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Reaction of Cytidine with Ethylating Agents†

L. Sun and B. Singer*

ABSTRACT: The products of the reaction of cytidine and deoxycytidine with stoichiometric amounts of ethyl iodide in anhydrous solution, with or without K_2CO_3, were isolated and characterized. They included 3-ethylcytidine, N^4-ethylcytidine, 3,N^4-diethylcytidine, N^4,N^4-diethylcytidine, and possibly 3,N^4,N^4-triethylcytidine as well as small amounts of unidentified products. The extent of reaction was 25–50%. Data are presented for the ultraviolet absorption spectra and acid dissociation constants of these derivatives. 3-Ethylcytidine, N^4-ethylcytidine, and N^4,N^4-diethylcytidine were obtained after neutral aqueous reaction of cytidine and poly(C) with [^{14}C]-ethyl methanesulfonate and [^{14}C]diethyl sulfate. One of the unidentified derivatives was a major product of the reaction of cytidine with ethyl methanesulfonate and diethyl sulfate. The extent of total ethylation with these reagents did not exceed 0.5%. The reaction of cytidine in poly(dG)·poly(dC) with [^{14}C]ethyl methanesulfonate in neutral aqueous solution was very limited and the products were 3-ethylcytidine and N^4-ethylcytidine in approximately equal amounts. Methylation of cytidine and deoxycytidine with stoichiometric amounts of methyl iodide, in anhydrous solution containing K_2CO_3, led to the almost quantitative formation of 3-methylcytidine only. When the amount of reagent and reaction time were increased, 3,N^4-dimethylcytidine and 3,N^4,N^4-trimethylcytidine were found. Similarly, 3-methylcytidine, 3-ethylcytidine, and N^4-ethylcytidine could be exhaustively methylated or ethylated with the alkyl iodides to form the corresponding derivatives. The direct ethylation of the exocyclic nitrogen of cytidine has not previously been observed in neutral aqueous solution.

Alkylation of cytidine at the 3 position has been shown to be mutagenic in intact TMV (Singer and Fraenkel-Conrat, 1969b) and to cause mispairing when copolymers of cytidine with 3-methylcytidine or 3-ethylcytidine were used as templates by DNA-dependent RNA polymerase (Singer and Fraenkel-Conrat, 1970; Ludlum, 1970; Ludlum and Magee, 1972). The importance of these observations has increased as it has become more apparent that the generally predominant reaction of alkylating agents with the N-7 of guanosine is of little or no importance in mutagenesis or carcinogenesis (Swann and Magee, 1968, 1971; Loveless and Hampton, 1969; Ludlum, 1970; Lijinsky *et al.*, 1972; Goth and Rajewsky, 1972; Kleihues and Magee, 1973; Craddock, 1973).

In the previous paper of this series the reaction of guanosine with several ethylating and methylating agents was examined and it was concluded that the nature of the alkylating group (ethyl *vs.* methyl) played an important role in determining the site and rate of alkylation (Singer, 1972). This approach has been continued in the present study of the alkylation of cytidine in which we present data on the characterization of alkyl derivatives formed after nonaqueous reaction with ethyl iodide and methyl iodide, as well as the detection of products upon alkylation with the less reactive ethyl methanesulfonate ($EtMeSO_3$) and diethyl sulfate (Et_2SO_4) in neutral aqueous solution. To our knowledge, the only previously described derivatives of cytidine found after direct alkylation are 3-methylcytidine (Brookes and Lawley, 1962), 3-benzylcytidine (Brookes *et al.*, 1968), 3-(2-morpholinoethyl)deoxycytidine, and 3,N^4-di(2-morpholinoethyl)deoxycytidine (Price *et al.*, 1968).

The present paper presents data on the isolation and characterization of products of the reaction of cytidine and ethyl iodide in nonaqueous media. These were 3-ethylcytidine, N^4-ethylcytidine, 3,N^4-diethylcytidine, N^4,N^4-diethylcytidine and possibly 3,N^4,N^4-triethylcytidine. The products of the reaction of cytidine, poly(C), and poly(dG)·poly(dC) in aqueous solution at neutrality with ^{14}C-labeled diethyl sulfate and ^{14}C-labeled ethyl methanesulfonate were found to include 3-ethylcytidine, N^4-ethylcytidine, and N^4,N^4-diethylcytidine. The relationship of the new finding, that the amino group of cytidine is alkylated at neutrality, to possible biological effects is discussed.

Experimental Section

Reaction of Cytidine with Alkyl Iodides. (a) One-hundred milligrams of cytidine or deoxycytidine was dissolved in 1 ml of dimethyl sulfoxide containing 60 mg of anhydrous K_2CO_3. Alkyl iodide (25 μl) was added and the mixture was stirred at room temperature. After 2 hr, 20 mg of K_2CO_3 and 10 μl of alkyl iodide were added. Stirring was continued for an additional 2 hr. The reaction mixtures were filtered through a 2-cm Celite column in a Pasteur pipet. The filtrate was then subjected to electrophoresis and chromatography to separate the reaction products. (b) The reaction conditions

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TABLE I: R_F Values of Alkylated Cytidines.

	R_F[a]		
	Solvent A	Solvent B	Solvent C
Cytidine derivatives			
3-Ethyl-	0.70	0.24	0.69
N^4-Ethyl-	0.74	0.49	0.69
N^4,N^4-Diethyl-		0.70	
3,N^4-Diethyl-		0.55	
3,N^4,N^4-Triethyl-		0.31	
Cytidine	0.51	0.15	0.47
Unidentified X		0.37	
Unidentified Y		0.46 (0.24)[b]	
Unidentified Z		0.59 (0.29)	
3-Methyl-	0.60	0.11	
3,N^4-Dimethyl-		0.43	
3,N^4,N^4-Trimethyl-		0.17	
Deoxycytidine derivatives			
3-Ethyl-	0.76	0.39	0.36
N^4-Ethyl-	0.83	0.70	0.36
Deoxycytidine	0.65	0.28	0.20
Uridine derivatives			
3-Ethyl-		0.62	
3-Methyl-		0.44	
Uridine	0.73	0.30	

[a] See Experimental Section for details; solvent A, isopropyl alcohol–H_2O (70:30, v/v); solvent B, butanol–ethanol–H_2O (80:10:25, v/v); solvent C, isopropyl alcohol–HCl–H_2O (68:17:15, v/v). [b] R_F's in parentheses are for the corresponding methylated derivatives.

