as shown by the presence of a single symmetrical peak when they are chromatographed on either OV-1 or OV-17. Cm and Gm form multiple derivatives, indicated by a trailing peak for Cm on both phases and two peaks for Gm on OV-17. The trailing peak for the ratio determination; the two peaks formed by the Gm derivatives may also be treated together since the first peak is much larger than the second and they are not well resolved.

Retention time is determined by the time of elution of the crest of the GLC peak (major peak for Cm or Gm). Table 5 contains the retention times of the TMS derivatives for the 2'-O-methylnucleosides as resolved on OV-17 or OV-1 under the conditions described

Nucleoside	Retention Time	
	OV-17	OV-1
	198°	177°
Um	6.4	6.1
Am	14.2	12.9
Cm	21.6	16.5
Gm	29.4	---

Table 5. The retention times of the BSA derivatives of the 2'-O-methylnucleosides.

The TMS derivatives were prepared separately for each 2'-O-methylnucleoside with BSA as described in Methods. Samples were injected onto a 1% OV-1 column or a 2% OV-17 column of the GLC. Retention times are given in minutes at the indicated column temperatures.

in the methods section. It is seen that there are more than 7 minutes between the closest two peaks when OV-17 was used. Separation was not successful on OV-1 since the Am and Cm derivatives are not resolved sufficiently to give discrete peaks.

Figure 2 is a composite drawing of the GLC tracing of a mixture of Um, Am, and Cm which was derivatized with BSA and injected on OV-17 and the GLC tracing of Gm which has been treated similarly. It was not possible to obtain a GLC tracing of a mixture of the four 2'-O-methylnucleosides at this time since the Gm is in short supply as discussed earlier. The composite was drawn to make the excellent resolution of the four peaks more obvious.

Stability and reproducibility of derivatives.

Another aspect of the GLC nucleoside ratio determination is the reproducible formation of derivatives which are stable over a reasonable period of time. The reproducibility and stability of a compound are traditionally studied using its absolute molar response.[1]

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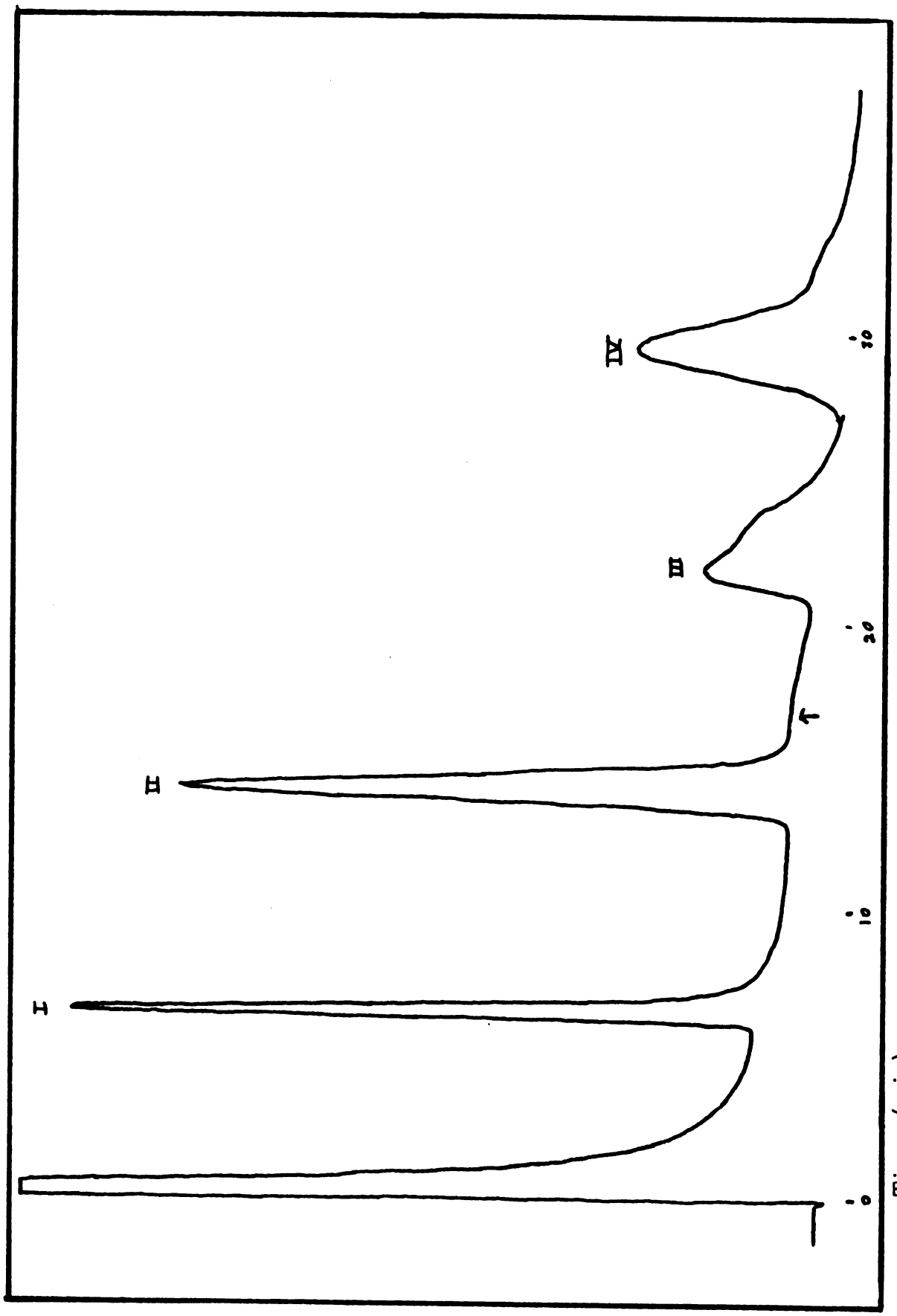
[1]The absolute molar response is calculated as the ratio of the response of a known amount of a compound to the response of an equimolar amount of an internal standard, an added compound which is known to give a stable and uniform response. The reproducibility of derivative formation is established by obtaining a constant value for the absoluet molar response of a compound in a series of derivatizations. The stability of a derivative is similarly established by obtaining a constant absolute molar response value from a series of injections of one sample over the time interval of interest.

Figure 2. A gas-liquid chromatogram of the TMS derivatives of the 2'-O-methylnucleosides on an OV-17 column.

