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DERIVATIZATION AND CHROMATOGRAPHY OF NUCLEOSIDES AND NUCLEOTIDES*

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SUMMARY

The aims of this study were to determine the effect of levels of various substances and reaction by-products, which are formed during hydrolysis of nucleic acids, on the derivatization and chromatography of nucleosides; and to investigate the silylation of mono- and dinucleotides.

The effect of NaCl, KCl, $MgCl_2$, NH_4Cl, and $(NH_4)_2SO_4$ on silylation and chromatography of nucleosides was studied at various molar excesses of salt. The response values for all nucleosides were studied at various molar excesses of salt. The response values for all nucleosides were significantly affected at molar excess salt present values (MSP) between 1 and 10 for KCl, NaCl, NH_4Cl, $(NH_4)_2SO_4$ and between 0.1 and 1 for $MgCl_2$. It was noted that thymidine was more sensitive than other nucleosides if silylated in presence of these salts. Two chromatographic peaks at retention temperatures (*RT*) 240 and 251 were obtained for cytidine at MSP values of 10^{-3} for NaCl, KCl, and $MgCl_2$, and 10^{-4} for NH_4Cl and $(NH_4)_2SO_4$. In a mixture of nucleosides the $RT = 251$ peak was used for quantitative analysis of cytidine as the $RT = 240$ peak elutes with guanosine. Thus, these salts have a significant effect on the gas–liquid chromatography of trimethylsilyl (TMS) cytidine in a mixture of nucleosides, especially the $RT = 241$ peak. The effect of salts on derivatization can be explained in part as follows: (a) reduced derivatization of nucleosides due to a decreased solubility in the solvent system; (b) formation of TMS anion derivatives, *e.g.* TMS-SO_4, TMS-PO_4, with a reduced molar excess of BSTFA; (c) metal chelation by Mg ions or other divalent cations with nucleosides or BSTFA; and/or (d) an increased breakdown of TMS derivatives in presence of salt in the sample or on the top 3 in. of the column packing.

Also, experiments were made on the effect of other substances such as Tris, phosphate, alkaline phosphatase, and KCl on completeness of silylation. The indi-

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vidual impurities showed no significant effect on the relative weight response (RWR) values of nucleosides; however, when a mixture was used, significantly lower RWR values were observed for all nucleosides except thymidine when using 1000 molar excess bis(trimethylsilyl)trifluoroacetamide (BSTFA). It was concluded that a molar excess of BSTFA greater than 1000 should be used for silylation and chromatography of nucleosides in an RNA hydrolysate.

As reported earlier the best derivatization of nucleosides was achieved using closed tube silylation at 150° for 15 min with 225 molar excess BSTFA and chromatography on 4% OV-11 on Supelcoport. In general, the presence of salts and other substances can be significant in quantitative work, thus it is suggested that they be removed using chromatographic cleanup methods.

The stability of nucleosides as a function of concentration of HCl, at room temperature was studied and very low RWR values for nucleosides were obtained when stored for 48 h in >0.001 *N* HCl.

Trimethylsilylation of various nucleotides and dinucleotides were made at 15 min as a function of temperature, and at 150° at different times. It was concluded that the optimal derivatization conditions were different for different nucleotides and dinucleotides. At 150° the best silylation time varied from 15 min to 4 h depending on the base attached. The multiple peaks obtained from dinucleotides corresponded to the TMS-nucleotide peaks, thus a breakdown of dinucleotides occurred.

INTRODUCTION

A variety of hydrolysis procedures have been used for the base composition analysis of nucleic acids[1,2]. The most commonly used methods are (a) hydrolysis to free purine and pyrimidine bases with concentrated perchloric acid, formic acid, and trifluoroacetic acid, (b) hydrolysis to mixtures of 2′- and 3′-nucleotides with KOH and piperidine, and (c) enzymatic hydrolysis to nucleosides using a mixture of ribonuclease, phosphodiesterase, and alkaline phosphatase.

Other methods for quantitative hydrolysis of RNA with NH_4OH, pyridine, and H_2SO_4 have been used with little success[2].

Early in 1951, Marshak and Vogel[3] developed a procedure in which 12 *N* perchloric acid was used for hydrolysis of RNA and DNA to the bases. This procedure was modified[4] by adding a short ion-exchange cleanup step, and then analyzed for the purine and pyrimidine bases after forming the N-methyl derivatives. This method of derivatization was not satisfactory, as multiple peaks resulted for adenine and cytosine. Other procedures suitable for gas–liquid chromatography (GLC) derivatization and chromatography were tried. The acetyl and isopropylidine derivatives of some of the nucleic acid bases and nucleosides were reported[5]. Later, the analysis of the base composition of RNA was determined by trimethylsilylation[6–8] after perchloric acid hydrolysis[3]. RNA was hydrolyzed to 2′- and 3′-nucleotides with 1 *N* KOH at 100° for 2 h, and the nucleotides obtained were converted to nucleosides by alkaline phosphatase in the presence of Tris buffer[9]. The minor nucleosides were qualitatively examined by trimethylsilylation with bis(trimethylsilyl)trifluoroacetamide (BSTFA) and chromatographed using a 1.5% SE-30 on Anakrom column. This two-step hydrolysis procedure was reduced to one step[10] using a mixture of ribonuclease, snake