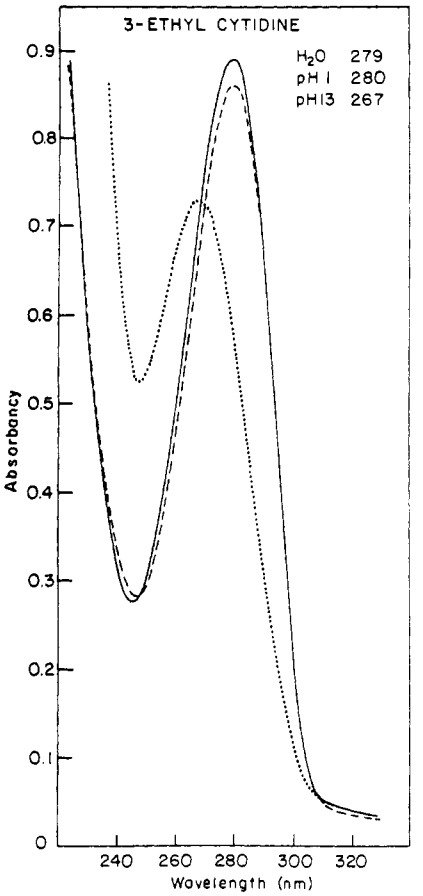

FIGURE 1: Ultraviolet absorption spectra of 3-ethylcytidine in water (—), 0.1 N HCl (- -), and 0.1 N KOH (· · · · ·). The absorption maximum at each pH is given in the figure.

were identical with (a) except that no K_2CO_3 was added. (c) Reaction mixtures prepared as in (a) were reacted with 400 μl of alkyl iodide in 2 ml of dimethyl sulfoxide containing 60 mg of K_2CO_3 for 18 hr at 37°. Alternatively 10 absorbancy units of the isolated reaction products from reaction (a), 3-methylcytidine, 3-ethylcytidine, and N^4-ethylcytidine were individually reacted with 35 μl of alkyl iodide in 0.1 ml of dimethyl sulfoxide containing 6 mg of K_2CO_3 at 37° for 18 hr.

Reaction of Cytidine, Poly(C), and Poly(dG)·Poly(dC) with [^{14}C]Ethyl Methanesulfonate and [^{14}C]Diethyl Sulfate. (a) Cytidine (50 mg) in 2 ml of H_2O was reacted at pH 7.0 (the pH was maintained by the addition of NaOH in a Radiometer pH-Stat) with 10 μl of [^{14}C]Et_2SO_4 (specific activity 0.02 Ci/mol) dissolved in 40 μl of dimethyl sulfoxide. After 8-hr reaction an additional 10 μl of [^{14}C]Et_2SO_4 in 40 μl of dimethyl sulfoxide was added and the reaction was continued for a total of 24 hr at room temperature. The cytidine was freed of excess reagent by repeated ether extraction. The ^{14}C-ethylated cytidine (as well as the ^{14}C-labeled nucleosides from the polymers in sections b and c) was mixed with a sufficient carrier amount of the reaction mixture from cytidine and ethyl iodide so that the various possible ethylated cytidines could be identified and quantitated after electrophoresis and chromatography.

The same reaction conditions were used for the reaction with [^{14}C]$EtMeSO_3$ except that the $EtMeSO_3$ (specific activity 1 Ci/mol) was dissolved in four volumes of ethanol.

(b) Poly(C) (1 mg; Miles Laboratories) was reacted with 2 μl of [^{14}C]Et_2SO_4 [specific activity 0.2 Ci/mol, dissolved in four volumes of dimethyl sulfoxide] in 0.4 ml of 0.5 M pH 7 cacodylate buffer for 18 hr at room temperature, the pH remaining constant. The polymer was precipitated three times with three volumes of ethanol at 0° and the final precipitate was washed with cold ethanol. The precipitate was dissolved in 0.2 ml of 0.01 M pH 7.6 Tris buffer and 0.1 mg of ribonuclease was added. After 1 hr at 37°, $MgCl_2$ was added to a concentration of 0.01 M and the polymer was further digested with 0.1 mg of snake venom phosphodiesterase and 0.1 mg of alkaline phosphatase for 18 hr at 37°. The digestion was judged complete since no absorbancy or counts were found at the origin when the digest was chromatographed. The same reaction and digestion conditions were used for the reaction with [^{14}C]$EtMeSO_3$ except the specific activity was 1 Ci/mol.

(c) Approximately 12 absorbancy units of poly(dG)·poly(dC) (Miles Laboratories) in 2 ml of H_2O was reacted at pH 7.0 in the pH-Stat with [^{14}C]$EtMeSO_3$ (specific activity 1 Ci/mol) under the same conditions as for cytidine in section a. The polymer was precipitated three times with three volumes of ethanol at 0° to free it of excess reagent, then depurinated by heating in 1 N HCl at 100° for 10 min. The depurinated polymer was centrifuged, washed several times with 0.1 N HCl, and redissolved in 0.1 ml of 0.5 M ammonium bicarbonate containing 0.01 M $MgCl_2$. DNase (50 μg) was added and the sample was digested at 37° for 30 min. Snake venom phosphodiesterase (25 μg) and alkaline phosphatase (30 μg) were added and the digestion was continued 4 hr at 37°. The same quantities of phosphodiesterase and phosphatase were again added and the digestion was allowed to continued 18 hr at 37°.

Methods for the Separation and Characterization of Alkylated Cytidines. Paper electrophoresis on Whatman No. 3MM in

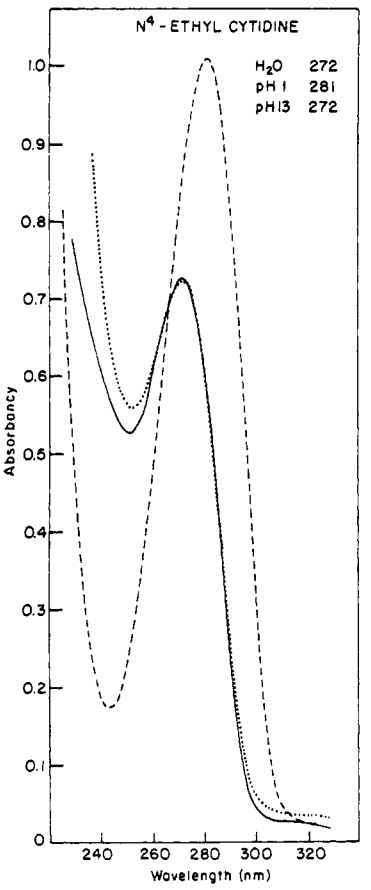

FIGURE 2: Ultraviolet absorption spectra of N^4-ethylcytidine in water (—), 0.1 N HCl (- -), and 0.1 N KOH (· · · · ·). The absorption maximum at each pH is given in the figure.