The TMS derivatives were formed for a mixture of Um, Am, and Cm and chromatographed on a 2% OV-17 GLC column as described in Methods. A sample of Gm was treated similarly and chromatographed under identical conditions on the same column. The chromatograms were then drawn as the composite to indicate relative retentions and separations as discussed in the text. Peak I is TMS-Um, peak II is TMS-Am; peak III is TMS-Cm; and peak IV is TMS-Gm. The attenuation was increased by a factor of 4 at the point indicated by the arrow.


Detector Response
Time (min)
0
10
20
30
I
II
III
IV

The absolute molar response is not necessary for a ratio determination since the relative amounts of the nucleosides will suffice. In this case, a stable relative molar response for the nucleoside derivatives may be used to establish their stability and reproducibility.[2] The relative molar response values may also be used to convert experimentally determined peak weight ratios into molar ratios for the nucleoside ratio determination.

The stability and reproducibility of the TMS derivatives of the 2'-O-methylnucleosides were checked by comparing their relative molar response values over a period of time and from one derivatization sample to another. Table 6 gives the values obtained for the molar response of Um with respect of Am and the molar response of Cm with respect to Am in that experiment. The values for both responses are grouped for easy comparison.

The relative molar response values are internally consistent within one sample over a time span of two and one-half hours, and externally consistent from one sample to another. There was not sufficient quantity of Gm at this time to test the stability and reproducibility of its response as the other nucleosides were tested.

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[2]The relative molar response is the relative response of one compound in a mixture with respect to an equimolar amount of another which is chosed as standard. The reproducibility and stability of derivative formation are established by obtaining constant values for the relative molar response values in the same manner as with the absolute molar response.

Table 6. The stability and reproducibility of the TMS derivatives of the 2'-O-methylnucleosides.

Three samples of a mixture of Am (0.351 umole), Um (0.328 umole), and Cm (0.634 umole) were treated with BSA at two day intervals as described in Methods. They were maintained under anhydrous conditions at room temperature and an aliquot was periodically injected onto a 2% OV-17 column of the GLC. The peaks were cut out, weighed, and the weights were corrected for differences in attenuation and chart speed. These corrected peak weights were used to calculate a peak weight ratio of Um with respect to Am and Cm with respect to Am. The relative molar responses were determined by correcting the peak weight ratio for the actual molar ratios present in the mixture of nucleosides.

Time (min)	Relative molar response of Um:Am		
	I	II	III
Zero	0.74	----	0.81
30	0.78	0.80	----
60	0.73	0.79	0.80
90	----	0.81	0.81
120	----	0.77	0.79
150	0.84	----	----

	Relative molar response of Cm:Am		
	I	II	III
Zero	0.14	----	0.02
30	0.17	0.16	----
60	0.21	0.15	0.17
90	----	0.18	0.18
120	----	0.14	0.22
150	0.17	----	----

Preliminary results indicate that the peak size remains fairly constant upon repeated injection of aliquots of the same volume from one sample.

Table 7 contains the mean relative molar response values for TMS-Um, TMS-Am, and TMS-Cm with their standard deviations. The relative molar response for Gm will be determined at a later time. A comparison of the relative molar responses reveals that TMS-Cm given a much poorer response than earlier TMS-Am or TMS-Um, which may partially account for the slightly higher variation in the TMS-Cm response indicated by the higher standard deviation. The values are in a workable range, although the low response of the Cm derivative raises the minimum amount of 2'-O-methylnucleosides required to determine a ratio.

Nucleoside ratio determination of synthetic copolymers

Nucleoside ratios were determined for synthetic copolymers prepared with polynucleotide phosphorylase in our laboratory (Rottman and Johnson, 1969). The ratio of the nucleosides resulting from an enzymatic digestion of each copolymer was obtained by GLC of their TMS derivatives. A paper chromatographic ratio determination was also done as a check on the accuracy of the GLC determination.

For the GLC determination, the relative molar response values reported in Table 8 were obtained from mixture of known amounts of standard nucleosides as described earlier. The artifical mixtures were prepared to approximate the ratios expected in the

	Um	Am	Cm
Actual Molar Ratio	0.94	1.00	1.81
Mean Peak Weight Ratio	0.74	1.00	0.31
Relative Molar Response	0.79	1.00	0.17
Standard Deviation	0.03	----	0.02

Table 7. The relative molar response values for 2'-O-methyl-nucleosides.

The values presented above represent the mean of the 12 values obtained from the experiment discussed in Table 6.

	U	A	Am
Actual Molar Ratio	1	0.0572	0.0481
Mean Peak Weight Ratio	1	0.0847†	0.0574*
Relative Molar Response	1	1.48	1.19
Standard Deviation	-	0.04	0.02

†n= 7; * n=5

Table 8. The relative molar response values for the nucleosides in synthetic copolymers.

Mixtures of U and A or U and Am were prepared in the molar ratios indicated above. TMS derivatives were prepared with BSA and GLC was performed on an OV-17 column. The relative molar response of A and Am were determined with respect to U as described in the footnote to Table 7.

copolymers. The corrected peak weight ratios were calculated for the nucleosides in enzymatically digested samples of poly (U, A) and poly (U, Am) after their separation on GLC. The relative molar response values discussed above were used to calculate the molar ratios of the nucleosides from the peak weight ratios for these synthetic copolymers.[3]

The molar ratios of the nucleosides in the two copolymers are given in Table 9. Part A contains the values obtained by GLC and Part B contains the values obtained by paper chromatography. The two techniques give values which are in good agreement.

Alternative derivatization techniques

BSTFA derivatives of nucleosides

There are some practical difficulties to be faced in using BSA as a TMS donor for nucleosides. Both BSA and its by-product mono (trimethylsilyl) acetamide, have longer retention times and higher detector responses than is desirable, which restricts the lower limit for elution times of peaks. The detector must be frequently cleaned since silicon dioxide, one of the combustion products of the hydrogen flame, rapidly coats the detector resulting in

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[3]For example, the poly (U, Am) polymer gave a corrected peak weight ratio of 11.8 for U with respect to Am. Since a mole of Am gave 1.19 times as much response as a mole of U, the response of U must be increased by a factor of 1.19 to obtain the molar ratio. The molar ratio is therefore 14.1 moles of U per mole of Am.