venom phosphodiesterase, and alkaline phosphatase in Tris–HCl buffer (pH 8.3) for 16 h at 37°. The solution was then evaporated to dryness and the trimethylsilylation of nucleosides was carried out in the presence of salt and proteins with bis(trimethylsilyl)acetamide (BSA), 100 molar excess, at 120°, for 2 h. It was observed by Gehrke and Ruyle[8] that the amount of salt present in the reaction mixture was critical with cytosine, adenine, and especially guanine as complete derivatization of the bases in their presence was questionable.

Gehrke and Patel[11] studied the reaction conditions needed for quantitative derivatization of the nucleosides to the trimethylsilyl (TMS) derivatives. The optimal derivatization conditions were found to be at 150°, for 15 min with closed tube silylation using 225 molar excess of BSTFA and chromatographic separation using a 4% OV-11 on Supelcoport column. Later silylating strengths of BSA, BSTFA, and TMSI were compared. BSTFA was found to be superior to BSA and TMSI (trimethylsilyl imidazole) for derivatization of the nucleosides[12]. Silylations were also studied in various solvents, and acetonitrile was selected as a suitable solvent for further experimental work.

The purpose of this investigation was to study the effect of levels of salt and hydrolysis byproducts on the derivatization and chromatography of nucleosides. The effect of NaCl, KCl, $MgCl_2$, NH_4Cl, Na_2SO_4, $CaCl_2$, $CaSO_4$, and $(NH_4)_2SO_4$ on silylation and chromatography of nucleosides was investigated at various molar excesses of salt. Effect of substances such as Tris (used for pH 10 adjustment), phosphate (hydrolysis of byproduct), alkaline phosphatase (hydrolytic reagent), and KCl (formed during neutralization) on completeness of derivatization was studied. Also, the stability of TMS nucleosides at room temperature as a function of concentration of HCl was investigated.

Experiments were included to determine the optimal silylation conditions, retention time and temperature, and relative weight response (RWR) values of the nucleotides and dinucleotides and the effect of other substances as alkaline phosphatase, phosphate, Tris and KCl on completeness of silylation.

EXPERIMENTAL

Reagents

The purine and pyrimidine nucleosides were obtained from Mann Labs. (New York, N.Y., U.S.A.) and were chromatographically pure. The BSTFA was purchased from Regis (Morton Grove, Ill., U.S.A.) and stored at 4°. Acetonitrile of "Nanograde" purity was purchased from Mallinckrodt (St. Louis, Mo., U.S.A.). The water was double distilled from an all-glass distillation apparatus. The salts used in the experiments, KCl, NaCl, $MgCl_2$, NH_4Cl, $(NH_4)_2SO_4$, $CaCl_2$, and $CaSO_4$ were of primary standard grade and were obtained from Fisher Scientific (Rochester, N.Y., U.S.A.). Alkaline phosphatase from calf intestine was obtained from Sigma (St. Louis, Mo., U.S.A.).

Apparatus and chromatographic conditions

Apparatus and glassware, instrumental and chromatographic conditions, the internal standard method of calculation, and the chromatographic column preparations were the same as reported earlier[11].

Effect of salts on trimethylsilylation and chromatography

The effect of NaCl, KCl, $MgCl_2$, NH_4Cl, $(NH_4)_2SO_4$, $CaSO_4$, $CaCl_2$, and Na_2SO_4 on trimethylsilylation and chromatography of the nucleosides was studied. By using tenfold serial dilutions, the concentrations for various salts ranging from 1 to 10^{-7} mmoles/ml were obtained. The standard solutions contained 1 μmole/ml of each nucleoside in distilled water. One milliliter of nucleoside standard, and 1 ml of salt solution at concentrations of 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} mmoles/ml were placed in the reaction tube to obtain the salt–nucleoside molar ratio of 10^3, 10^2, 10, 1, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}, respectively. The samples were dried with nitrogen gas and then silylated at 150° for 15 min using 0.25 ml BSTFA and 0.50 ml CH_3CN containing 250 μg of pyrene. Then, 5 μl of the derivatized samples were chromatographed using 4% (w/w) OV-11 on Supelcoport (100–120 mesh) 1 m × 4 mm I.D. glass columns.

Effect of other impurities

Two nucleoside standards (a) uridine, adenosine, and guanosine, and (b) thymidine, adenosine, and cytidine containing 250 μg/ml each were prepared. To 1 ml of each standard solution, the following substances were added: 1 ml of 0.1 *N* HCl, or 1 ml of 0.1 *N* Tris, or 1 ml of 1 mg alkaline phosphatase per ml water, or 1 ml of 0.005 *M* NaH_2PO_4, or 1 ml of all above mentioned combined in one solution.