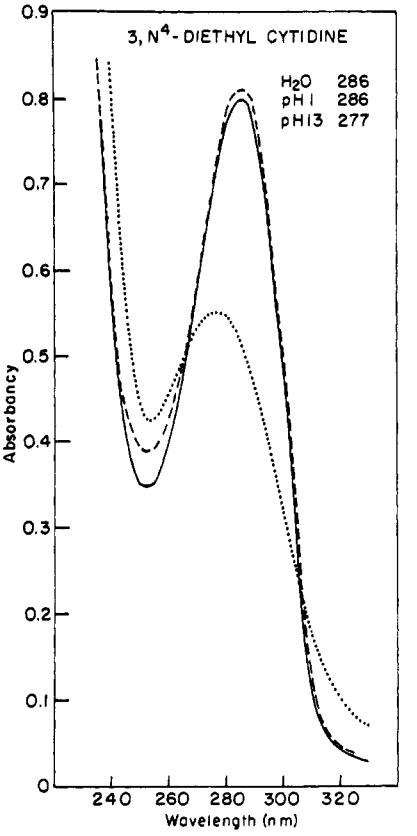

FIGURE 3: Ultraviolet absorption spectra of 3,N^4-diethylcytidine in water (—), 0.1 N HCl (- -), and 0.1 N KOH (· · · · ·). The absorption maximum at each pH is given in the figure.

0.05 M ammonium formate buffer (pH 5.7) separates the products with quaternary N and pK higher than 5.7 (the 3-, 3,N^4-, or 3,N^4,N^4-alkylcytidines) from cytidine and N^4- or N^4,N^4-alkylcytidine whose pK's are lower than 5.7.

After elution of the two areas of the electropherogram, termed "high pK" and "low pK," further separation was accomplished by descending paper chromatography on Whatman No. 3MM in either of two solvent systems. Solvent A was isopropyl alcohol–H_2O (7:3, v/v). Solvent B was butanol–ethanol–H_2O (8:1:2.5, v/v). A third solvent system C was also used occasionally as an additional means of resolving cytidine or deoxycytidine and their N^4-ethyl derivatives. Solvent C was isopropyl alcohol–HCl–H_2O (68:17:15, v/v). R_F values are given in Table I including those of 3-methyl- and 3-ethyluridine which are deamination products of 3-alkylcytidine.

Chromatography in butanol–0.8 M boric acid–ammonia (100:13.5:0.4) (Al-Arif and Sporn, 1972) was used to determine whether any derivatives were alkylated on the ribose moiety.

Paper electropherograms or chromatograms were observed under ultraviolet light to detect various derivatives. After elution with water by capillarity, the spectra were plotted using a Cary 15 recording spectrophotometer. HCl (6 N) was added to the same solution to a final concentration of 0.1 N and the spectra were replotted. KOH (6 N) was added to a final concentration of 0.1 N and the spectra were again plotted. The spectra of several ethylcytidines are shown in Figures 1–5.

A spectrophotometric method was used for the determination of the acid dissociation constants, similar to that of

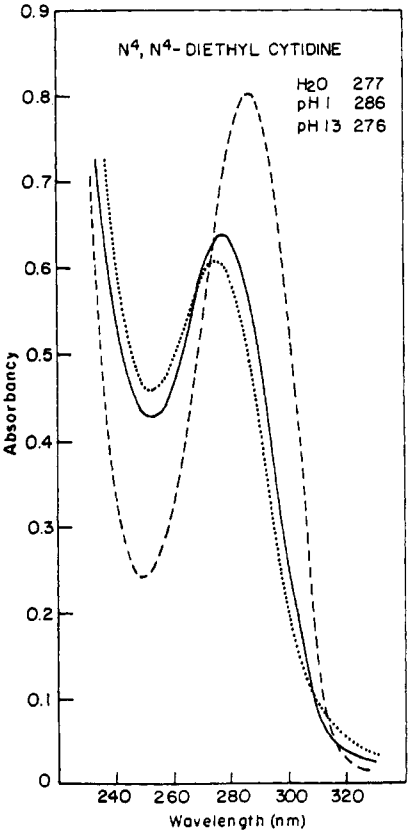

FIGURE 4: Ultraviolet absorption spectra of N^4,N^4-diethylcytidine in water (—), 0.1 N HCl (- -), and 0.1 N KOH (· · · · ·). The absorption maximum at each pH is given in the figure.

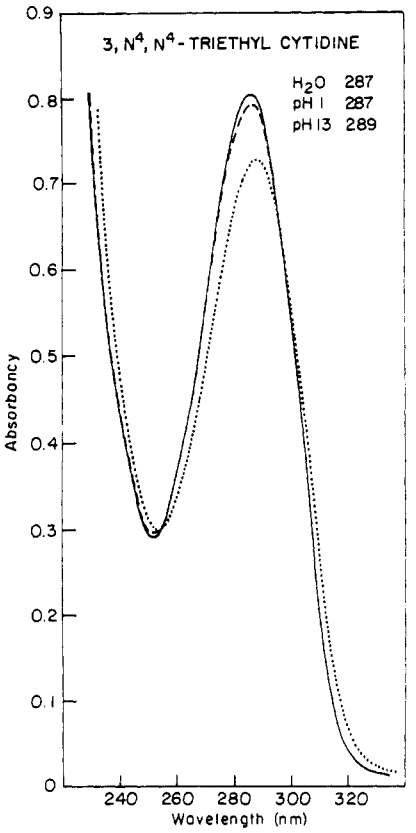

FIGURE 5: Ultraviolet absorption spectra of presumed 3,N^4,N^4-triethylcytidine in water (—), 0.1 N HCl (- -), and 0.1 N KOH (· · · · ·). The absorption maximum at each pH is given in the figure.

Singer (1972), except that the pH range was from pH 1 to 13. Apparent pK values are given in Table II.

