Methylation and Breakdown of Microsomal and Soluble Ribonucleic Acid from Rat Liver by Diazomethane

E. KRIEK AND P. EMMELOT

From the Department of Biochemistry, Antoni van Leeuwenhoek-Huis: The Netherlands Cancer Institute, Amsterdam

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Treatment of aqueous solutions of microsomal or soluble RNA with diazomethane dissolved in ether resulted in an increase of the molar extinction coefficient and a decrease of the $A_{(260)}/A_{(280)}$ ratio,[1] the hyperchromicity, and the protamine titration of treated as compared with control RNA. These changes, which were counteracted by Mg^{++} ions, were due to the methylation of the RNA-guanine and the esterification of the secondary phosphate groups leading to the breakdown of RNA. 7-Methylguanine was the main methylation product next to lower amounts of 1,7-dimethylguanine and (1-methyl-)2,4-diamino-5-methylamino-6-hydroxypyrimidine resulting from opening of the imidazolyl ring of the former two compounds. The breakdown of the diazomethane-treated RNA was ascertained by gel filtration, dialysis and ultracentrifugation. The pattern of base methylation following treatment of an ether suspension of RNA with diazomethane was completely different from that observed under aqueous conditions. No 7-methylguanine, but an appreciable amount of 1-methyluracil and a lesser amount of 1-methylguanine, were now formed. Evidence was obtained for a difference in RNA breakdown following methylation in ether and in water. It was further shown that, under aqueous conditions, much more diazoethane than diazomethane was needed to induce a comparable degree of change in microsomal RNA. The significance of these results is discussed in relation to the mechanism of interaction between nucleic acids and diazoalkanes, which yield alkylcarbonium ions under aqueous conditions and are metabolic derivatives of the hepatotoxic and carcinogenic *N*-nitrosodialkylamines.

Evidence has previously been presented that the *in vivo* effects of the hepatotoxic and carcinogenic *N*-nitrosodialkylamines are not due to the parent compounds per se, but to their metabolites (Magee and Vandekar, 1958; Brouwers and Emmelot, 1960; Hultin *et al.*, 1960; Mizrahi and Emmelot, 1962, 1963; Heath, 1962). Following *N*-demethylation of dimethylnitrosamine (*N*-nitrosodimethylamine) by the liver microsomes, the monomethylnitrosamine (methyldiazohydroxide) thus produced will yield methyldiazonium ions, either directly (compare Austin, 1960) or via the intermediary formation of diazomethane. The diazonium ions decompose to carbonium ions which may alkylate nucleophilic components of the liver cell. The methylcarbonium ions and not the diazoalkanes are the metabolites most likely to be responsible for the *in vivo* effects of the *N*-nitrosodialkylamines (Emmelot *et al.*, 1962).

As regards the fine structure of the liver cell, dimethylnitrosamine has been found (Emmelot and Benedetti, 1960, 1961) to affect primarily the endoplasmic reticulum (microsomes). The concomitant inhibition of microsomal amino acid incorporation (Magee, 1958; Brouwers and Emmelot, 1960; Hultin *et al.*, 1960) may result from the methylation of the protein synthetic machinery. It has been shown recently (Mizrahi and Emmelot, unpublished) that the lesion is located in the ribonucleoprotein particles of the endoplasmic reticulum. Since the latter particles are composed of protein and high molecular RNA, the methylation of either one or both of these compounds may account for the inhibition of amino acid incorporation produced by dimethylnitrosamine. Methylation of liver proteins and nucleic acids by C^{14}-dimethylnitrosamine *in vitro* and *in vivo*, respectively, has recently been reported by Magee and Hultin (1962) and Magee and Farber (1962). An analogous lesion as operative in the toxic effect but presumably of a less indiscriminate type, perhaps involving nucleic acid only, may also underly the carcinogenic effect of dimethylnitrosamine.

In the experiments reported in the present paper, the reaction of methyl- and ethylcarbonium ions with nucleic acid has been studied using a model system consisting of diazomethane and diazoethane (which under aqueous conditions yield CH_3^+ and $C_2H_5^+$), and ribonucleic acids obtained from the microsomes and soluble fraction of normal rat liver.

EXPERIMENTAL

Diazoalkanes.—Solutions of diazomethane in ether were prepared according to the method of de Boer and Backer (1954) from *p*-toluylsulfonylmethylnitrosamide, recrystallized from diethyl ether (mp 61–62°). Solutions of diazoethane in ether were obtained from *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chem. Co.) by the distillation method of McKay *et al.* (1950). The diazoalkane concentrations were measured using the method of Marshall and Acree (1910). Ether solutions containing freshly prepared diazoalkane were used in each experiment.

Ribonucleic Acids.—Microsomal and soluble RNA were prepared from rat liver by phenol extraction according to Kirby (1956). Yeast s-RNA was prepared by the method of Monier *et al.* (1960). In order to obtain RNA free from nucleotides and oligonucleotides, the RNA preparations were chromatographed over columns (30 × 2 cm) of Sephadex G50 (Pharmacia AB., Uppsala, Sweden) (compare Bosch *et al.*, 1961). Water (twice distilled in all experiments) was used as eluent and fractions (5 ml) 4–10 were collected and used in the experiments.

The RNA content was estimated by the method of Volkin and Cohn (1954); organic phosphate was converted to inorganic phosphate by digestion with 10 N H_2SO_4 and the inorganic phosphate was determined by the Fiske-Subbarow method. Protamine titration

[1] Abbreviations used: m- and s-RNA = microsomal and soluble ribonucleic acid; $A_{(260)}$ = absorbancy at 260 mμ; $\epsilon(P)_{260}$ = molar extinction coefficient expressed per mole RNA-phosphate per liter; I = ionic strength.