A. Determined by GLC of TMS derivatives.

	poly (U, A) U:A ratio	poly (U, Am) U:Am ratio
Mean Peak Weight Ratio	4.04†	11.8*
Molar Ratio	6.0	14.1
Standard Deviation	0.2	0.4

†n=10; *n=3

B. Determined by Paper Chromatography

	poly (U, A) U:A ratio	poly (U, Am) U:Am ratio
Molar Ratio ‡	6.6	13.9

‡Based on duplicates

Table 9. Nucleoside ratios of synthetic copolymers.

Samples of poly (U, A) and poly (U, Am) were digested with Russell's viper venom phosphodiesterase as described in Methods.

For the GLC ratio determination, the incubation mixtures were lyophilized and silylation was achieved with BSA as described in Methods. GLC was done on a 2% OV-17 column. Following the measurement of the peak weights, the molar ratios were calculated using the relative molar response values from Table 8.

For the paper chromatographic ratio determination, duplicate samples of each enzymatically digested copolymer were spotted on Whatman #1 (acid washed) with standard nucleosides and the chromatogram was developed with n-butanol, ammonia, water (86:5:14; v:v:v) for 16 hours. The spots representing the nucleosides derived from the copolymers were eluted in 2.0 ml 0.1N HCl. The absorbance at 260 nm was recorded, molar values were calculated using the appropriate extinction coefficients, and the molar ratios were determined.

baseline noise and reduced response of the desired peaks. These difficulties can be reduced by use of BSTFA, a BSA analogue, since its retention time as well as that of its by-product, mono (trimethylsilyl) trifluoroacetamide, are greatly reduced. Since the BSTFA combustion product, silicon tetrafluoride, passes through the detector to a greater extent than its BSA analogue, there is much less coating action.

It was for these reasons that Hancock (1969) tried unsuccessfully to use a mixture of BSTFA and pyridine for the nucleoside silylation. The use of pyridine in silylation of nucleosides has led to unsatisfactory derivatives with BSA and TSIM as well as with BSTFA in our experience. Um and Am derivatives form trailing peaks and Cm does not give a derivative which elutes at all under our usual GLC conditions. The use of BSTFA was therefore investigated without added solvent in a manner similar to the BSA silylation.

Initially, the ribonucleosides were used as model compounds. Table 10 contains the retention times for the BSTFA formed TMS derivatives of the ribonucleosides when chromatographed on OV-1. The peaks are respectable and resolution is good. The 2'-O-methylnucleosides were then derivatized with BSTFA; the retention times of their TMS derivatives are also reported in Table 10. Um and Am form satisfactory derivatives with retention times similar to those formed with BSA, but Cm forms two derivatives with widely spaced retention times on both OV-1 and OV-17. Use of both TMS-

Nucleoside	Retention Time			
	OV-17		OV-1	
			173°	
U			7.0	
A			14.6	
C			17.5	
G			30.4	
	199°		161°	
Um	5.6		13.2	
Am	12.5		31.6	
Cm	19.2	38.0	21.3	35.7

Table 10. The retention times of the BSTFA derivatives of the ribonucleosides and the 2'-O-methylnucleosides.

The TMS derivatives were prepared separately for each ribonucleoside and 2'-O-methylnucleoside with BSTFA as described in Methods. Samples were injected onto a 1% OV-1 column or a 2% OV-17 column of the GLC as indicated above. Retention times are given in minutes at the indicated column temperatures.

Cm peaks for quantitation was not practical since the first peak interferred with the elution of TMS-Um and the second peak on OV-17 was very broad (it spanned several minutes) and it would interfer with the elution of TMS-Gm. The first TMS-Cm peak on OV-1 disappeared upon addition of one microliter of water to the reaction mixture. The parallel experiment was not attempted with OV-17, since the second peak is not desirable anyway.

BSTFA was not used for silylation of the nucleosides since Cm formed two unusable derivatives. Jacobson et al (1968) reported a similar formation of two derivatives for C when trimethylsilylation was done with BSA at higher temperatures. Since BSTFA is a more potent TMS donor, a similar situation has arisen, even at the lower temperature at which the derivatization was performed.

Mass spectral analysis of A and Am derivatives formed with BSTFA

Mass spectra were obtained for a group of trimethylsilyl nucleoside derivatives. The extent and position of hydrogen replacement by TMS groups was examined to characterize the derivatives. The mass spectral analyses of BSTFA formed TMS-A and TMS-Am are presented here to illustrate the method by which the derivatives were characterized.

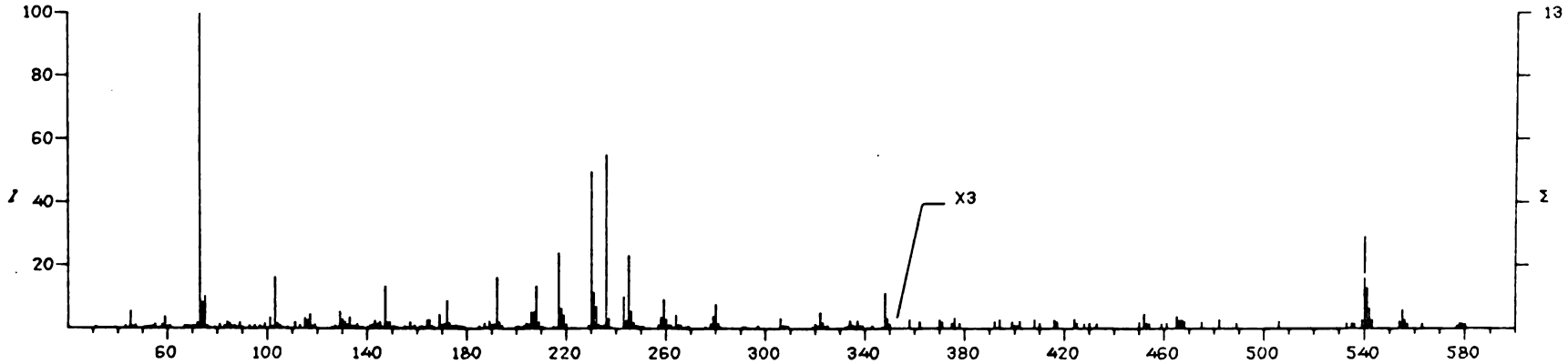
The molecular weight of adenosine is 267; the molecular weight of the TMS derivative would therefore be 267+72n with n representing the number of added TMS groups. A molecular ion with a mass to charge ratio (m/e) 33, 411, 483, 555, or 627 would

be expected for the substitution of 1 through 5 hydrogens respectively. The m/e 555 ion on the mass spectrum of TMS-A in Figure 3 represents the molecular ion for TMS-A indicating the substitution of four hydrogens by TMS groups. A molecular weight of 555 is supported by the presence of ions at M-15 representing the loss of a methyl group, M-73 representing the loss of TMS group, M-90 representing the loss of trimethylsilanol, and M-107 representing the loss of the 5'carbon with its constituents (CH_2-0-TMS).