Treatment with nitrous acid was used as a method of detecting primary amino groups. Alkylated cytidines were reacted with 1 N $NaNO_2$ in 25% acetic acid for 2 days at room temperature (Kušmierek *et al.*, 1973), or with 1 N $NaNO_2$ in 0.12 M pH 4 acetate buffer for 4 days at room temperature, and chromatographed in solvent B and the spectra of the uv-absorbing products were studied. Alkylcytidines were converted to the corresponding cytosines by digestion in 70% perchloric acid at 100° for 1 hr.

Materials. [^{14}C]Ethyl methanesulfonate (5.3 Ci/mol) was obtained from Amersham/Searle. [^{14}C]Diethyl sulfate (0.9 Ci/mol) was obtained from ICN. Both reagents were diluted with unlabeled reagent before use. 3-Methylcytidine was obtained from Calbiochem. All enzymes used were obtained from Worthington Biochemical Corp.

Results

Reaction of Cytidine and Deoxycytidine with Ethyl Iodide and Methyl Iodide. The reaction of cytidine with ethyl methanesulfonate or diethyl sulfate was first attempted since we wanted to compare the reaction products of $EtMeSO_3$ which is both mutagenic and carcinogenic (Singer and Fraenkel-Conrat, 1969c; Swann and Magee, 1969) with those of Et_2SO_4 which appears to be neither. However, in confirmation of Ludlum (1970), the rate and extent of reaction with $EtMeSO_3$ and Et_2SO_4 are extremely low so that it did not appear feasible to study these cytidine alkylations except with a radioactive label. For this purpose one needs known derivatives to use as markers. We therefore reacted cytidine (and deoxycytidine) with approximately stoichiometric amounts of ethyl iodide and methyl iodide in dimethyl sulfoxide containing K_2CO_3, conditions favoring considerably more reaction than obtainable with $EtMeSO_3$ and Et_2SO_4.

TABLE II: Acidic Dissociation Constants of Alkylcytidines.[a]

	pK_a
Cytidine derivatives	
3-Ethyl	8.4
N^4-Ethyl	4.2
3-Methyl	8.3
Deoxycytidine derivatives	
3-Ethyl	8.6
N^4-Ethyl	4.2

[a] See Experimental Section for experimental procedure.

After reaction with methyl iodide, 88% of the cytidine was converted to 3-methylcytidine, with 2.5% 3-methyluridine also found as a deamination product. Under the same conditions, ethyl iodide yielded 15% 3-ethylcytidine, 5% N^4-ethylcytidine, and smaller amounts of N^4,N^4-diethylcytidine, 3,N^4-diethylcytidine, possible 3,N^4,N^4-triethylcytidine, and unidentified products. The experimental evidence for the identification of these alkylcytidines is given in a later section of Results.

Although the evidence for direct aminoalkylation by ethylating as contrasted to methylating agents seemed clear, the alkylation of cytidine was repeated in dimethyl sulfoxide in the absence of K_2CO_3, conditions more similar to the aqueous conditions used by other investigators for alkylating cytidine (Brookes and Lawley, 1962; Brookes *et al.*, 1968; Price *et al.*, 1968; Singer and Fraenkel-Conrat, 1969a; Ludlum, 1970). This reaction proceeded more rapidly and yielded 48% 3-ethylcytidine and 2.3% N^4-ethylcytidine. In contrast, methylation under the same conditions yielded 96% 3-methylcytidine with no N^4-methylcytidine detected. The anhydrous neutral reaction with ethyl iodide also produced three additional unidentified products ranging from 3 to 0.4% of the total reaction. These three unidentified products (termed X, Y, and Z) were also obtained when cytidine was reacted with an excess amount of ethyl iodide. Their R_F values and spectral data are given in Tables I and III. Methylation produced two corresponding additional products (0.5 and 0.2%).

To test the possibility that N^4-ethylcytidine resulted from a rearrangement of 3-methylcytidine, in a manner analogous to that known for the formation of N^6- from 1-alkyladenosine, control experiments were performed by treating 3-methyl- and 3-ethylcytidine in dimethyl sulfoxide containing K_2CO_3 for up to 18 hr at 37°. No new products were detected except a small amount of 3-alkyluridine due to deamination. The deamination of 3-methylcytidine was slightly more rapid than that of 3-ethylcytidine. 3-Methyl- and 3-ethylcytidine as well as N^4-ethylcytidine were also completely stable in 1 N HCl at 100° for 1 hr.

The striking qualitative difference in the reactivity of the amino group of cytidine to ethylation as contrasted to methylation was investigated by reacting 3-methylcytidine and 3-ethylcytidine with excess amounts of alkyl iodide, all in dimethyl sulfoxide containing K_2CO_3, for 18 hr at 37°. These reaction conditions are extreme when compared to the reaction of stoichiometric amounts of alkyl iodides with cytidine at room temperature for 4 hr. Under these conditions, both 3-methylcytidine and 3-ethylcytidine reacted slowly with both

TABLE III: Spectral Identification of Alkylcytidines.[a]

Compound	Neutral λ_{max} (nm)	Neutral λ_{min} (nm)	Acidic λ_{max} (nm)	Acidic λ_{min} (nm)	Basic λ_{max} (nm)	Basic λ_{min} (nm)	Source[b]
Cytidine[c]			280	241	271	249	Kuśmierek *et al.* (1973)
3-Ethylcytidine	279	246	280	247	267	248	Experimental
3-Methylcytidine	278	243	278	243	267	245	Experimental
3-Methylcytidine	278	243	278	243	267	246	Calbiochem
3-Ethyldeoxycytidine	280	245	280	245	268	247	Experimental
N^4-Ethylcytidine[d]	272	252	281	244	272	253	Experimental
N^4-Methylcytidine[d]	270	250	280	242	273	250	Szer and Shugar (1966)
N^4-Ethyldeoxycytidine	270	251	279	247	272	253	Experimental
N^4-Methyldeoxycytidine	270	248	282	242			Wempen *et al.* (1961)
N^4,N^4-Diethylcytidine	277	252	286	249	276	252	Experimental
N^4,N^4-Dimethylcytidine[e]	278	238	287	246			Wempen *et al.* (1961)
N^4,N^4-Dimethylcytidine	278	239	285	246	279	247	Szer and Shugar (1966)
$3,N^4$-Diethylcytidine	286	253	286	252	277	253	Experimental
$3,N^4$-Dimethylcytidine	286	249	286	249	277	249	Experimental
$3,N^4$-Di(DEAE)deoxycytidine[f]			284	245			Price *et al.* (1968)
$1,3,N^4$-Trimethylcytosine			287	248			Price *et al.* (1968)
$3,N^4,N^4$-Triethylcytidine[g]	287	252	287	252	289	253	Experimental
Cytosine	276	238	276	238	281	249	P-L Biochemicals
3-Ethylcytosine			275	242	294	254	Experimental
3-Methylcytosine			274	240	294	250	Brookes and Lawley (1962)
N^4-Ethylcytosine			277	244	284	253	Experimental
N^4-Methylcytosine	277	240	277	240	286	256	Szer and Shugar (1966)
N^4,N^4-Dimethylcytosine	275	235–250	283	242	290	260	Szer and Shugar (1966)
N^4,N^4-Dimethylcytosine	272	245–250	282	243	289	258	Wempen *et al.* (1961)
3-Ethyluridine	262	245	261	245	264	247	Experimental
3-Methyluridine[h]	262	240	262	238	263	241	Experimental
3-Methyluridine			263	233	262	233	Hall (1971)
Unidentified ethyl-X	263	246	263	247	267	250	Experimental
Unidentified ethyl-Y	270	243	270	243	271	248	Experimental
Unidentified ethyl-Z	276	246	277	246	269	247	Experimental