After drying, silylation was conducted at 150° for 15 min with 0.8 ml BSTFA (1000 molar excess), 0.5 ml CH_3CN containing 250 μg of pyrene as internal standard (IS), and 0.7 ml CH_3CN; then 5 μl were chromatographed using a 4% (w/w) OV-11 on Supelcoport column.

Stability of nucleosides in HCl

Two milliliters of nucleoside standards (a) and (b) containing 1 μmole/ml of each nucleoside and 2 ml of 10^{-6}–1 *M* HCl solution were mixed and allowed to stand for 48 h at room temperature, then neutralized with 10^{-6}–1 *M* NH_4OH. The control experiments were made by immediate neutralization with dilute NH_4OH at 0 h. The samples were then dried with nitrogen gas at 75° and silylations were carried out using 0.5 ml BSTFA and 0.5 ml CH_3CN containing 500 μg pyrene at 150° for 15 min, then 5 μl were chromatographed.

Silylation conditions for nucleotides and dinucleotides

One milligram of nucleotide or dinucleotide, 0.5 ml BSTFA, and 0.5 ml CH_3CN containing 1 μmole pyrene were silylated at 25°, 75°, 100°, 150°, 175° and 200° for 15 min. Then trimethylsilylation as a function of time was carried out at 150° for 0.25, 0.75, 1, 2, 3, and 4 h. Samples (5 μl) were chromatographed using 2% (w/w) SE-30 on Supelcoport (100–120 mesh) and 4% (w/w) OV-11 on Supelcoport 1 m × 4 mm I.D. glass columns.

RESULTS AND DISCUSSION

Effect of salts on silylation and chromatography of nucleosides

The effect of NaCl, KCl, NH_4Cl, $(NH_4)_2SO_4$, $MgCl_2$, $CaSO_4$, $CaCl_2$, Na_2SO_4 on silylation and chromatography of nucleosides was studied. The presence of these

salts occurs in the neutralization of excess KOH or NaOH after alkaline hydrolysis of nucleic acids, or on the addition of $MgCl_2$ necessary for maximum enzymatic hydrolytic activity of alkaline phosphatase. In each of these experiments 1 μmole of nucleoside was used and the micromolar amounts of salts were varied.

The term molar excess of salt present (MSP) is defined as follows:

$$\text{MSP} = \frac{\mu\text{moles of salt present during silylation and chromatography}}{\mu\text{moles of each nucleoside}}$$

The RWR values for the nucleosides were plotted as a function of the MSP values for the various salts (Figs. 1–6).

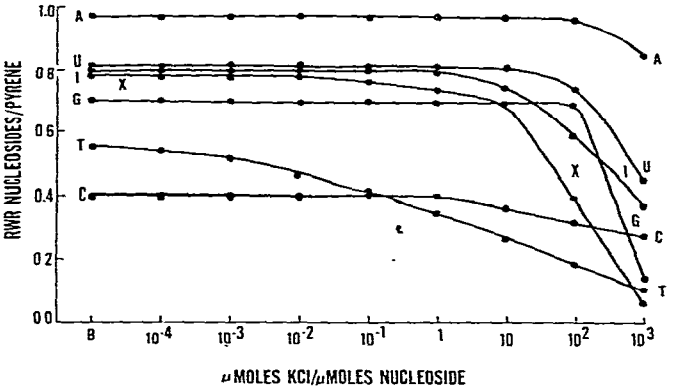

Fig. 1. Effect of KCl on silylation of nucleosides. Plot of RWR values as a function of MSP values.

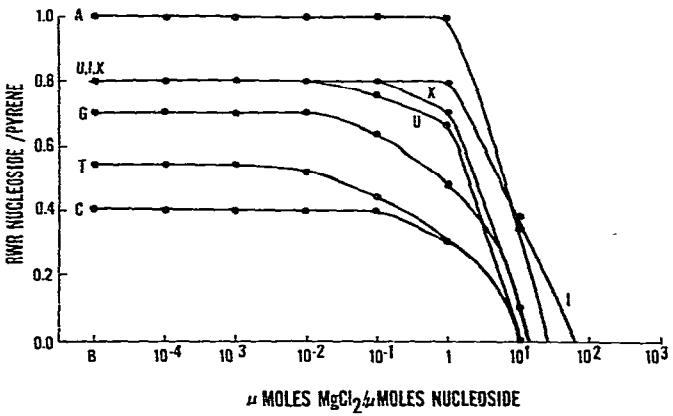

Fig. 2. Effect of $MgCl_2$ on silylation of nucleosides. Plot of RWR values as a function of MSP values

In initial experiments, 5 μl (5 μmoles) of each salt solution in water were injected (ten times) directly onto the column maintained at 300°, followed by injection of the TMS nucleoside standards. The average decrease in RWR values was from 20 to 35% when $MgCl_2$ was pre-injected, and from 5 to 15% when NaCl or KCl were pre-injected. Replacing the glass wool plug and refilling the top 3 in. of column