The molecular weight of Am is 281, 14 more than the molecular weight of A since a methyl group has been substituted for the hydrogen of the 2'hydroxyl. Since the 2'hydroxyl is no longer available as a site for TMS substitution, the molecular weight would increase 216 mass units by the addition of only three TMS groups if the Am silylation parallels that of A. As seen in Figure 4, the expected molecular ion m/e 497 is present on the TMS-Am mass spectrum. There are also ions at M-15 representing the loss of a methyl group. M-31 representing the loss of an OCH_3 group, and M-103 representing loss from the molecular ion of C-5' with its constituents.

There are four potential silylation sites on the adenosine molecule; the three hydroxyl groups of the ribose moiety and the amino group on the purine ring. The fragmentation patterns discussed below indicate that each of these four sites is silylated. The structure proposed for TMS_4-A, shown in Figure 5, is consistent with the structure proposed by Lawson et al (1971) for the structure of TMS_5-

Figure 3. The mass spectrum of TMS_4-adenosine.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580

Figure 3. The mass spectrum of TMS_4-adenosine.

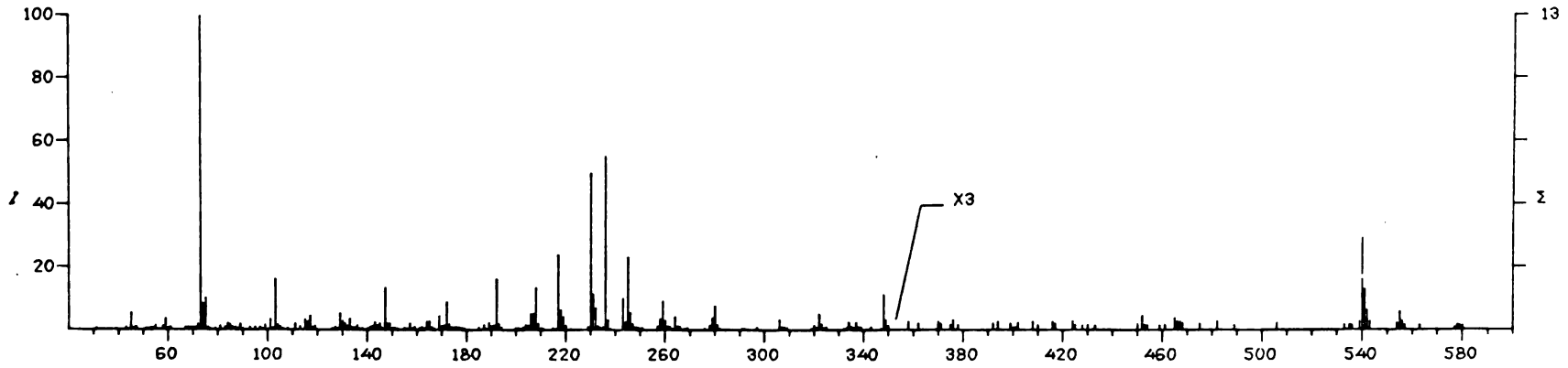
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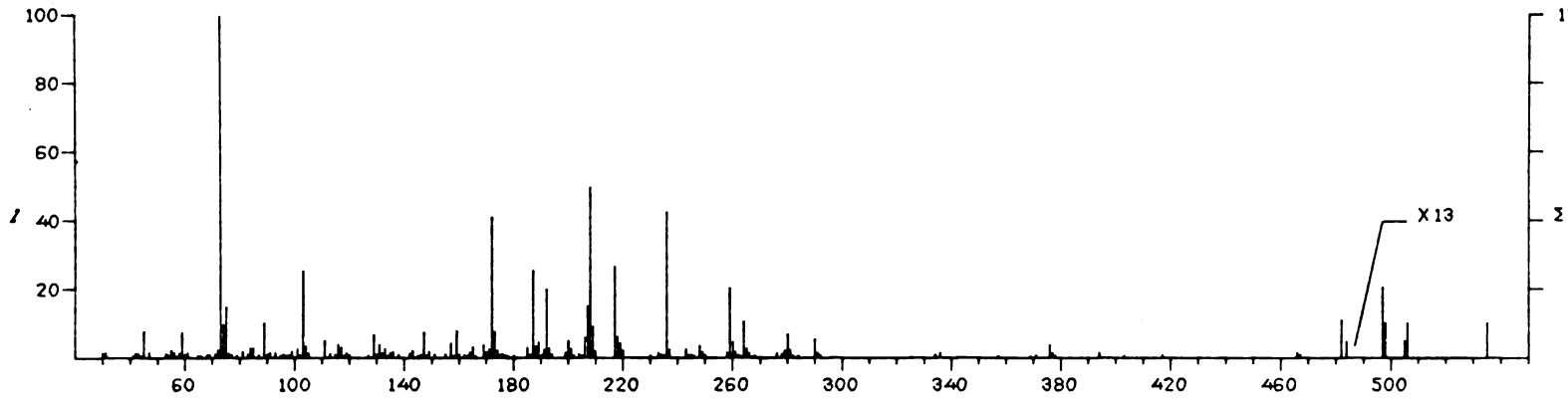
Figure 4. The mass spectrum of TMS_3-2'-O-methyladenosine.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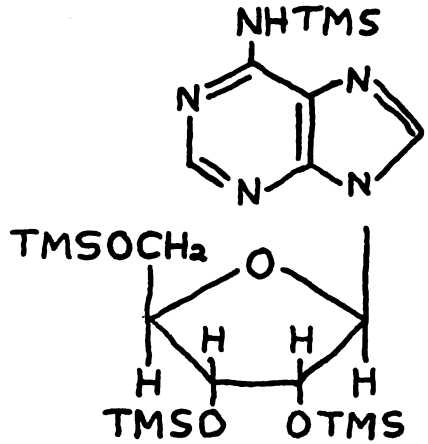