[a] The spectra of the experimental and commercial samples were plotted in H_2O, 0.1 N HCl, and 0.1 N KOH. The corresponding literature data was obtained under a variety of neutral, acidic, and basic conditions. [b] Experimental samples were isolated from paper chromatograms after prior electrophoretic separation. [c] All *O'*-alkyl derivatives have identical spectra to that of cytidine (Tazawa *et al.*, 1972; Kuśmierck *et al.*, 1973). [d] N^4-Methyl-2'-*O*-methylcytidine (Robins and Naik, 1971) or N^4-methyl-2',3',5'-*O*-trimethylcytidine (Kuśmierek *et al.*, 1973) exhibit in acidic and neutral solution an λ_{max} 280–281 nm and in basic solution, λ_{max} 271 nm. [e] N^4,N^4-Dimethyl-2'-*O*-methylcytidine (Robins and Naik, 1971) exhibits in acidic solution λ_{max} 287 nm and in basic solution, λ_{max} 278 nm. [f] DEAE is the abbreviation for 2-diethylaminoethyl. [g] This compound was isolated in extremely small amounts and the identification is tentative. [h] Obtained by deamination of authentic 3-methylcytidine.

alkyl iodides to form small amounts of $3,N^4$-di-, and presumed $3,N^4,N^4$-trialkyl derivatives. N^4-Ethylcytidine also reacted slowly with both alkyl iodides to form the $3,N^4$-, N^4,N^4, and presumed $3,N^4,N^4$ derivatives.[1] These experiments indicate that both the 3 and N^4 positions are available to both methylation and ethylation but that owing to the high reactivity of the 3 position to methylation this group is preferentially methylated, while the comparative slowness of 3-ethylation permits substantial reaction at the less preferred amino group.

Reaction of Cytidine, Poly(C), and Poly(dG)·Poly(dC) with $[^{14}C]Et_2SO_4$ *and* $[^{14}C]EtMeSO_3$. With the information and markers gained from the study of the reaction of alkyl iodides with cytidine, it was possible to study the products of reaction of cytidine, as monomer or polymer, with the biologically interesting ethylating agents, diethyl sulfate and ethyl methanesulfonate. These reactions were all performed in neutral aqueous solution, the pH being maintained at 7.0 in a pH-Stat or by cacodylate buffer. After removal of excess reagent and, in the case of polymers, enzyme digestion to nucleosides, enough of the cytidine–ethyl iodide reaction mixture was added to act as unlabeled ultraviolet-absorbing markers, and the various alkylcytidines were separated by chromatography, either alone or after electrophoresis.

The yields of each derivative from ethylated cytidine and poly(C) as per cent of total recovered ^{14}C are shown in Table IV. Figure 6 shows the radioactivity profiles, from a

[1] When cytidine is reacted with ethyl iodide under the same conditions, a maximum of 5% ribose alkylation is found in addition to the base alkylated derivatives.