TABLE I

PROPERTIES OF m-RNA AFTER TREATMENT WITH DIAZOMETHANE

Exp. 1: 4.2 μmoles RNA-P in 6 ml water, treated with increasing amounts of diazomethane (0.13 mmole portions/0.5 ml ether); exp. 2: 2.1 μmoles RNA-P in 4 ml water treated with one portion of 0.37 mmole diazomethane in 1 ml ether; both at 0°.

Exp. No.	CH_2N_2 (mmoles)	Addition	Protamine Titration (% of control)	$A_{(260)}/A_{(280)}$	$\epsilon(P)_{260}$	Hyperchromicity (%)
1	—	—	100	2.18	7800	22
	0.13	—	80	1.79	8000	—
	0.26	—	74	1.75	8500	—
	0.52	—	64	1.67	8700	5.5
	1.04	—	33	1.59	8800	0
	0.13	0.001 M $MgCl_2$	100	2.18	7800	18.5
	0.26	0.001 M $MgCl_2$	94	2.04	8000	14.7
2	—	—	100	2.04	8500	24 (11)[a]
	0.37	0.01 M KCl	64	1.70	9050	0
	0.37	0.01 M KBr	57	1.43	10200	—
	0.37	0.01 M KJ	57	1.45	11000	—
	0.37	0.005 M K_2SO_4	66	1.59	9700	0
	0.37	0.001 M $MgSO_4$	88	1.89	8800	18 (10)[a]
	0.37	0.001 M $MgCl_2$	87	1.92	9200	19 (12)[a]

[a] Values in parentheses represent precentage hypochromicity after cooling.

was carried out by the method of Terayama (1952) as modified by Reiner and Zamenhof (1957).

For measurement of the hyperchromicity the RNA was dissolved in 0.1 M potassium acetate buffer of *p*H 7.4, and the absorbancy read at 260 mμ during gradual heating of the solution from 20° to 90° in 120 minutes.

Reaction between RNA and Diazoalkane.—One or successive portions (0.5 or 1.0 ml) of an ethereal solution of diazoalkane were added to an aqueous solution (*p*H 6.5–7.0) or ether suspension of RNA under vigorous stirring. The temperature and the concentrations of the reactants are indicated in the legends of the tables. When the yellow color of the diazoalkane had disappeared, which took a few minutes for each aliquot of ethereal diazoalkane, the ether was blown off by a stream of nitrogen. In each experiment control RNA was treated in the same manner except for the presence of diazoalkane. Samples were withdrawn for protamine titration, and paper- and ion-exchange chromatography. For measurement of absorption spectra and hyperchromicity the samples were diluted with 0.1 M potassium acetate buffer of *p*H 7.4 to equal concentrations.

Chromatography.—Control and diazoalkane-treated RNA were hydrolyzed with 1 N HCl or 70% $HClO_4$, as indicated under Results, at 100° for 1 hour. The hydrolyzates were diluted with water and applied to a column (15 × 1.0 cm) of Dowex-50 × 8 (200–400 mesh) in the H^+-form, equilibrated with 0.5 or 1 N HCl, or water in the case of the $HClO_4$ hydrolyzate. The column was eluted with 0.5 N, 1 N, and 2 N HCl. Perchlorate ions were removed by washing with 0.1 N HCl. Fractions of 5 ml were collected; flow rate was about 12 minutes per fraction. Pyrimidine nucleos(t)ides, obtained from the HCl hydrolyzates by passing over the above column, were separated on Dowex-1 × 8 (200–400 mesh; 15 × 1.0 cm) in the formate form; nucleosides were collected by washing with water. Elution was performed using the method of Spahr and Tissières (1959), as modified by Ingram and Pierce (1962). The dialyzate of methylated RNA was also separated on Dowex-1 × 8, the oligonucleotides being eluted with 0.5 N HCl according to Cantoni *et al.* (1962). 1-Methylcytidine-2′(3′)-phosphate was eluted from the Dowex-1 × 8 column by 0.15 M formic acid; 1-methyluridine-2′(3′)-phosphate was eluted before uridine-2′(3′)-phosphate with 0.05 M ammonium formate–0.01 M formic acid buffer.

The fractions containing material absorbing at 260 mμ were collected and evaporated under diminished pressure at about 40°. Alkylated bases were identified by their UV absorption spectra in 1 N HCl and 0.1 N NaOH (compare Brookes and Lawley, 1961a; Reiner and Zamenhof, 1957; Smith and Dunn, 1959) and by their chromatographic behavior on Whatman No. 1 filter paper with ethanol–water–25% ammonia as solvent (Brookes and Lawley, 1961a). 7-Methyl- and 7-ethylguanine, prepared according to Brookes and Lawley (1961a), were used as markers. The following R_F values (relative to guanine) were found: 7-methylguanine, 1.3; 7-ethylguanine, 1.9; 1-methylguanine, 1.5; 1,7-dimethylguanine, 2.3.

An $R_P/R_P(G)$ value of 2.3 was found for a compound which was identified as 1-ethylguanine according to the resemblance of its UV absorption spectrum (in 1 N HCl λ_{max} 251, λ_{min} 230; and in 0.1 N NaOH λ_{max} 275, λ_{min} 232; $A_{(280)}/A_{(260)}$ being 0.66 and 1.0, respectively) to that of 1-methylguanine. 1-Methylcytidine- and 1-methyluridine-2′(3′)-phosphate were identified by their UV absorption spectra in acid and alkali, which resembled those of 1-methylcytidine and 1-methyluridine, prepared according to the method of Brookes and Lawley (1962). 1-Methylcytidine and 1-methyluridine from the nucleoside fraction were identified by comparison of their chromatographic properties in ethanol–water–25% ammonia with those of the synthetic samples.

The base composition of control and diazomethane-treated RNA was calculated using the following millimolar extinction coefficients at 260 mμ in 1 N HCl: uracil and methyl derivative, 7.3; cytosine, 6.0; guanine, 8.0; alkylated guanines, 8.2; compound X, 10.0 (calculated on the assumption that X is derived from guanine, concentration of total guanines of treated RNA is that of guanine of control RNA); adenine 13.0.