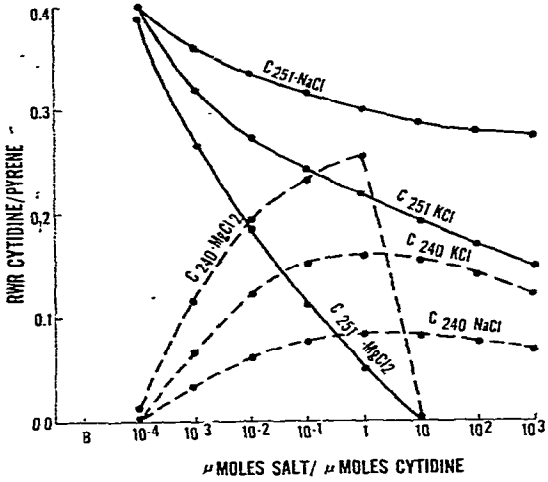

Fig. 3. Effects of various salts on silylation of cytidine. RWR values are plotted as a function of MSP values for two cytidine peaks at $RT = 240$ (– – –) and $RT = 251$ (———).

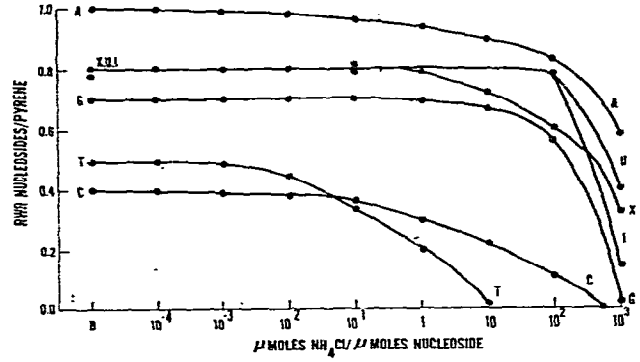

Fig. 4. Effect of NH_4Cl on silylation of nucleosides. RWR values are plotted as a function of MSP values.

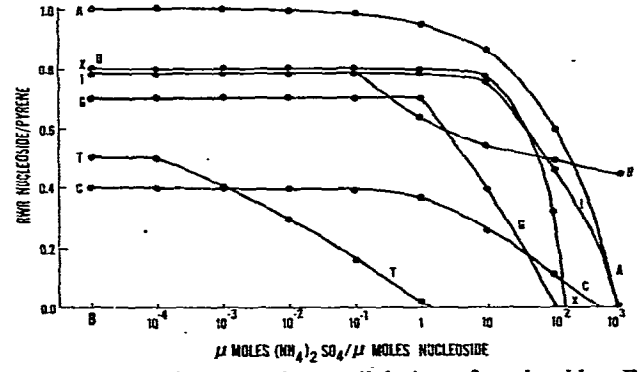

Fig. 5. Effect of $(NH_4)_2SO_4$ on silylation of nucleosides. RWR values are plotted as a function of MSP values.

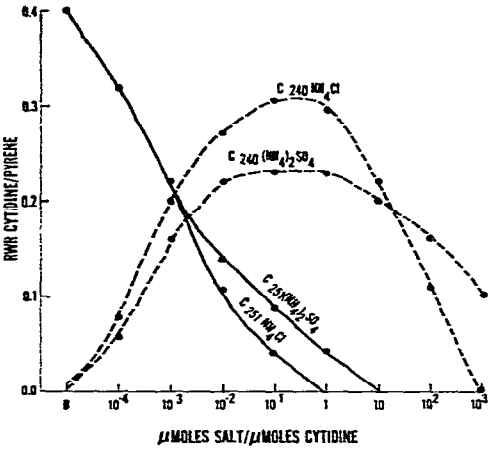

Fig. 6. Effect of ammonium salts on silylation of cytidine. RWR values are plotted as a function of MSP values for two cytidine peaks at $RT = 240$ (— — —) and $RT = 251$ (———).

packing, followed by re-conditioning of the column overnight completely restored the resolution of the column. The RWR values after re-conditioning of the column were comparable to the original values, indicating that breakdown of the TMS derivatives of nucleosides was caused by the salt deposits on the top 3 in. of column packing.

The nucleoside standards and samples containing salts at various MSP values were then derivatized. Five microliters of the derivatized sample were injected. The first injection on each day was a standard (*i.e.*, without salt) followed by a salt-containing sample, then the standard was re-injected. If the response values for the second standard failed to agree within experimental error of 3% with those for the first standard, then the glass wool and top one-half inch of column packing were replaced. The above injections were then repeated after overnight re-conditioning of the column. It was necessary to precede and follow each salt-containing sample with the chromatography of standards to prove that the column retained its efficiency and separation. The same concentrations of each nucleoside and internal standards were used in all derivatizations, and the same amount (5 μl) was injected each time onto the chromatographic column. Thus, the same corresponding area should be obtained for a sample containing salt and nucleoside standard. By doing the experiments in this way, it was determined whether the experimental areas for nucleosides or the IS were enhanced or reduced by the presence of salt. A reduced volatilization of the IS results in a reduced response for the IS and thus an apparent increase in the $RWR_{N/IS}$ for the nucleosides (N). It should be noted that no enhancement of response for any nucleoside was observed as the result of the presence of salt.