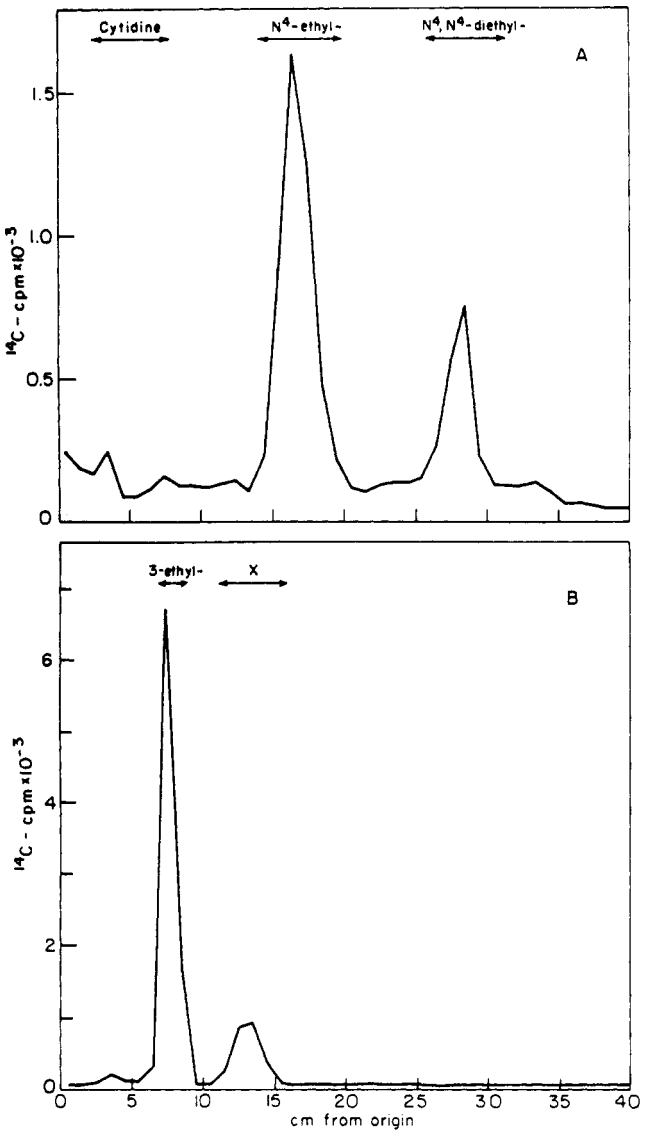

FIGURE 6: Radioactivity profiles of chromatograms of electrophoretic fractions of cytidine reacted with [^{14}C]EtMeSO$_3$. Cytidine (50 mg) was reacted at pH 7.0 with a total of 20 μl of [^{14}C]EtMeSO$_3$ (specific activity 1 Ci/mol) for 24 hr at room temperature. Paper electrophoresis of 20% of the ether-extracted solution was in 0.05 M ammonium formate buffer at pH 5.7. (A) Twenty-five per cent of the electrophoretic "low pK" fraction was chromatographed in solvent B. (B) Three per cent of the electrophoretic "high pK" fraction was chromatographed in solvent B. The chromatograms were cut into 1-cm strips and counted in toluene containing Omnifluor (14.7 g/3 l. of toluene) in a Beckman liquid scintillation counter. Double arrows indicate the positions of ultraviolet absorbing internal markers.

typical experiment, of the reaction of cytidine with [^{14}C]-EtMeSO$_3$. N^4-Ethylcytidine and N^4,N^4-diethylcytidine (from the "low pK" region of electrophoresis) are well resolved in solvent B (Figure 6A). Similarly, 3-ethylcytidine and an unknown derivative X (from the "high pK" region of electrophoresis) are resolved (Figure 6B). While the reaction of cytidine with either EtMeSO$_3$ or Et$_2$SO$_4$ yields approximately the same amounts of N^4-ethylcytidine, reaction with EtMeSO$_3$ produced more N^4,N^4-diethylcytidine than that with Et$_2$SO$_4$ (Table IV). The major product of high pK is 3-ethylcytidine. The other product of high pK differed chromatographically in solvent B from 3,N^4-diethyl-, 3,N^4,N^4-triethylcytidine, and 3-ethyluridine. However it was coincident with one of the three unidentified products (X) obtained when cytidine was

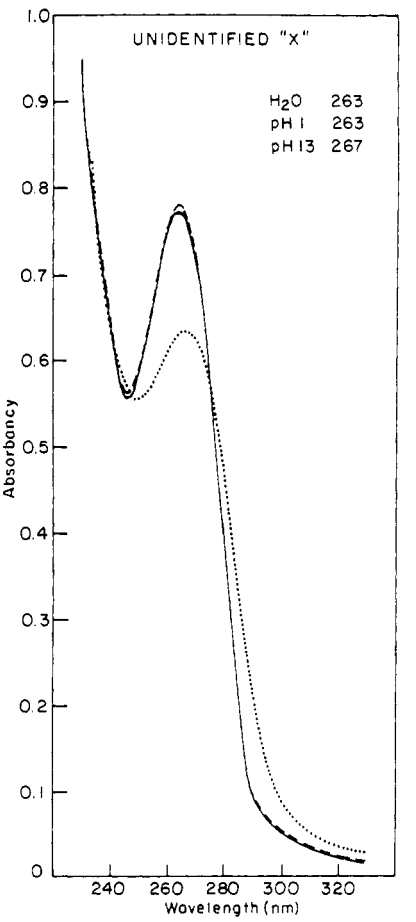

FIGURE 7: Ultraviolet absorption spectra of unidentified ethylcytidine "X" in water (—), 0.1 N HCl (- -), and 0.1 N KOH (. . . . .). The absorption maximum at each pH is given in the figure.

reacted with excess ethyl iodide in either aqueous or anhydrous neutral solution. This unidentified product, not found after reaction with methyl iodide, has a distinctive ultraviolet absorption spectrum, as shown in Figure 7. Its R_F is also give in Table I. The high pK (pK > 5.7) excludes its being 3-ethyluridine or any other uridine derivative and its spectrum and pK exclude derivatives alkylated solely on the 5 or 6 positions. No data have been found for O^2-alkylcytidine and the possibility remains that X may be alkylated on this site.

The radioactivity profile of the chromatographed enzyme digest from the reaction of poly(C) with [^{14}C]EtMeSO$_3$ without prior electrophoresis is shown in Figure 8. All four products (3; X; N^4; and N^4,N^4) are well separated. Because of the low reactivity of the polymer a large excess of reagent was used. The monosubstituted derivatives (3 and N^4) can, in this case, further react to give increased amounts of disubstituted derivatives, so that relatively less N^4-cytidine was found and more N^4,N^4-diethylcytidine than shown in the profiles of the reaction of cytidine with the same reagents (Figure 6). Similarly, a comparison of the products of the reaction of cytidine and poly(C) with [^{14}C]Et$_2$SO$_4$ shows more N^4,N^4-diethylcytidine formed in poly(C) (Table IV). With either reagent acting on cytidine, poly(C), or poly(dG)·poly(dC), N^4-ethylcytidine was found in addition to the major product, 3-ethylcytidine. Approximately 14–18% of the total ethylation of cytidine in poly(C) was on the exocyclic amino group (Table IV), a higher proportion than found after reaction with ethyl iodide in neutral dimethyl sulfoxide. The extent of ethylation by [^{14}C]Et$_2$SO$_4$ or [^{14}C]EtMeSO$_3$ was between 0.3 and 0.5% as calculated by specific radioactivity

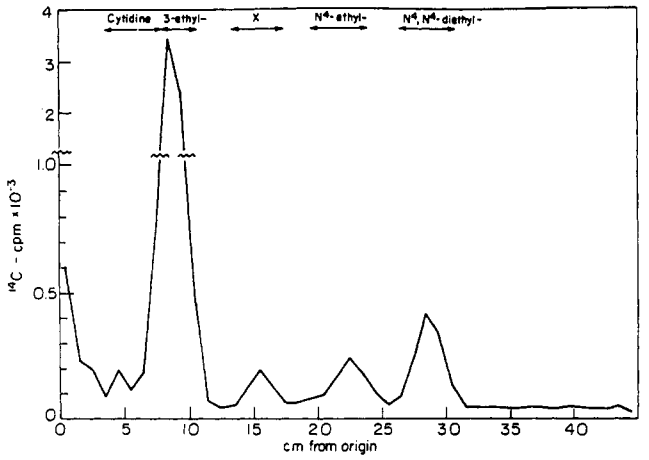

FIGURE 8: Radioactivity profile of a chromatogram of poly(C) reacted with [^{14}C]EtMeSO$_3$. See text for experimental details. One-third of the sample, after repeated alcohol precipitation, was enzyme digested to nucleosides and chromatographed in solvent B. Double arrows indicate the positions of ultraviolet-absorbing internal markers. Radioactivity was determined as in Figure 6.

while the reaction with ethyl iodide was about 50%. Thus, even when little cytidine was ethylated, the N^4 position was substituted.