For calculation of the nucleotide composition after chromatography on Dowex-1 × 8 formate the extinction coefficients according to Beaven *et al.* (1955) were used. UV spectra were measured with the Beckman potentiometric recorder operating in conjunction with a Beckman D.B. spectrophotometer.

TABLE II

ALKYLATION OF RNA-GUANINE BY DIAZOALKANES

m-RNA (23, 20.8, and 30 μmoles P in experiments 1, 2, and 3, respectively)/10 ml water or salt solution (as indicated) was incubated with 3 successive portions of 0.24 or 0.20 mmole CH_2N_2/0.5 ml ether in exp. 1, with 3 successive portions of 0.22 mmole $C_2H_4N_2$/1 ml ether in exp. 2, and with 6 successive portions of 1.2 mmoles $C_2H_4N_2$/5 ml ether in exp. 3, at 0°. Hydrolysis in 1 N HCl and separation of purines over Dowex-50 × 8. G = guanine; A = adenine; G(total) = G + 7-alkyl-G + X.

Exp. No.	Diazo-alkane (mmoles)	Addition	Protamine Titration (% of control)	A	G	7-Alkyl-G	X	A:G(total)
				(moles/mole RNA-P)				
1	—	—	100	0.15	0.33	0	0	10:22
	0.72	—	80	0.15	0.16	0.18[a]	Trace[c]	10:22
	0.72	$MgSO_4$ (I = 0.04)	98	0.14	0.23	0.08[a]	0	10:22
	0.60	K_2SO_4 (I = 0.04)	65	0.14	0.08	0.18[a]	0.05[c]	10:22
2	0.66	K_2SO_4 (I = 0.04)	97	0.15	0.33	0	0	10:22
3	—	—	100	0.135	0.26	0	0	10:19
	7.2	—	90	0.135	0.15	0.08[b]	0	10:19[d]

[a] 7-Methylguanine. [b] 7-Ethylguanine. [c] Including a trace of 1,7-dimethylguanine. [d] Including 0.03 mole 1-ethylguanine.

RESULTS

The Reaction between Diazomethane and RNA under Aqueous Conditions

Change in Properties of m- and s-RNA.—Increasing amounts of diazomethane, dissolved in ether, were allowed to act upon aqueous solutions of m-RNA at 0° as described under Experimental. This resulted in a decrease of the protamine titration, the hyperchromicity, and the $A_{(260)}/A_{(280)}$ ratio, and in an increase of the molar extinction coefficient of the treated as compared with the control RNA. The changes in the properties of RNA were progressive with increasing concentrations of diazomethane and were counteracted by Mg^{++} but not by K^+ ions (Table I). Similar changes were observed after incubation of s-RNA with diazomethane at 0° or 21°; phosphate ions (0.01 M phosphate buffer, pH 6.8) prevented the diazomethane effect at 21°.

Methylation of RNA-Guanine.—Methylated m- and s-RNA (Table II, Fig. 1) were hydrolyzed in 1 N HCl and the purines were separated on Dowex-50 × 8. No methylated derivatives of adenine could be detected under the conditions employed. A substantial amount of 7-methylguanine was always formed in the absence of Mg^{++} ions, but in their presence the formation of 7-methylguanine was markedly impaired. Other methylated products were 1,7-dimethylguanine and an unknown substance X. Both compounds were found in trace amounts in the present experiments with m-RNA carried out in the absence of added ions, but were lacking after reaction in the presence of Mg^{++}. A rather substantial amount of compound X was formed in the presence of K_2SO_4. In an experiment carried out with potassium acetate buffer (pH 7.4) at 35°, 36% of the RNA guanine was recovered as the 7-methyl derivative; no change in protamine titration could be observed.

Analogous results were obtained with s-RNA (Figs. 1a and b), except that in the absence of added ions much more 1,7-dimethylguanine and X were formed than in the former experiments. 1-Methylguanine, a minor constituent normally present in s-RNA, was absent in diazomethane-treated s-RNA (Fig. 1a) but present after reaction in the presence of Mg^{++} (Fig. 1b). In the absence of the latter ions, 1-methylguanine was apparently methylated by diazomethane to form 1,7-dimethylguanine. However, a comparison of the quantities of both compounds (Fig. 1a and b) indicates that 1,7-dimethylguanine is also formed by