Figure 5. The structure of TMS_4-adenosine

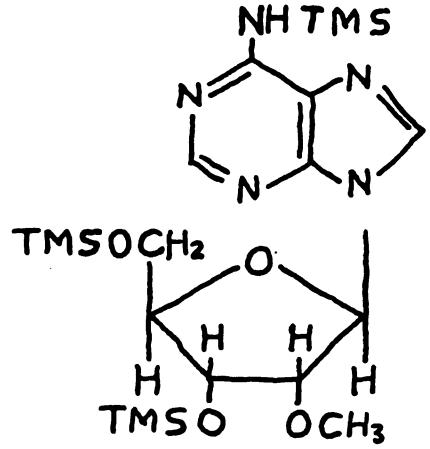
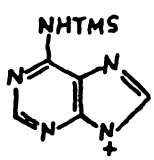


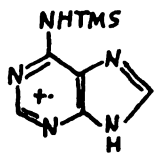
Figure 6. The structure of TMS_3-2'-O-methyladenosine

5'-AMP. Since the fragmentation patterns of TMS_3-Am indicate that each of the three remaining silylation sites of Am also contain a TMS group, the structure in Figure 6 is proposed for TMS_3-Am.

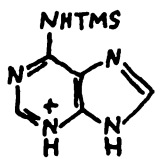
Fragmentation of the molecular ion is consistent with the stability of the purine ring as reported by others for the nucleoside (Shaw et al, 1970; Hecht et al, 1969) or nucleotide (McCloskey et al, 1968) and for TMS derivatives of the nucleotides (Lawson et al, 1971) Simple cleavage of the glycosidic bond leads to ion B, which represents the base fragment for either TMS_4-A or TMS_3-Am. This ion is



B
m/e 206



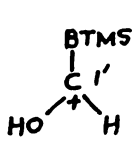
C
m/e 207



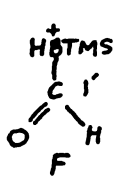
D
m/e 208

accompanied by ions C and D indicating the presence of one or two hydrogens which have been abstracted from the ribose moiety. Alternate fates of ion C are loss of a TMS methyl group giving rise to an ion at m/e 192 or the addition of a ribose TMS group resulting in the m/e 280 ion.

The ion at m/e 236 is produced by cleavage of the molecular ion to include C-1', O-4', and two hydrogens with the base fragment Either E or F may represent the structure of this ion since one of



E
m/e 236

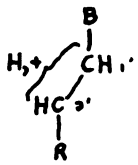


F
m/e 236

the hydrogens is of dubious origin and both of the structures would

be stable. Production of the ion is seen to be a major pathway for fragmentation of both molecules by the high relative intensity of their respective ions.

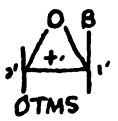
A diagnostic ion, G, is produced when the base fragment includes both C-1', and C-2'. A similar but less frequent ion, H, is produced when 0-4' is included with the base fragment containing C-1' and C-2'. The difference of 58 mass units between the TMS



G

m/e 398, R = OTMS

m/e 264, R = OCH_3



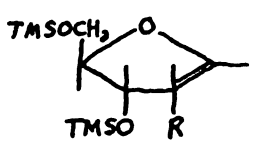
H

m/e 337, R = OTMS

m/e 279, R = OCH_3

group and the methyl group located on the 2'hydroxyl is reflected in the mass of the ions produced. A methyl group from TMS and a hydrogen may be lost from ion G, possible as methane, producing an ion at m/e 306 for TMS_4-A and an m/e 248 ion for TMS_3-Am.

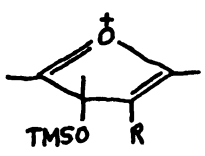
When the ribose moiety retains the charge upon cleavage of the glycosidic bond, a hydrogen is typically lost from the ribose ion leading to ion S. A series of related ions is produced by loss of portions of this parent ion. A TMS methyl group can be lost from



S

m/e 348, R = OTMS

m/e 290, R = OCH_3



J

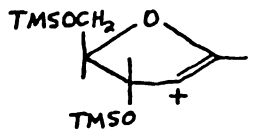
m/e 345, R = OTMS

m/e 187, R = OCH_3

the intact ribose producing ions at m/e 334 for TMS_4-A and m/e 276 for TMS_3-Am. Loss of C-5' with its constituents may

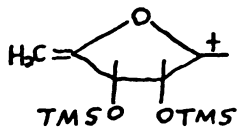
occur resulting an ion J.

Loss of the C-2' ether group from ion S produces ion K with both TMS_4-A and TMS3-Am. When the TMS ether group is lost



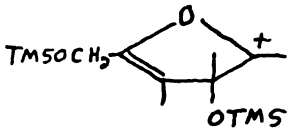
K

m/e 259



L

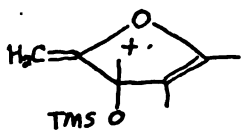
m/e 259



N

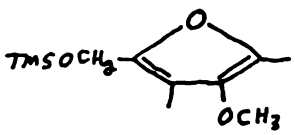
m/e 259

from C-5' or C-3' or ion S, ions L or N respectively are produced for TMS_4-A. The related loss of the TMS ether from ion S of TMS_3-Am usually occurs with a hydrogen yielding ion O or P, respectively.



O

m/e 200

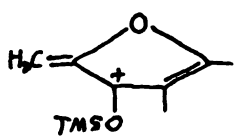


P

m/e 200

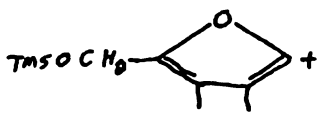
Loss of the C-2' ether group may be preferred since the relative intensity of the m/e 200 ion in the mass spectrum of TMS_3-Am.

Significant ions are also produced by the loss of two ether groups from ion S. Loss of the C-2' ether group yields ion R when the C-5' TMS ether is lost or ion T when the C-2' TMS ether is lost.