The effect of KCl and $MgCl_2$ on the silylation and chromatography of nucleosides is shown in Figs. 1 and 2. The response values for all nucleosides were significantly affected at MSP values between 1 and 10 for KCl and NaCl, and between 0.1 and 1 for $MgCl_2$. It was noted that the deoxyribonucleoside thymidine was more sensitive than other nucleosides if silylated in the presence of salts. Chromatographic peaks for all the nucleosides were not obtained if the MSP value was raised to 100 for $MgCl_2$, whereas, at the same MSP value of 100 for NaCl and KCl, most of the nucleosides were derivatized and chromatographed. The RWR values ob-

tained at MSP values of 100 for KCl were then compared to those for an MSP of 10 for $MgCl_2$. The nucleosides gave lower RWR values in presence of $MgCl_2$ even though the concentration was tenfold lower. The decrease in RWR values can be ascribed to the chelating ability of Mg^{2+} with the nucleosides. Two chromatographic peaks were obtained for guanosine and cytidine in the presence of NaCl, KCl, and $MgCl_2$ at MSP values above 0.001. $RWR_{C/P}$ values of cytidine (C) in the presence of salts is shown in Fig. 3 (P = pyrene).

It was observed that all of the nucleosides except thymidine were quantitatively silylated and chromatographed when the MSP values were between 0.1 and 1 for NH_4Cl and $(NH_4)_2SO_4$ (Figs. 4 and 5). The deoxyribonucleoside thymidine was found most sensitive to these salts and its RWR value was significantly affected at a salt–thymidine ratio of 0.1 with NH_4Cl and 0.001 with $(NH_4)_2SO_4$. In the presence of NH_4Cl and $(NH_4)_2SO_4$ (Fig. 6), two peaks for TMS cytidine were obtained. The RWR values for the TMS cytidine peak ($RT = 251$) decreased as the MSP values for NH_4Cl and $(NH_4)_2SO_4$ increased from 10^{-4} to 1. At MSP values above 1 with these salts only one TMS cytidine peak at $RT = 240$ was observed. A further decrease in the RWR value was noticed as MSP values increased to 1000. In a mixture of nucleosides, the $RT = 251$ peak was always used for quantitative analysis of cytidine, as the $RT = 240$ peak elutes with guanosine. It was concluded that ammonium salts have a significant effect on the derivatization of cytidine in a nucleoside mixture. The silylation of the nucleosides (500 μg each) in the presence of 10–100 μg of $CaCl_2$, Na_2SO_4, and $CaSO_4$ drying agents gave RWR values which were within experimental error of 5% when compared to nucleoside standards.

The effect of water on derivatization of TMS nucleosides was reported earlier[11], and the drying of the Nanograde CH_3CN solvent was found not necessary as long as the concentration of water was less than 10 ppt.

Some possible explanations for the decreased RWR values of nucleosides in the presence of salts follow:

(1) A reduced derivatization of nucleosides results due to a decreased solubility in the solvent system (salting-out or adsorption on salts phenomena).

(2) The formation of TMS anion derivatives: *e.g.*, TMS-SO_4, TMS-PO_4, thus a reduced molar excess of BSTFA; the TMS derivatives of various anions have been reported by Butts and Rainey[13].

(3) Formation of metal chelates by Mg^{2+} ions or other divalent cations with nucleosides.

(4) An increased breakdown of the TMS derivatives in presence of salts in the sample or on the top 3 in. of the column packing.

Effect of other substances on derivatization

When yeast RNA is hydrolyzed to nucleotides by alkaline hydrolysis followed by alkaline phosphatase hydrolysis to nucleosides, various substances and by-products are present KCl, Tris, $MgCl_2$, alkaline phosphatase, and phosphate. The effect of these substances, on derivatization and chromatography of the nucleosides, were studied: (a) 100 μmoles of KCl formed by neutralization; (b) 100 μmoles of Tris added to achieve pH 10 for optimal alkaline phosphatase activity; (c) 5 μmoles of phosphate formed on hydrolysis of nucleotides; (d) 1 mg of alkaline phosphatase enzyme added to hydrolyze RNA.

The effect of these substances on the RWR values of various nucleosides is given in Table I. The TMS derivatives of phosphate and Tris eluted at 75° and 120°, respectively, on a 4% OV-11 on Supelcoport column. When silylations were carried out using a 225 molar excess of BSTFA, at 150° for 15 min in presence of these impurities (singly and mixture), lower response values were obtained, thus the silylations were next made in the presence of 1000 molar excess BSTFA (Table I). Even at 1000 molar excess of BSTFA a lower RWR value for cytidine was observed in presence of alkaline phosphatase. When a mixture of salts and enzyme was used, significantly lower RWR values for all ribonucleosides were observed, except thymidine. Thus, it was concluded that a molar excess of BSTFA $\gg$ 1000 must be used for silylation and chromatography of nucleosides in RNA hydrolysates.