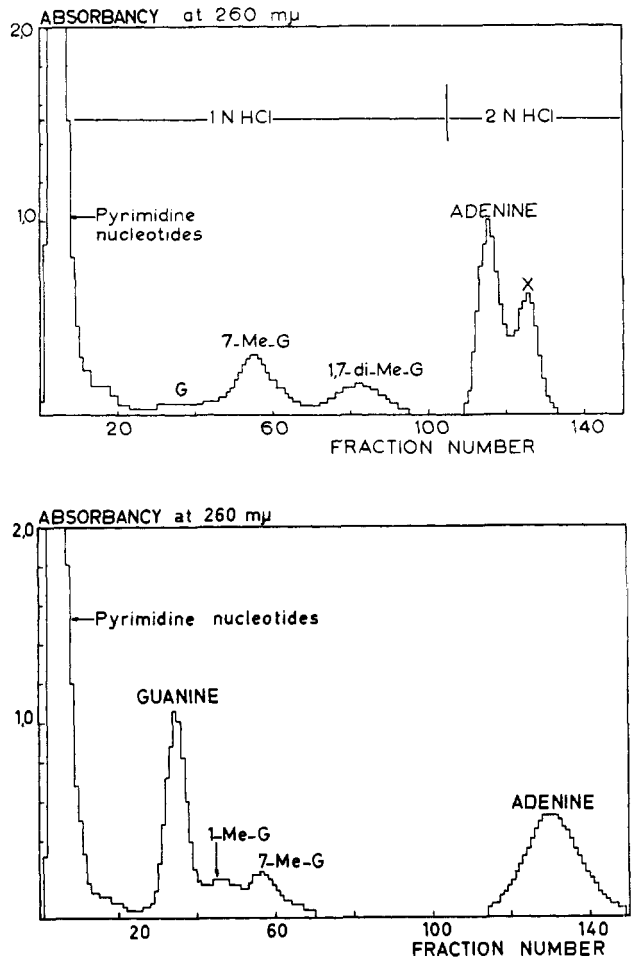


FIG. 1.—*Separation on Dowex-50-8 of the purines present in hydrolyzates of diazomethane-treated s-RNA.* Ten ml water containing 18 μmoles RNA-P (a) and 10 ml 0.01 M $MgSO_4$ containing 20 μmoles RNA-P (b) were incubated with four successive portions of each 0.26 mmole diazomethane in ml ether at 0°. Hydrolysis in 1 N HCl. Yield of purines in exp. a: 0.30 μmoles guanine, 2.20 μmoles 7-methylguanine, 1.32 μmoles 1.7-dimethylguanine, and 1.9 μmoles of compound X; G (total) = 5.72 μmoles; 0.32 mole G/mole RNA-P. Yield of purines in exp. b: 4.05 μmoles guanine, 0.82 μmole 1-methylguanine, and 1.33 μmoles 7-methylguanine; G (total) = 6.20 μmoles; 0.31 mole G/mole RNA-P. In experiment (b) compound X was not detected in the fractions eluted with 1 N HCl after adenine.

TABLE III

BASE COMPOSITION OF THE NONDIALYZABLE PART OF DIAZOMETHANE-TREATED s-RNA

Yeast s-RNA (138 μmoles P) in 25 ml of 0.05 M K-acetate buffer of *p*H 7.2 was treated with 4 successive portions of each 3.75 mmoles diazomethane in 8 ml ether at 0°. After removal of the ether the *p*H, which had risen to 8.5, was readjusted to *p*H 7.2 with acetic acid. The reaction mixture was dialyzed against 250-ml portions of twice-distilled water until no more ultraviolet-absorbing material was released. The contents of the dialysis bag were freeze-dried and dissolved in 5 ml 0.05 M NH_4-acetate, followed by gel filtration over Sephadex (see Fig. 2). Fractions 4–11 of control RNA and fractions 12–22 of the treated RNA were collected, freeze-dried, and dissolved in 5 ml water. Two ml of this solution containing 32.3 μmoles P in the case of treated RNA were acidified with concentrated HCl to 1 N and kept at 100° for 1 hour. The solution was diluted to 20 ml and applied to a column of Dowex-50 × 8 in the H^+ form. The column was eluted with 0.5 N HCl to give the pyrimidine nucleotides which were subsequently separated on Dowex-1 × 8, and with 1 N and 2 N HCl for separation of the purines. The base composition of the control RNA was determined following the separation by high-voltage paper electrophoresis in 0.05 M ammonium formate buffer, *p*H 3.5, according to Markham and Smith (1952), of the nucleotides obtained after hydrolysis in 0.3 N KOH for 18 hours at 37° (procedure I), and by two-dimensional paper chromatography following hydrolysis in 1 N HCl (II). The latter but not the former procedure separates G from 1-MeG.

Purines	Moles/ Mole P	Pyrimidine Nucleos(t)ides	Moles/ Mole P
G	0.011	1-Methyl cytidine	0.065
1-MeG	0.016	1-Methyl uridine	0.013
7-MeG	0.052	5-Hydroxymethylcytidylic acid	0.002
1,7-di-MeG	0.079	Cytidylic acid (2′ + 3′)	0.077
A + 1-MeA	0.096	1-Methyl uridylic acid (2′ + 3′)	0.093
X	0.194	Uridylic acid (2′ + 3′)	0.186
G (total including X)	0.352	C (total)	0.144
		U (total)	0.292
A (total):G (total)	10:35	C (total):U (total)	10:20

Control s-RNA

	(I)	(II)		(I)	(II)
A	0.194	0.191	C	0.257	0.290
G	0.335	0.320	U	0.214	0.180
1-MeG	—	0.019	C:U	10:8	
A:G	10:17				

a reaction other than methylation of 1-methylguanine (compare Discussion).

Compound X was eluted from the Dowex columns with 2 N HCl after the adenine peak, indicating that the basicity of X was higher than that of adenine. Compound X is a derivative of guanine since it was also detected, next to 1-methyl-, 7-methyl-, and 1,7-dimethylguanine, after treatment of guanosine-2′(3′)-phosphate with diazomethane in aqueous methanol. X was identified as follows. 7-Methylguanosine was prepared according to Bredereck (1947; compare Haines *et al.*, 1962) by reaction between triacetylguanosine and diazomethane in methanol, and was treated with 0.1 N NaOH at 37° during 30 minutes to open the imidazolyl ring (Lawley, 1957; Haines *et al.*, 1962). The 2-amino-4-(*N*-β-ribofuranosylamino)-5-*N*-methylformamido-6-hydroxypyrimidine thus formed was subsequently hydrolyzed during 1 hour in 1 N HCl at 100° to yield 2,4-diamino-5-methylamino-6-hydroxypyrimidine. A compound similar to X in chromatographic behavior after acid hydrolysis was also isolated from the reaction mixture according to Bredereck. The absorption spectra of this X and that isolated from diazomethane-treated s-RNA, and of the synthetic 2,4-diamino-5-methylamino-6-hydroxypyrimidine were recorded and compared with the data on the latter compound as obtained by Haines *et al.* (1962) following a milder hydrolytic procedure involving methanolic HCl at 0°. Only slight differences were noted in spectral properties between X and the synthetic compound, which were taken to mean that X consisted of 2,4-diamino-5-methylamino-6-hydroxypyrimidine and, possibly, the 1-methyl derivative of the latter arising from the opening of the imidazolyl ring of the 1,7-dimethylguanosine (phosphate moieties of RNA). The identity of X was ascertained by ring closure of the 4,5-diamino groups of its suspected components (15-minute reflux with formamide). Paper chromatography showed the presence of 7-methyl-, 1,7-dimethyl-, and some 1-methyl-guanine and guanine, the latter two resulting from the release of methylamine (instead of ammonia) during ring closure.