Poly(dG)·poly(dC) was reacted with [^{14}C]EtMeSO$_3$ and, after depurination to remove both alkylated and nonalkylated guanine residues, the remaining poly(dC) chain was enzyme digested. There was very little alkylation of C compared to that of G (0.2% of the total). However of the radioactivity due to C-ethylation almost half was coincident with the N^4-ethyldeoxycytidine marker. It was previously shown by us that in poly(G)·poly(C) the methylation of the 3 position of cytidine is largely suppressed due to its involvement in hydrogen bondings (Singer and Fraenkel-Conrat, 1969a). However, it now appears that the N^4 position is reactive toward ethylating agents.

Identification of Ethylcytidines. The identification of 3-ethylcytidine (and 3-ethyldeoxycytidine) was based on its almost identical properties when compared to authentic 3-methylcytidine in the following respects: (1) uv spectrum of the nucleoside (Figure 1); of the base obtained by perchloric acid digestion; and of the ethyluridine obtained by deamination (Table III); (2) pK_a of 8.4 (Table II) in agreement with previous values of 8.3–8.9 for 3-methylcytidine (Brookes and Lawley, 1962; Szer and Shugar, 1966; Hall, 1971). The chromatographic behavior in solvents containing borate, which distinguishes base alkylation from ribose alkylation (Al-Arif and Sporn, 1972), showed that no ribose alkylation had occurred. Nitrous acid deamination further showed that this compound possessed a free primary amino group, thus not being additionally N^4 ethylated. Neither 3-ethyl- nor 3-methylcytidine was deaminated with HNO_2 to 3-alkyluridine as rapidly as cytidine was deaminated to uridine.

The identification of aminoethylated cytidines, possibly the most unexpected result of ethylation under our conditions, was based on the following considerations. The spectra of our presumed N^4-ethyl- and N^4,N^4-diethylcytidines (Figures 2 and 4) corresponded to the published spectra for the synthetic methyl derivatives (Szer and Shugar, 1966; Wempen *et al.*, 1961) (Table III). The spectrum of N^4-ethylcytosine, the product of perchloric acid digestion of the nucleoside, is very similar to that of authentic N^4-methylcytosine (Szer and Shugar, 1966) (Table III). It can be noted that the λ_{max} and λ_{min} of N^4-methylcytidine is almost indistingiushable from that of

TABLE IV: Distribution of Ethylated Cytidines Found after Reaction with [^{14}C]Ethyl Methanesulfonate or [^{14}C]Diethyl Sulfate at Neutrality.

Cytidine Derivatives	% Total Ethylation[a] Cytidine, EtMeSO$_3$	Cytidine, Et$_2$SO$_4$	Poly(C), EtMeSO$_3$	Poly(C), Et$_2$SO$_4$
3-Ethyl-	69	69	78	82
Unknown X	18	20	4	4
N^4-Ethyl-	9	11	7	1
N^4,N^4-Diethyl-	4	1	11	13

[a] The total ethylation of cytidine, as monomer or polymer, is between 0.3 and 0.5% as calculated from the specific radioactivity. See Experimental Section for conditions of reaction of cytidine and poly(C) with [^{14}C]EtMeSO$_3$ and Et$_2$SO$_4$.

cytidine (and the same is true for N^4-methylcytosine and cytosine). However our compound can not be unreacted cytidine since it is labeled in experiments with [^{14}C]EtMeSO$_3$ or [^{14}C]Et$_2$SO$_4$ and moreover differs in R_F from cytidine in three solvent systems (Table I). The compound was not a ribose alkyl derivative on the basis of its chromatography in a borate containing solvent. Lastly, substitution of the amino group was verified by the fact that it was not deaminated after nitrous acid treatment. Nitrous acid however caused the formation of small amounts of other products, possibly nitroso compounds.

When N^4-ethylcytidine was further ethylated with ethyl iodide (see Experimental Section) one of the products, of p$K < 5.7$ (thus lacking a quaternary nitrogen) not found as a product of further alkylation of 3-alkylcytidine, corresponds in spectrum and chromatographic behavior to the N^4,N^4-diethylcytidine separately isolated from the reaction of cytidine with the various ethylating agents.

The assignment of the structure, 3,N^4-dialkylcytidine, is based on the fact that it is a product from the further ethylation of N^4-ethylcytidine and has a quaternary nitrogen (p$K > 5.7$) showing that substitution of the 3 position has occurred. It is also, as expected, a product of further alkylation of 3-alkylcytidine. Its spectrum (Figure 3) is in accord with the limited data available for 3,N^4-di(2-diethylaminoethyl)deoxycytidine and 1,3,N^4-trimethylcytosine (Price *et al.*, 1968) (Table III). A very minor product, also quaternary, of the ethylation of either 3-alkylcytidine or N^4-ethylcytidine was presumed to be 3,N^4,N^4-trialkylcytidine. Its spectrum is shown in Figure 5. No comparative spectral data have been found.

Discussion

Cytidine was directly alkylated to an appreciable extent on the exocyclic N^4-amino group when reacted with a stoichiometric amount of ethyl iodide in either alkaline or neutral dimethyl sulfoxide. Under the same conditions there was no detectable N^4-methylation with methyl iodide. However a large excess of methyl iodide for long reaction times did lead to the formation of small amounts of 3,N^4-dimethylcytidine and possibly 3,N^4,N^4-trimethylcytidine. Thus, we are dealing with a matter of relative rates of alkylation of the 3 and N^4 positions, rather than absolute differing specificities of ethyl iodide and methyl iodide.