TABLE I

EFFECT OF IMPURITIES IN HYDROLYSATES ON NUCLEIC ACID ANALYSIS

Column, 4% OV-11 (methyl phenyl silicone, 35% phenyl) on Supelcoport (100–120 mesh). $RWR_{N/IS} = \frac{\text{area (N)/grams (N)}}{\text{area (IS)/grams (IS)}}$; N = nucleoside. Each value is a single determination on an independent sample; silylation at optimal conditions 150°, 15 min, 1000 molar excess BSTFA. KCl: 100 μmoles formed from 0.1 *N* KOH solution when neutralized with HCl. Tris: 100 μmoles added to achieve alkaline pH of 10.2 needed for optimal activity of alkaline phosphatase. Enzyme: 1 mg present only when free enzyme is added to achieve hydrolysis. Phosphate: arises from nucleic acid hydrolysis. Mixture: KCl, Tris, enzyme and phosphate.

Nucleoside	*Control RWR*	*KCl*	*Tris*	*Enzyme*	*Phosphate*	*Mixture*	
Uridine	0.81	0.75	0.80	0.79	0.80	0.56	0.59
	0.80	0.76	0.78	0.80	0.81	0.51	0.62
Guanosine	0.70	0.62	0.69	0.69	0.69	0.48	0.52
	0.69	0.64	0.70	0.68	0.68	0.43	0.47
Thymidine	0.30	0.34	0.34	0.45	0.34	0.48	0.46
	0.28	0.32	0.35	0.43	0.35	0.43	0.42
Adenosine	0.95	0.89	0.82	0.77	0.83	0.59	0.58
	0.93	0.90	0.81	0.76	0.82	0.60	0.55
Cytidine	0.50	0.50	0.48	0.35	0.50	0.20	0.25
	0.47	0.51	0.49	0.34	0.48	0.21	0.23

As reported earlier[11] the best derivatization of nucleosides was achieved with closed-tube silylation at 150° for 15 min using 225 molar excess BSTFA and chromatography using 4% OV-11 on Supelcoport. In general, the presence of salts and other substances can be significant in quantitative work, thus it is suggested that they must be removed by the use of ion-exchange chromatography or other means.

Stability of Nucleosides in Acids and Bases

Hydrolysis of ribonuclease with 0.1 *M* KOH at 100° for 2 h gives nucleotides, however, the excess base must be neutralized with 0.1 *M* HCl before hydrolysis with alkaline phosphatase can be carried out as the excess hydrochloric acid in the reaction mixture converts nucleotides and nucleosides in part to their bases.

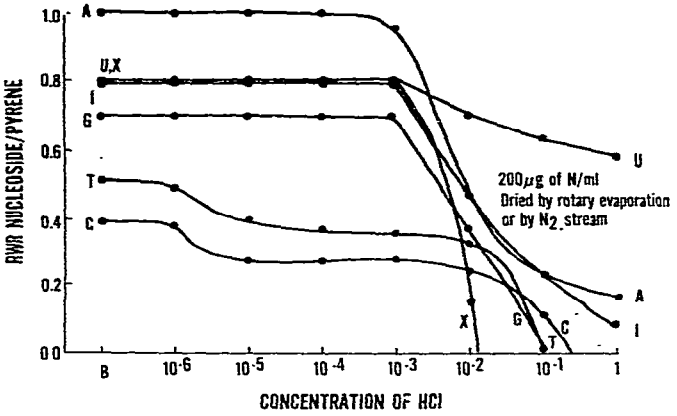

Fig. 7. Recovery of nucleosides from acid solutions. RWR values are plotted as a function of HCl concentration. Conditions: see Experimental.

The stability of nucleoside standards as a function of acid concentration (10^{-6}–1 *M* HCl) was studied after standing 48 h at room temperature (Fig. 7). The samples containing the nucleosides in HCl were neutralized using equimolar NH_4OH, dried, silylated, and chromatographed. Control experiments were done, where nucleoside standards in 0.01 *M* HCl were immediately neutralized with 0.01 *M* NH_4OH. Recovery of various nucleosides from 0.01 *M* HCl after 48 h are presented in Table II and extremely low RWR values were obtained for all nucleosides when stored in HCl.

TABLE II

RECOVERY OF NUCLEOSIDES

Recovery from acids and bases. RWR = relative weight response of nucleoside. Column, 4% OV-11 on Supelcoport (100–120 mesh). HCl: nucleoside in 0.01 *M* HCl, let stand for 48 h, then neutralize with equal volume of 0.01 *M* NH_4OH, dry, silylate, and chromatograph. NH_4Cl: control experiments containing equimolar amount (0.01 *M*) of NH_4Cl.