Breakdown of RNA.— Diazomethane-treated m-RNA (as indicated in Table II) was dialyzed against 10^{-3} M NaCl at room temperature for 24 hours. Organic phosphate (about 12% of the RNA-P) and UV-absorbing material, $\epsilon(P)_{260}$ = 12,000, appeared in the dialyzate, indicating the release of low molecular weight material; no breakdown of control m-RNA was observed.

After no further release of UV-absorbing material from the dialysis bag containing the methylated RNA could be detected, part of the latter material was freeze-dried and hydrolyzed with 70% perchloric acid, and the purines and pyrimidines were separated on Dowex-50 × 8. Cytosine (2.20 μmoles), guanine (0.84 μmole), 7-methylguanine (1.75 μmoles), 1,7-dimethylguanine (0.40 μmole), and adenine (1.10 μmoles) were recovered from the Dowex-50 × 8 eluates; uracil was not measured. The change observed in adenine-guanine(total) ratio of the diazomethane-treated nondialyzable RNA core (10:27), as compared with that of the untreated m-RNA (10:20), prompted a search for the base composition of the dialyzable material. To this end a large amount of s-RNA, prepared from baker's yeast, was treated with diazomethane in high excess as indicated in the legend of Table III. After termination of the reaction and dialysis, the dialyzate was concentrated and applied to a Dowex-1 × 8 column. The following components of the dialyzate were identified in the eluates: adenosine, 1-methyladenosine (tentative), and the 2′- and 3′-phosphates of cytidine, 1-methylcytidine, uridine, and 1-methyluridine. The E_{260} positive material, eluted by 0.5 N HCl, yielded no diphosphates after alkaline hydrolysis, but yielded nucleosides and nucleotides of adenine, cytosine, uracil, and the 1-methyl derivatives of the latter two com-

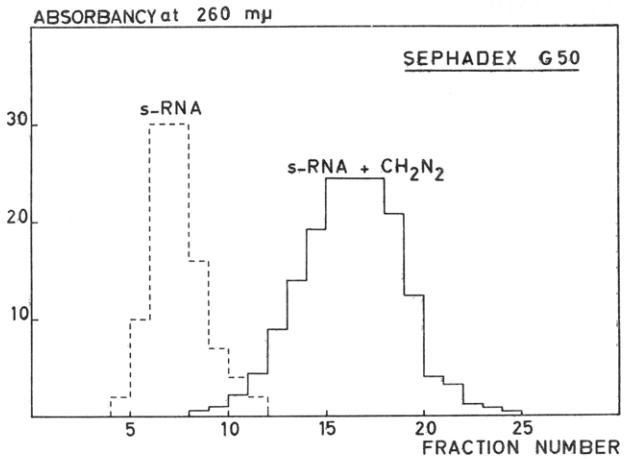


FIG. 2.—*Gelfiltration of control s-RNA from yeast and diazomethane-treated nondialyzable s-RNA over Sephadex G 50.* The Sephadex (30 × 2 cm) was equilibrated and eluted with 0.05 M ammonium acetate (*p*H 7.4.)

pounds, in about the same proportion as found in the previous fractions; the 0.5 N HCl fraction apparently consisted of di- and/or trinucleotides lacking the 5′-phosphate end-group. Neither guanine nor methylated guanine derivatives could be detected in any of the fractions. Of the nucleic acid bases (parent plus methylated) present in the dialyzable material, 45% consisted of adenine, 35% of cytosine, 11% of uracil, and, os roughly estimated, 9% of two unidentified bases.

Gel filtration over Sephadex G50 of the contents of the dialysis bag yielded RNA in fractions 12–22 (Fig. 2). Since the control s-RNA was recovered in fractions 4–11, and the release of the terminal CpCpA residues from the s-RNA by the action of diazomethane could not have been the only factor responsible for the former effect, it follows that the diazomethane-treated nondialyzable s-RNA was broken down to smaller molecules. Moreover, base analysis of the latter material confirmed that much more adenine- and cytosine-containing material had been released than could be accounted for by the terminal residues. The results of this experiment are listed in Table III; in terms of nucleic acid bases about 90% of the RNA-P used for the base analysis was recovered. The diazomethane-treated nondialyzable RNA was markedly deficient in adenine and cytosine.

Control and methylated s-RNA from rat liver were sedimented in the Spinco E analytical ultracentrifuge to give, respectively, a sharp symmetrical peak (Fig. 3a; s_{20w} = 4.0 S) and a more diffuse peak (Fig. 3b; s_{20w} = 2.7 S), thus presenting independent evidence for the breakdown of RNA.

The Reaction between Diazomethane and RNA in Ether

The residue left after removal of ether and diazomethane from an ethereal suspension of m-RNA, which had been incubated with an ethereal solution of diazomethane at 30° for one hour, was dissolved in potassium acetate buffer (*p*H 7.4) and dialyzed against water at room temperature. The dialyzate contained 21% of the organic phosphate originally present in the RNA preparation. After hydrolysis in 1 N HCl the base composition of an aliquot of the contents of the dialysis bag was determined (Table IV) by paper chromatography. A fifth spot was observed on the chromatograms of the methylated RNA hydrolyzate with an R_F of 1.17 relative to that of uridylic acid. This material was characterized as 1-methyluridine-