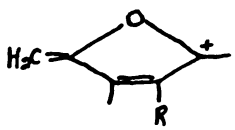
R

m/e 169



T

m/e 169



U

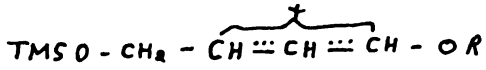
m/e 169 , R = OTMS

m/e 111 , R = OCH_3

Loss of both the C-5' and C-3' TMS ether groups produces ion U. Apparently the coordinate loss of two ether groups is more easily achieved from C-5' and C-3' than from either of the other two

combinations since the relative intensity of the m/e 169 ion (U) is larger than the relative intensity of the m/e 111 ion (R T) in the mass spectrum of TMS_3-Am. This hypothesis is supported by the unpublished mass spectrum of TMS_3-3'-O-methyladenosine for which the relative intensity of the m/e 169 ion (R+T) is larger than the relative intensity of the m/e 111 ion (U).

The charge may also reside with the ribose moiety following the cleavage of the molecular ion in which C-1' and 0-4' reside with the base. When this occurs, the very stable ion W is produced in



W

m/e 230 , R = OTMS

m/e 172 , R = OCH_3

good yield as shown by its high relative intensity. Distribution of the charge over C-4', C-3' and C-2' in this four carbon remnant of the ribose moiety probably accounts for its stability.

Characteristic ions are present on both spectra resulting from fragmentation of the ribose ring leaving three carbon pieces with their constituents. Ion X represents C-5', C-4' and C-3', an m/e 217 ion for both TMS_4-A and TMS_3-Am. Ion Z represents C-4', C-3' and C-2', also an m/e 217 ion for TMS_4-A, but an m/e 159 ion for TMS_3-Am. Fragmentation which results in the production

X

m/e 217

Z

m/e 217 , R = TMS

m/e 159 , R = CH_3

of ion X seems to be preferred since this ion has a higher relative intensity than does the Z ion in the mass spectrum of TMS_3-Am. This preference may be explained on the basis of the stability of the X ions resulting from distribution of the charge over three carbons as in the case of ion W.

An m/e 103 ion is produced if the charge resides with C-5' and its constituents when it cleaves from the molecular ion or from ion S. Several ions are characteristically produced on mass spectra of TMS derivatives from anomolies of the trimethylsililanol molecule. The TMS ion at m/e 73 is produced in sufficient quantities to be the base peak in these spectra. Other examples are the m/e 89 ion ($\overset{+}{O}$TMS) and the m/e 147 ion (DMS=$\overset{+}{O}$-TMS) formed by loss of a methyl group from the di-TMS ether.

Effect of methoxyamine-hydrochloride pretreatment on the nucleoside derivatives formed by BSA.

The response ratio for TMS-Cm with respect to TMS-Am displays more variation than the response ratio for TMS-Um with respect to TMS-Am as seen in Table 7. Butts (1970) reported improved gas chromatographic behavior of cytidine and deoxycytidine upon treatment with methoxyamine hydrochloride in pyridine to form the methoxime derivatives prior to silylation. He further reported that the reaction to form methoximes is unique to cytidine and its derivatives; other nucleosides do not react. In an attempt to avoid difficulty with the TMS-Cm response, the TMS-methoxime-

Cm derivative was formed. The structure shown in Figure 7 assumes that methoxime-Cm is silylated at both available sites.

Table 11 contains retention times for 2'-O-methylnucleosides which were treated with methoxyamine hydrochloride prior to silylation or which were silylated only. Methoxyamine hydrochloride treatment does not alter the retention time of either the Um or Am derivatives, but there is an obvious difference in the retention times of the Cm derivatives. TMS-methoxime-Cm has a retention time of less than one-third the retention time of TMS-Cm on either OV-1 or OV-17. The derivative formed is superior to TMS-Cm on both liquid phases since it forms a single sharp peak with little trailing. TMS-methoxime-Cm cannot be used to determine the 2'-O-methylnucleoside ratio in an RNA molecule however, since it is not well resolved from TMS-Um on either liquid phase as shown in Table 11.

TSIM derivatives of nucleosides.

In addition to the TMS-methoxime-Cm derivative, two other approaches were used in an attempt to form a more satisfactory derivative of Cm. Both approaches were based on the assumption that the available sugar hydroxyls of a nucleoside are rapidly silylated and the subsequent partial silylation of the amino or keto groups on the purine or pyrimidine rings is responsible for multiple derivatization. The first approach was treatment of the nucleosides with trimethylsilylimidazole (TSIM), which should silylate hydroxyl groups only (Pierce, 1970).

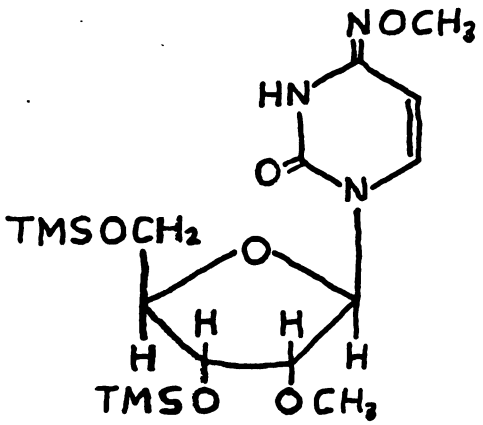


Figure 7. The structure of the TMS_2-methoxime derivative of 2'-O-methylcytidine.

Nucleoside	Retention Times	
	OV-1	OV-17
	182°	
Am	11.2	
Am (methoxyamine treated)	11.2	
	171°	186°
Um	10.8	14.9
Um (methoxyamine treated)	11.2	14.9
	169°	200°
Cm	3.18	22.6
Cm (methoxyamine treated)	8.8	7.6
	169°	186°
Um	10.5	14.9
Cm (methoxyamine treated)	8.8	15.8

Table 11. The effect of pretreatment with methoxyamine-hydrochloride on the retention times of the BSA derivatives of the 2'-O-methylnucleosides

Samples of Um, Am, and Cm were treated with methoxyamine-hydrochloride in pyridine as described in Methods. These samples and similar untreated samples were then silylated with BSA and G. C was performed on a 1% OV-1 column or on a 2% OV-17 column. Retention times are given in minutes at the indicated column temperatures.

The TMS derivatives of Um and Am formed by treatment with TSIM have retention times which are very similar to those formed by treatment with BSA on both OV-1 and OV-17. The peaks are symmetrical and display a minimum of trailing. However, the silylation of Cm is less satisfactory than treatment with BSA since a series of peaks was formed. The peaks were not well resolved on either liquid phase. They overlapped on OV-1 and a trailing peak resulted; they were spread out on OV-17 resulting in a broad plateau effect rather than well defined peaks. Since silylation of the 2'-O-methylnucleosides with TSIM does not result in a satisfactory derivative of Cm, the procedure was not used.