Nucleoside	*RWR*	
	HCl	*NH_4Cl*
Uridine	0.60	0.80
	0.62	0.82
Thymidine	0.00	0.36
	0.01	0.35
Cytidine	0.10	0.38
	0.12	0.39
Adenosine	0.20	0.95
	0.22	0.96
Inosine	0.20	0.80
	0.21	0.82
Xanthosine	0.00	0.81
	0.01	0.80
Guanosine	0.00	0.70
	0.02	0.72

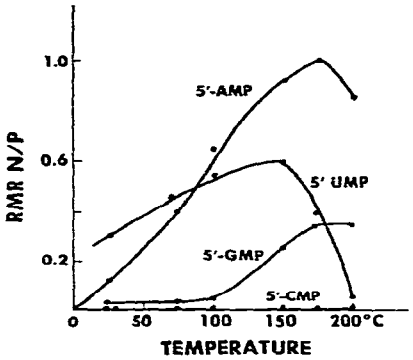

Fig. 8. Silylation of nucleotides (RMR value) as a function of temperature. For silylation and GLC conditions: see Experimental. GLC initial temperature was 100°, then programmed at 7.5°/min.

Silylation of nucleotides and dinucleotides

The trimethylsilylation of various 5'-nucleotides was conducted for 15 min and studied as a function of temperature (Fig. 8). Most complete derivatization was achieved at 150° for 15 min for 5'-UMP, at 175° for 5'-AMP, and at 200° for 5'-GMP. As shown in Fig. 9, the trimethylsilylation of various 2', 3', 5'-, and cyclic 2',3'-nucleotides were carried out at 150° as a function of time. The uridine nucleotides gave the best derivatization on reaction for 15 min, the adenosine nucleotides at 45 min, and guanosine nucleotides after about 3 h. When 5'-UMP was silylated at 150° for 3 h, and the $RWR_{N/P}$ values obtained compared to those for silylation at 150° for 15 min a 90% decrease in response was noted. This decrease in RWR value was due to thermal decomposition, which occurred on long reaction at 150°, also peaks corresponding to uridine, and uracil were observed. Similarly, when 2'- and 3'-GMP's were derivatized at 150° for 15 min and the RWR values compared to those for silylation at 150° for 3 h, it was observed that only 15% of the 2'- and 3'-GMP's were derivatized at reaction conditions of 150° and 15 min, chromatographic peaks were obtained for cyclic 3'-, 5'-AMP, cyclic 2'-, 3'-AMP, cyclic 2'-, 3'-UMP. The retention temperatures, relative molar response (RMR) values, and optimal reaction conditions

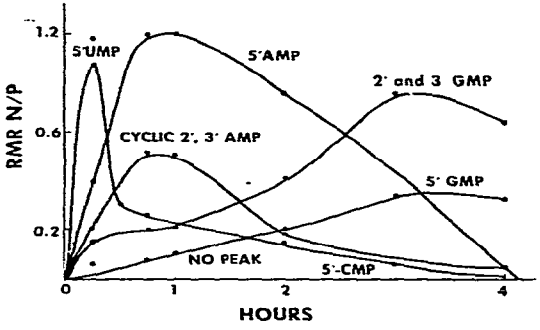

Fig. 9. Silylation of nucleotides (RMR value) as a function of time at 150°. Silylation and GLC conditions are the same as in Fig. 8.

for various 2'-, 3'-, 5'- and cyclic 2'-, 3'-nucleotides are given in Table III. These values are for chromatography using a 4% OV-11 on Supelcoport column, which is the column used for the chromatography of the nucleosides. The derivatization of 5'-CMP and deoxy 5'-CMP results in a cytosine peak at $RT = 150$ and another peak at $RT = 120$ (Table III).

TABLE III

RELATIVE MOLAR RESPONSE (RMR) OF NUCLEOTIDES AND DINUCLEOTIDES

RMR = nucleotide or dinucleotide per μmole pyrene. Column, 4% OV-11 on Supelcoport (100–120 mesh), 1 m × 4 mm I.D., Glass. RT = retention temperature.

Dinucleotide	*RT*	*RMR*		*Optimal silylation conditions*	
				Temp. (°C)	*Time*
2'-UMP	243	0.55	0.56	150	15 min
3'-UMP	247	0.61	0.60		
Cyclic 2',3'-UMP	248	0.38	0.36		
5'-UMP	250	0.62	0.59		
2'-AMP	257	0.90	0.89	150	45 min
3'-AMP	261	0.91	0.90		
Deoxy 5'-AMP	264	0.88	0.85		
Cyclic 2',3'-AMP	265	0.42	0.44		
5'-AMP	268	1.01	1.02		
Cyclic 3',5'-AMP	271	0.45	0.44		
2'GMP	271	0.64	0.63	150	3 h
3'GMP	274	0.65	0.67		
5'GMP	277	0.35	0.34		
Deoxy 5'GMP	278	0.29	0.30		
5'-CMP	120	0.40	0.45		
5'-CMP (cytosine)	153	0.20	0.21	150	45 min
Deoxy 5'-CMP	120	0.45	0.47		
Deoxy 5'-CMP (cytosine)	153	0.20	0.21		
Ap (2' → 5') A	265	0.30	0.28	150	15 min
	278	0.20	0.19		
Ap (3' → 5') U	250	0.72	0.70	150	2 h
	261	0.30	0.31		
Ap (3' → 5') A	250	0.42	0.41	150	15 min
	288	0.35	0.34		
Up (3' → 5') U	241	0.11	0.12	150	15 min
	266	0.08	0.09		
	280	0.06	0.05		