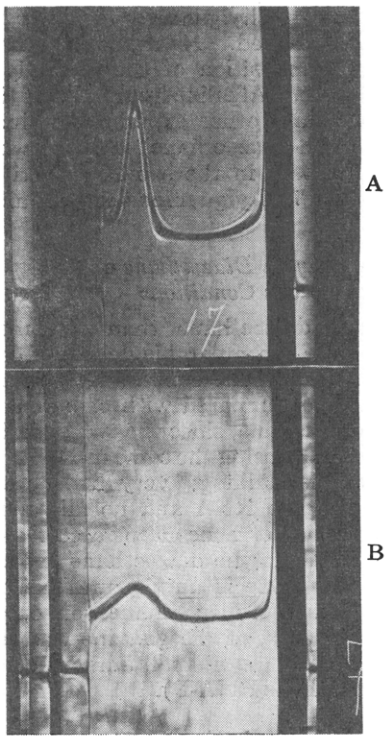


FIG. 3.—A. *Sedimentation pattern of control s-RNA in 0.2 M NaCl.* 41 minutes after reaching maximum speed (59,780 rpm). B. *Sedimentation pattern of diazomethane-treated RNA at 20° in 0.2 M NaCl,* 86 minutes after reaching maximum speed. (Protamine titration: 68% of control). *Procedure:* s-RNA (25 μmoles P), dissolved in 20 ml water, was treated with 2 portions of each 0.3 mmoles diazomethane in 1 ml ether at 20°. After the ether had been blown off, RNA was precipitated with 2 volumes of ethanol containing 2% K-acetate, was washed with 70% ethanol, and was dissolved in 1 ml 0.2 M NaCl. A similar amount of control s-RNA was treated in the same manner except for the presence of diazomethane.

2′(3′)-phosphate on the following account. After perchlorate hydrolysis of the remaining part of the methylated RNA, chromatography over Dowex-50 × 8 yielded in the 0.1 N HCl eluate (fractions 4–10) a compound, corresponding to about 40% of the total uracil present, that was identified as 1-methyluracil by its spectral properties (compare Shugar and Fox, 1952). Further elution with 1 N HCl yielded 1-methylguanine, corresponding to 25% of the total

TABLE IV

BASE COMPOSITION OF M-RNA AFTER TREATMENT WITH DIAZOMETHANE IN ETHER SUSPENSION

m-RNA (30 μmoles P) was precipitated with ethanol containing 2% K-acetate and washed with absolute ethanol and ether. The precipitate was suspended in 10 ml ether. A solution of 1.20 mmole CH_2N_2 in 2.5 ml ether was added and the mixture was stirred during 1 hour at 30°. After removal of the ether, the precipitate was dissolved in 0.04 M K-acetate buffer of *p*H 7.4 and dialyzed against twice-distilled water at room temperature for 24 hours. The base composition of the nondialyzable RNA was determined after hydrolysis in 1 N HCl by the paper chromatographic method of Kirby (1955).

m-RNA	Guanine	Cytosine	Uracil
	(moles/10 moles adenine)		
Control	18.9	16.4	9.0
CH_2N_2-Treated	19.0[a]	14.3	4.5[b]

[a] Including 1-methylguanine. [b] Excluding 1-methyluracil (3.5 moles).

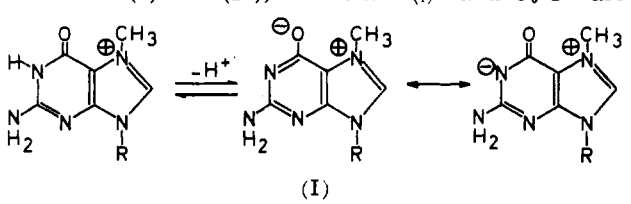
guanine. No 7-methylguanine or other methylated base could be detected. No significant changes in the relative base composition of the nondialyzable RNA core were evident. Alkaline hydrolysis of the dialyzate of the present and other experiments (including one carried out with diazoethane) also showed the four bases to be present in the same proportion as found in intact RNA; 7-alkylguanine was also absent in this fraction.

The Reaction between Diazoethane and RNA under Aqueous Conditions

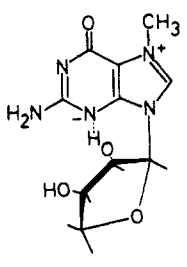
Much more diazoethane than diazomethane was needed to induce a comparable degree of change in m-RNA. The diazoethane experiment (experiment 2 of Table II) can be compared with experiment 1 carried out with diazomethane, since the two experiments were run simultaneously using the same m-RNA preparation. The difference is striking; very little change in the protamine-titratable RNA and no alkylated guanine, following the assay of nonconcentrated Dowex eluates, could be detected in the diazoethane experiment. In experiment 3 of Table II another m-RNA preparation and an 11-fold dose of diazoethane were employed. In this case about 30% of the guanine was recovered as 7-ethylguanine, next to a small amount of 1-ethylguanine (0.03 mole/mole RNA-P).

Discussion

If available protons are taken up by the nucleophilic diazoalkanes under formation of alkyldiazonium ions, the latter decompose to nitrogen and electrophilic alkylcarbonium ions which react with water or other bases of sufficient nucleophilicity to compete with water (Huisgen, 1955). The sites of reaction in RNA are the guanine and phosphate moieties. The formation of 7-methylguanine is due to a nucleophilic substitution by the tertiary $N_{(7)}$ of guanine at CH_3^+ according to a S_N1 mechanism and is in accord with the formation of similar products by the action of other alkylating agents (compare for review: Wheeler, 1962; Ross, 1962). Next to this main methylation product, lesser amounts of 1,7-dimethylguanine, but no 1-methylguanine, were formed. At neutral *p*H the $N_{(1)}$-H of guanine is largely in the undissociated form (pK_a = 9.2; Levene and Simms, 1925) and, therefore, unsuitable for reaction with CH_3^+, but the introduction of a methyl group at $N_{(7)}$ will result in a lowering of the acidic dissociation constant of $N_{(1)}$-H (pK_a = 7; Lawley and Brookes, 1961). Since 7-methylguanine will be present largely in the form of zwitterions of structures (I) and (II), both the $N_{(1)}^{\ominus}$ and C_6-$O^{\ominus}$ are



(I)



(II)

susceptible to alkylation by CH_3^+, leading to, respectively, 1,7-dimethylguanine and 2-amino-6-methoxy-7-methylpurine; the latter will escape detection since the methoxy is reconverted to a hydroxy group on acid hydrolysis (Ulbricht, 1962). The opening of the imidazolyl ring of a small amount of 7-methyl- and 1,7-dimethylguanine must have been brought about by the OH^- ions (or CH_3O^- in the case of methanol), arising from the capture of H^+ by diazomethane. Enhancement of ring opening by potassium acetate was observed in the present and in other experiments not reported here, and apparently resulted from the competition between the added anions of OH^- for CH_3^+, thus leaving a higher effective OH^- concentration; the amounts of X produced at 0° decreased in the order acetate buffer (*p*H 7.4) > water > phosphate buffer (*p*H 6.5), as shown by a comparative experiment.