Effect of methanolic acetic anhydride pretreatment on the nucleoside derivative formed by BSTFA.

The second approach to more clearly defining the sites of trimethylsilylation of the nucleosides was an initial treatment with acetic anhydride in methanol. Selective N-acetylation on the presence of hydroxyl groups is routinely achieved with sphingolipids by this treatment (Gaver and Sweeley, 1966; Carter and Gaver, 1967). Acetylation of the amino group of the purine ring was therefore attempted prior to silylation of the sugar hydroxyl group.

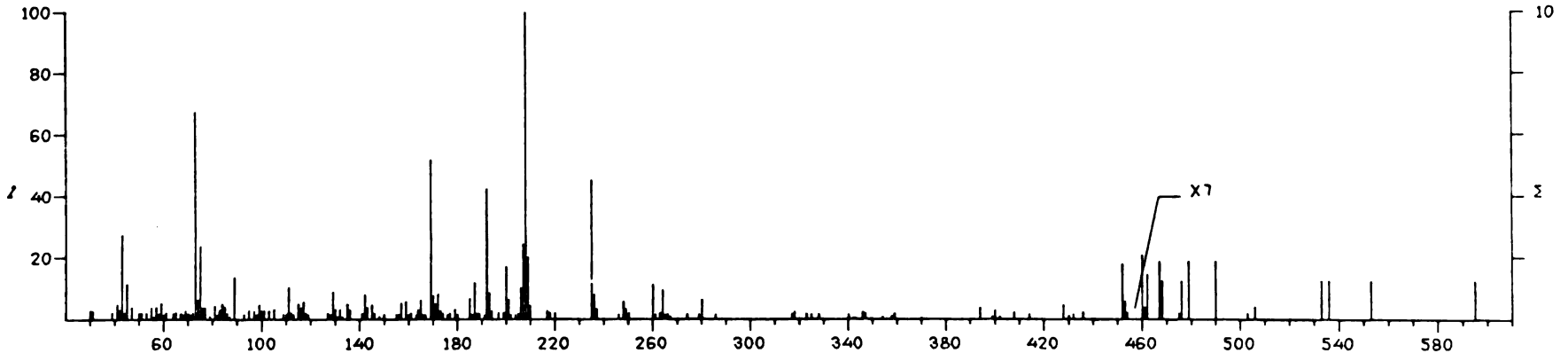
Two derivatives of Am were obtained by acetylation with acetic anhydride in methanol followed by silylation with BSA. The first peak eluted with a retention time similar to that of TMS_3-Am (prepared by treating Am with BSTFA) indicating that no acetylation

has occurred. The other peak has a retention time about one-third longer than TMS_3-Am, suggesting that acetylation has occurred. There was some indication that higher yields of acetylated Am were obtained if room temperature acetylations were performed over longer periods of time (two days or longer) or if short term acetylations were carried out at elevated temperatures (90°).

Mass spectral analysis of the two peaks confirmed the conclusion that acetylation of Am produced the second peak. The mass spectrum of the first peak eluted was identical to that of TMS_3-Am, shown in Figure 4. The mass spectrum of the second peak is shown in Figure 8. Since an acetyl group has a molecular weight of 43, 30 mass units less than a trimethylsilyl group, the number of acetyl groups present on the molecule can be deduced from the molecular weight change. The difference in molecular weight between TMS_3-Am and the compound in the second peak is 30 units, indicating the presence of one acetyl group.

The location of this acetyl group can be determined by comparison of the fragmentation pattern of TMS_2-acetyl-Am with the fragmentation patterns of TMS_3-Am and TMS_4-A discussed earlier. The purine ring has a strong m/e 208 ion in both TMS_3-Am and TMS_4-A which would be changed to an ion at m/e 178 if the acetylation has occurred on the amino group of carbon six. The 2'-O-methylribose moiety has a m/e 290 ion in TMS_3-Am, the expected 58 mass units lower than the ribose moiety ion of m/e 348 in TMS_4-A.

Figure 8. The mass spectrum of TMS_2-5'-acetyl 2'-O-methyladenosine.


100
80
60
I
40
20
60
100
140
180
220
260
300
340
380
420
460
500
540
580
X7
10
Σ

Acetylation of the 2'-O-methylribose moiety would result in a further reduction of 30 mass units giving an ion at m/e 260 for TMS_2-acetyl-Am. As seen on the mass spectrum in Figure 8, the m/e 208 ion has a high relative intensity, indicating that the base remains unaltered. The ion representing the ribose moiety has shifted from m/e 290 to m/e 260, however, indicating that the acetylation has occurred on the sugar.

Acetylation of the 5'hydroxyl is indicated by loss of the ion at m/e 217 ($TMSO-\overset{+}{\frown}CH=CH=CH-OTMS$) which originates from C-5', C-4', and C-3' of TMS-Am as discussed earlier. The m/e 187 ion ($CH_3-COO-\overset{+}{\frown}CH=CH=CH-OTMS$) replaces this ion as would be expected for a C-5' acetylation. The m/e 103 ion ($\overset{+}{C}H_2OTMS$) representing C-5' and its constituents is also missing from the mass spectrum of TMS_2-acetyl-Am. Its replacement ion at m/e 73 ($\overset{+}{C}H_2-OOC-CH_3$) cannot be distinguished from the ion produced by TMS alone without high resolution mass spectroscopy.

The complete structure of TMS_2-5'-acetyl-Am is given in Figure 9. Since the acetylation occurred at the 5' hydroxyl rather than at the amino group on the purine ring of Am, this derivatization procedure was not applied to Cm. Incomplete acetylation was another difficulty to be faced if the investigation was to continue.

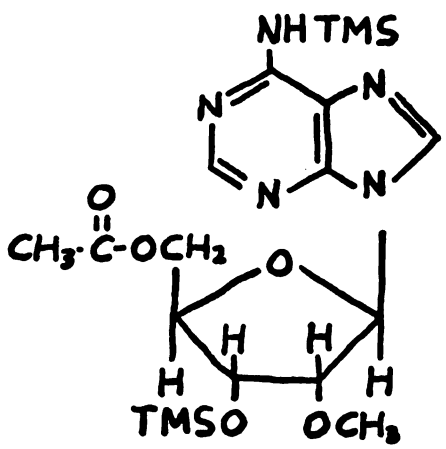


Figure 9. The structure of TMS$_2$-5'-acetyl 2'-O-methyladenosine.[2]

DISCUSSION

A study of the distribution of 2'-O-methylnucleosides in an RNA molecule can be approached from several levels. The first is quantity, a determination of the percent which the combined 2'-O-methylnucleosides represent of the total nucleosides (Abbate and Rottman, 1971). These data are easily obtained and are informative. Although differences may be more subtle, comparisons of RNA molecules can be made on the basis of the percentage of the nucleosides which are 2'-O-methylated.