Very little information on GLC of dinucleotides is reported in the literature. Several dinucleotides were derivatized at 150° as a function of time (Fig. 10), and also at various temperatures for 15 min (Fig. 11). Multiple peaks were obtained for the dinucleotides (dN) when silylated under the above conditions. The retention temperatures, $RMR_{dN/P}$ values, and reaction conditions for various TMS derivatives of dinucleotides are given in Table III. Silylation of dinucleotides for 15 min as a function of temperature gave the best derivatization at 100° for Up (3' → 5') U, 150° for Ap(2'→5')A, and 200° for Ap(3'→5')U and Ap(3'→5')A. In general, the silylation of dinucleotides at 150° for various times gave the best derivatization after reaction for

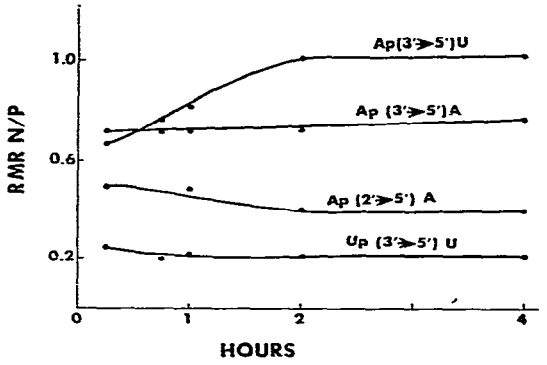

Fig. 10. Silylation of dinucleotides (RMR value) as a function of time at 150°. Silylation and GLC conditions are the same as in Fig. 8.

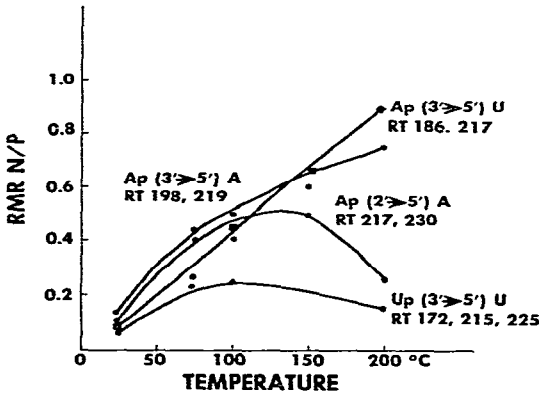

Fig. 11. Silylation of dinucleotides (RMR value) as a function of temperature. Silylation and GLC conditions are the same as in Fig. 8.

2 h. It was noted that the formation of multiple derivatives varied if the time was increased from 15 min to 4 h (Fig. 12). These multiple peaks obtained from the dinucleotides corresponded to the TMS-nucleotide peaks, thus a breakdown of dinucleotides occurred.

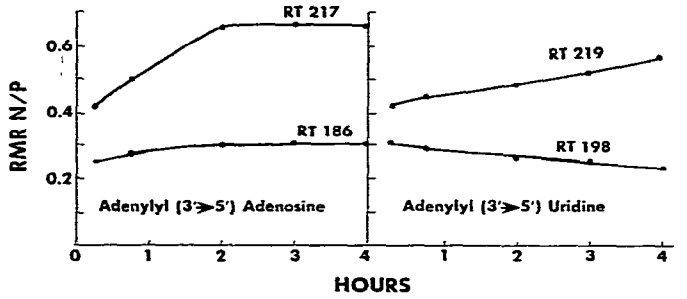

Fig. 12. Silylation of dinucleotides (RMR value) as a function of time at 150°. Silylation and GLC conditions are the same as in Fig. 8.

It was concluded that the optimal derivatization conditions were different for different nucleotides and dinucleotides. At 150° the best silylation time varied from 15 min to 4 h, depending on the base attached.

CONCLUSIONS

The derivatization of nucleosides and nucleotides is significantly affected by salts, hydrolysis reaction by-products, temperature, and time. BSTFA is a good silylation reagent and a 1000 molar excess is recommended. Temperatures and times are given for optimal silylation of 22 nucleotides and dinucleotides.

In general, biological samples as urine, serum, and cell cultures should be prefractionated by chromatographic and solvent extraction methods before GLC analysis for nucleosides, deoxynucleosides, and methylated nucleosides is performed. Successful derivatization and chromatography of the mono- and dinucleotides was not encouraging, thus nucleotides in biological samples should be converted to the corresponding nucleosides or bases, then chromatographed.

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