The pattern of base methylation of RNA suspended in ether was completely different from that obtained under aqueous conditions and showed that in the former case diazomethane itself reacted with acidic groups. Taking this and the nonreactivity of the $N_{(1)}$-H of guanine under aqueous conditions into account, the presence of 1-ethylguanine (Table II) and of 1-methyluracil (Table III), following reaction between the diazoalkanes and RNA under aqueous conditions, is believed to have resulted from a direct reaction between the diazoalkane itself and the $N_{(1)}$H's at the interphase ether (containing diazoalkane)/water (containing RNA). In both experiments more ether had been present than in any other. The high excess of diazomethane and the almost complete alkylation of guanine (the complementary base of cytosine) is probably responsible for the formation of 1-methylcytosine in the experiment of Table III, the tertiary $N_{(1)}$ of cytosine being susceptible to alkylation by carbonium ions (compare Brookes and Lawley, 1962).

Conclusive evidence has come forward from the present experiments for the breakdown of RNA following treatment with diazomethane. Reaction of CH_3^+ with the secondary phosphate groups of RNA leads to phosphate triesters and some of the latter types are known to be readily hydrolyzed by water. (Barnard *et al.*, 1955, 1961). Hydrolysis in the present case may proceed via the same labile intermediate, involving the vicinal 2′-hydroxyl groups, as proposed by Brown and Todd (1955a and b), thus accounting for the presence of nucleoside-2′- and 3′-phosphates in the dialyzate of the diazomethane-treated RNA. Our results indicate that methylation of RNA under aqueous conditions leads to a nonrandom breakdown, whereas methylation in ether, followed by introduction of the reaction product into water, leads to a random breakdown. We suggest that the former phenomenon may be due, at least partly, to the presence of a strong internal hydrogen bond (II) between the 2′-hydroxyl group and the $N_{(3)}$ of 7-methylguanine or compounds X, which position shows a higher electron density than the corresponding ones in the other bases, thus hampering the 2′-hydroxyl group's participation in the formation of the cyclic intermediate during the spontaneous hydrolysis of the phosphate triesters (compare also Zamecnik, 1962, and Kaltreider and Scott, 1962). Although such hydrogen bonds may be less tight in the case of the 1,7-dimethylguanosine moieties, the present view may account for the absence of guanine-containing compounds in the dialyzate of RNA treated with diazomethane under aqueous conditions, and for the presence of such derivatives after diazomethane treatment of an ether suspension of RNA. In the latter case no 7-methyl-, but 1-methylguanine is formed, and the electron density at $N_{(3)}$

of 1-methylguanine is not markedly different from that of guanine.

As far as the present authors are aware, a breakdown of RNA by alkylating agents has not been observed previously. The difference between the present results and those of Brookes and Lawley (1961b,c) may be due to the various degrees of instability of the phosphate triesters depending on the nature of the alkyl groups introduced (Ross, 1962).

In the presence of Mg^{++}, the alkylation by diazomethane of both the RNA guanine and phosphate groups was markedly diminished, especially at low molar ratios diazomethane/RNA. This protection is probably caused by complex formation between Mg^{++} and phosphate groups, respectively, purine nitrogens (Shack *et al.*, 1953; Zubay and Doty, 1958; Hotta *et al.*, 1961).

Finally, the significance of the present results for the proposed mechanisms of action of the biologically active *N*-nitrosodialkylamines must be briefly assessed. Insofar as the diazoalkanes are considered to be the active metabolites of the former compounds (Hultin *et al.*, 1960; Magee and Farber, 1962; Heath, 1962; Emmelot *et al.*, 1962; Mizrahi and Emmelot, 1962), the results of the present model experiments are in complete agreement with those obtained with the parent compounds *in vivo*. Magee and Farber (1962) reported the presence of radioactive 7-methylguanine in nucleic acids isolated from the livers of rats treated with C^{14}-dimethylnitrosamine and observed (Magee, personal communication) much less incorporation of C^{14} in the case of C^{14}-diethylnitrosamine. The finding that much more diazoethane than diazomethane is needed to induce a comparable degree of alkylation at the $N_{(7)}$ of RNA guanine, may be due to the fact that diazoethane, being more basic than diazomethane, will capture a proton more rapidly and, consequently, penetrate less far than diazomethane from the lipophylic (in the model experiments: ether; *in vivo:* the membranes of the endoplasmic reticulum including the nuclear envelope) into the aqueous phase with a greater chance of being decomposed by water. Material to this argument for the *in vivo* situation is our previous finding (Mizrahi and Emmelot, 1962, 1963) that the microsomal *N*-de-ethylating enzyme, which initiates the conversion of diethylnitrosamine to diazoethane, is at least as active as the *N*-demethylating enzyme, if not more, indicating that at least as much diazoethane as diazomethane may arise *in vivo*. Since, according to the present view, the effective *in situ* concentration of $C_2H_5^+$ will be less than that of CH_3^+, the toxicity of diethylnitrosamine (diazoethane) will be less than that of dimethylnitrosamine (diazomethane), but the chance of obtaining a specific effect will be higher in the former than in the latter case. This is what has actually been observed, dimethylnitrosamine being much more toxic but less efficient as a liver carcinogen than diethylnitrosamine (compare Mizrahi and Emmelot, 1962, 1963).