A more complete analysis, short of the complete sequence of the RNA, would include characterization of the 16 alkaline stable dinucleotides. Lane and his co-workers have perfected this technique; very complex contrasts and similarities become evident when comparisons are made on this basis (Lane and Lane, 1971). Unfortunately, isolation and resolution of the 16 dinucleotides is tedious and involved (Gray and Lane, 1967). Digestion of this fraction with snake venom phosphodiesterase and separation of the eight resulting nucleosides is nearly as involved and gives less information.

The research presented here makes possible an RNA characterization which is intermediate to the two discussed above--with respect to sophistication of the information obtained as well as the ease with which it is done. The objective was a more rapid technique by which one could obtain the 2'-O-methylnucleoside ratio of an RNA

molecule for comparative purposes.

Isolation of the 2'-O-methylnucleoside fraction from an enzymatic digest of RNA as described in this research utilizes the borate-complex formed with ribonucleosides to retain them on an AG-1 column. Hall (1964) also used this principle, but he used a diatomaceous earth partition column which is more difficult to work with than the AG-1 column. Complete recovery of the 2'-O-methylnucleoside standards was achieved on the AG-1 column.

The amount of RNA which can be applied to the AG-1 column under the reported conditions is restricted. Although the amount of 2'-O-methylnucleosides obtained from 200 A_{260} units of digested RNA will suffice for most determinations, it would be convenient if larger amounts could be used in some cases (eg. with RNA molecules containing very low percentages of the 2'-O-methylnucleosides). An initial measurement of the total percentage of 2'-O-methylation in an RNA sample (Abbate and Rottman, 1971) should be done to establish the amount of RNA required for the ratio determinations.

The AG-1 column isolation requires extra caution to insure that deoxyribonucleosides do not contaminate the 2'-O-methylnucleoside fraction. Extensive treatment of RNA samples with deoxyribonuclease during their preparation should minimize the danger of contamination. If contamination is suspected, the 2'-O-methylnucleoside fraction may be analyzed for deoxyribonucleosides by

paper chromatography following the GLC determination. To this end, the TMS groups may be hydrolyzed from the nucleosides with water or methanol.

The above research describes the successful separation of the TMS derivatives of the 2'-O-methylnucleosides on a 2% OV-17 column of the GLC. The use of GLC for separation of the isolated 2'-O-methylnucleosides takes advantage of the sensitivity of the hydrogen flame ionization detector for quantitation. Model studies by Jacobson et al (1968) showed that ratio determinations of ribonucleosides can be done with as little as 0.2 ug of each nucleoside onto the GLC column. An even lower limit is indicated from the studies done with standard nucleosides in determining the copolymer ratios reported above. As little as 0.02 ug of Am may be quantitated successfully. The other nucleosides would require larger amounts of material since they have relative molar response ratios below one with respect to Am. Cm is the limiting nucleoside with respect to detection; a minimum injection of 0.1 ug would be required under the conditions presented.

Since the minimum volume of BSA which can be handled conveniently is in excess of the volume which must be injected, a single ratio determination requires more material. The extra volume permits multiple injection of a sample, a process which results in statistically reliable data. In addition, the unused portion of a sample may be saved for subsequent checking of an analysis by evaporating

the BSA and storing the nucleoside mixture.

Both Hall and Lane made use of paper chromatographic separations. Quantitation of unlabeled nucleosides was accomplished by eluting the spots corresponding to each nucleoside (or dinucleotide) and measuring the ultraviolet absorbance of the material recovered from the chromatogram. The sensitivity of this approach is limited by the amount of material necessary to obtain an accurate absorbance reading (ca 7 ug of each nucleoside). The material eluted from the chromatogram gives a single absorbance reading and there is no recourse for checking its validity (i. e. the spots may not be completely eluted).

The sensitivity of paper chromatographic determinations may be greatly increased through the use of radiochemically labeled RNA. Lane and Tamoaki (1969) have made successful use of this technique with their characterization of ^{14}C-methyl labeled rRNA molecules from L-cells.

Unfortunately, the use of labeled RNA may often require a sacrifice in convenience. Labeled precursors must be obtained, a process which may involve several complicated synthetic steps or a great deal of expense. Specific samples must be prepared to contain the label, when the isolation steps are difficult this process may become tedious.

In several instances labeled RNA may be difficult if not impossible to obtain. The brine shrimp cyst is almost completely impervious

to RNA precursors (McClean and Warner, 1971). Since the oocyte of the South African clawed toad, Xenopus laevis, undergoes oogenesis eight weeks prior to ovulation, the labeled precursors must be administered at this time to obtain labeled rRNA (Brown and Littna, 1964). RNA obtained from the organs of higher eukaryotes (eg. mammals) cannot be labeled without complicated experimental designs.

There are some difficulties to be faced with the GLC analysis of the 2'-O-methylnucleoside derivatives. From the low relative molar response of Cm, it is concluded that only a small portion of the Cm is being derivatized. Multiple derivatives form under more severe conditions than those reported, which no derivative forms a t all if the temperature if lower.

The formation of the methoxime derivative of Cm prior to its trimethylsilylation with BSA results in a derivative which gives a good response with a single, symmetrical peak on the OV-17 GLC column. This derivative can be used very successfully for quantitation with synthetic copolymers which do not contain Um or U. Since the retention times of TMS-Um and TMS-methoxime-Cm nearly coincide, the derivative cannot be used with natural RNA molecules.

The technique is designed for the calculation of the 2'-O-methyl-nucleoside ratio since the percent of the nucleosides which are 2'-O-methylated can be obtained rapidly and accurately with small amounts of RNA as described by Abbate and Rottman (1971). An attempt to obtain absolute amounts of the 2'-O-methylnucleosides would complicate

the procedure since an internal standard would have to be added and since the handling of the 2'-O-methylnucleoside fraction would have to be quantitative.

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