Acknowledgments

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The Stability and Rearrangement of ε-*N*-Glutamyl-Lysines*

M. L. KORNGUTH, A. NEIDLE, AND H. WAELSCH

From the New York State Psychiatric Institute and the College of Physicians and Surgeons, Columbia University, New York City

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Two model compounds, ε-*N*-(α-glutamyl)-lysine and ε-*N*-(γ-glutamyl)-lysine, have been synthesized and their stability has been studied in acid solutions. At intermediate stages of hydrolysis each compound forms the isomeric ε-*N*-glutamyl-lysine through the same transient intermediate, ε-*N*-(αγ-glutamyl)-lysine. This cyclic imide has been isolated from the partial hydrolysate of ε-*N*-(α-glutamyl)-lysine and obtained by chemical synthesis. ε-*N*-(α-Glutamyl)-lysine is hydrolyzed more slowly at all HCl concentrations and yields optimally 25 mole per cent of the cyclic imide and 10 mole per cent of ε-*N*-(γ-glutamyl)-lysine in 11 N HCl. The γ-compound forms only up to 2 mole per cent of the cyclic imide in 11 N HCl, but its product of rearrangement, ε-*N*-(α-glutamyl)-lysine continues to persist throughout the course of hydrolysis because of its greater stability. Evidence of an α,γ-rearrangement has also been obtained with ε-*N*-(glycyl-α-L-glutamyl)-α-*N*-L-toluene-sulfonyl-lysine, its γ-isomer, α-glutamyl glycine and α-glutamyl alanine. It is concluded that in view of the possible α,γ-glutamyl rearrangement and γ-glutamyl bond instability, partial acid hydrolysis is not suitable for the investigation of the presence of an ε-*N*-glutamyl-lysine sequence in protein.

Work in this laboratory has shown that the enzyme transglutaminase catalyzes the hydrolysis of the amide group of protein-bound glutamine or its exchange with ammonia, hydroxylamine, and a variety of primary amines (Clarke *et al.*, 1959; Mycek *et al.*, 1959; Mycek and Waelsch, 1960). There is also tentative evidence indicating that the ε-amino group of protein-bound lysine can act as a replacing amine, resulting in the crosslinking of proteins (Neidle *et al.*, 1958). These and other experiments[1] suggest the possibility that an amide linkage, involving the γ-carboxyl of glutamic acid and the ε-amino group of lysine, may occur in native proteins. It has been suggested that a linkage between one of the carboxyl groups of glutamic acid and the ε-amino group of lysine occurs in collagen (Mechanic and Levy, 1959).

Prior to an investigation of the possible occurrence of an ε-*N*-(γ-glutamyl)-lysine sequence in proteins, a study of the relative stability of the γ-glutamyl bond under various hydrolytic conditions was undertaken. Such a study was indicated particularly since an α,γ-glutamyl rearrangement under conditions of hydrolysis could be visualized on the basis of behavior of aspartyl peptides (Swallow and Abraham, 1958). In addition, the cyclization of the glutamyl residue, a likely intermediate step in an α,γ-rearrangement, has been observed in nonaqueous systems (Sondheimer and Holley, 1954; Kovacs *et al.*, 1955; Clayton *et al.*, 1956).

In order to study glutamyl amide bond stability and possible rearrangement, two model compounds, ε-*N*-(α-glutamyl)-lysine and ε-*N*-(γ-glutamyl)-lysine were synthesized and their properties examined in acid solutions. The present communication describes the interconversion of the two model compounds via a common cyclic intermediate, ε-*N*-(αγ-glutamyl)-lysine, and presents a comparison of their rates of hydrolysis.

Experimental

α-N-Cbz-ε-N-(cbz-α-L-Glutamyl)-L-Lysine Dibenzyl Ester.[2]—Cbz-γ-L-glutamyl benzyl ester, prepared from γ-benzyl-glutamate (Hanby *et al.*, 1950) by the procedure described for cbz-α-glutamyl benzyl ester (Sachs and Brand, 1953), and α-cbz-L-lysine benzyl ester benzene sulfonate (Bezas and Zervas, 1961) were coupled by the mixed anhydride procedure (Boissonnas, 1951). Ethyl chlorocarbonate, 0.72 ml., was added dropwise to a cooled solution of cbz-γ-L-glutamyl benzyl ester, 2.8 g (0.0075 mole), and tri-*n*-butylamine, 1.78 ml (0.0075 mole), in 10 ml dimethylformamide–10 ml tetrahydrofuran. After 20 minutes at 0°, a chilled solution containing 4.0 g (0.0075 mole) of α-cbz-lysine benzyl ester benzene sulfonate and 1.78 ml of tri-*n*-butylamine (0.0075 mole), in 20 ml of the same solvent was added. The reaction mixture was kept for 30 minutes at 0° and for 2 additional hours at room temperature. The solvent was evaporated *in vacuo*. The residue was taken up in ethyl acetate, and the organic solution was extracted twice with 2 N HCl, once with water, twice with 5% K_2CO_3 solution, and with water. The ethyl acetate was evaporated *in vacuo*. The yield was 4.2 g (78%). The product, after recrystallization from ethanol-water, melted at 103–105°.

Anal. Calcd. for $C_{41}H_{45}O_9N_3$: C, 68.03; H, 6.27; N, 5.80. Found: C, 68.31; H, 6.38; N, 5.92.[3]

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[1] Korngut, S. E., and Waelsch, H., manuscript in preparation.

[2] Abbreviations used: cbz, carbobenzoxy; tosyl, *para*-toluene sulfonyl.

[3] We are indebted to Mr. T. Zelmenis for the nitrogen analyses. Carbon, hydrogen, and bromine analyses were carried out by Schwarzkopf Microanalytical Laboratory, Woodside, New York.