Previously N^4-methylation or ethylation of cytidine with alkyl iodides or alkyl sulfates has been found only under

strongly alkaline conditions primarily favoring ribose alkylation (Kuśmierek and Shugar, 1971; Kuśmierek *et al.*, 1973; Giziewicz and Shugar, 1973), or after exhaustive treatment with diazomethane (Szer and Shugar, 1966). Our usual anhydrous reaction in the presence of K_2CO_3 (room temperature, 4 hr) yielded little, if any, ribose alkyl derivatives although under aqueous alkaline conditions where an N^4-methylcytidine has been found, the major product was 2′-*O*-methylcytidine (Tazawa *et al.*, 1972) or other mono-, di-, or tri-*O*′-alkylcytidines (Kusmierek *et al.*, 1973). Moreover, N^4-ethylcytidine was also found in neutral anhydrous solution, although to a lesser extent. One other instance of N^4 substitution is reported by Price *et al.* (1968) who, on extensive neutral reaction of deoxycytidine with morpholine mustard (10-fold excess of reagent, 40°, 48 hr), found the 3,N^4-dialkyl derivative as a minor product.

Ethylation of cytidine, as monomer or polymer, with both Et_2SO_4 and $EtMeSO_3$ in neutral aqueous solution was slow and the total extent in our experiments was 0.3–0.5% in contrast to 20–100% reaction obtained with the anhydrous alkyl iodides. Surprisingly, at such low levels of reaction both Et_2SO_4 and $EtMeSO_3$ ethylated the N^4 position to a greater proportion than did ethyl iodide in neutral anhydrous solution. In cytidine and poly(C) reacted with Et_2SO_3 and $EtMeSO_3$, 12–18% of the total ethylation was found as N^4-ethylcytidine and N^4,N^4-diethylcytidine. No 3,N^4-diethyl- or 3,-N^4,N^4-triethylcytidine was detected although these derivatives were products of the extensive reaction of cytidine with ethyl iodide. It should be pointed out that N^4-ethylation of cytidine is, under all conditions, much lower than 3-ethylation and that 3-alkylcytidine is not readily alkylated at N^4 by alkyl iodides. The preferred site of reaction is the 3 position, followed by reaction at the exocyclic amino group which is then dialkylated to N^4,N^4 more readily than is 3-alkylcytidine further alkylated to the 3,N^4 derivative. One might then make the assumption that the quaternary structure of 3-ethylcytidine, leading to a pK of 8.4, diminishes the reactivity of the exocyclic amino group.

The possibility that the aminoethylation was due to a rearrangement of 3-alkylcytidine was considered. Ueda and Fox (1964) found a rearrangement of 3-methylcytosine to N^4-methylcytosine in acetic anhydride involving acetylation of both the 1 and N^4 positions. However Kuśmierek and Shugar (1971) exclude rearrangement in alkali since they obtain an N^4,N^4-dialkylated cytosine with dimethyl sulfate in 10 N NaOH. In our experiments we have found 3-alkylcytidine to be unchanged after incubation in dimethyl sulfoxide containing K_2CO_3, conditions under which, in the presence of ethyl iodide, about 20% of ethylation is on the amino groups of cytidine.

The reactivity of cytidine in poly(dG)·poly(dC) is extremely low compared to poly(C). However, in line with expectation, relatively more N^4-ethylation occurred, amounting to half the alkylation, the rest being 3-ethylation. The fact that the amino group of cytosine reacts with ethylating agents at neutrality to a significant extent has not previously been recognized. Similar reactivity of the exocyclic amino group of adenosine has also recently been reported (Singer *et al.*, 1974).

Since poly(C) and poly(dG)·poly(dC) can be considered as models for cytidine in biological systems we can examine the possible biological consequences of N^4-ethylation. It has previously been shown that the mutagenicity of the 3-methylation of cytidine could be demonstrated both biologically, in TMV, and by the *in vitro* template activity of copolymers of cytidine and 3-methylcytidine (Singer and Fraenkel-Conrat, 1969b). Similar template studies have not yet been performed with cytidine polymers containing N^4-substituted cytidine residues. However the binding studies of Brimacombe and Reese (1966) suggest, in support of what might be surmised on chemical grounds, that N^4-methylcytosine residues do not represent obvious mutagenic events, but that N^4,N^4-dimethylcytosine residues are unable to bind to G or I residues and might thus resemble the 3-alkylated cytosine residues in allowing the incorporation of any base. However, we find no significant differences in the extent of N^4-ethylation in cytidine and poly(C) by Et_2SO_4 and $EtMeSO_3$, while finding a significant difference in their mutagenicity toward TMV and TMV-RNA (Singer and Fraenkel-Conrat, 1969c). It is not excluded by these data that $EtMeSO_3$ and Et_2SO_4 could differ in their reactivity toward the exocyclic amino group of cytidine in TMV-RNA, accounting for the observed difference in biological effect.

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Reaction of Adenosine with Ethylating Agents†

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ABSTRACT: The products of ethylation of adenosine with diethyl sulfate and ethyl methanesulfonate in neutral aqueous solution are 1-ethyladenosine, N^6-ethyladenosine, and 7-ethyladenosine. In addition, lesser amounts of unidentified compounds are found which may be degradation products of 3-ethyladenosine. Reaction with anhydrous ethyl iodide or with methylating agents alkylates the 1 and 7 positions but not the exocyclic amino group. The new finding that up to half of the total ethylation was direct substitution of the N^6 position is paralleled by the recent finding that cytidine is also directly ethylated at the N^4 position (Sun, L., and Singer, B. (1974), *Biochemistry 13*, 1905). 7-Ethyladenosine and N^6,7-dialkyladenosine (obtained from the alkylation of N^6-methyladenosine) were isolated and characterized for the first time. They are both brightly fluorescent under ultraviolet light and the imidazole ring is rapidly opened in neutral or alkaline solution. The relative amount of 7-alkylation by both ethylating and methylating agents is higher than previously reported and it is suggested that the great lability of 7-alkyladenosine, like that of 3-alkyladenosine, has led to erroneously low values for alkylation at these sites. The extent of ethylation of poly(A) and poly(A)·poly(U) was extremely low and even the use of ^{14}C-labeled reagents did not permit detection of the products of their reaction with poly(A)·poly(U). N^6-Ethyladenine and a lesser amount of 3-ethyladenine were identified as products of the reaction of [^{14}C]diethyl sulfate and [^{14}C]-ethyl methanesulfate with poly(A), although the presence of other products was not excluded.

Adenosine, guanosine, and cytidine are the three nucleosides generally alkylated by mutagenic or carcinogenic alkylating agents. In order to understand the mechanism of the biological effect of alkylation on RNA and DNA, and particularly the differing effects of their methylation and ethylation, we have first isolated and characterized the products of ethylation of nucleosides by reagents such as ethyl iodide, diethyl sulfate, methyl methanesulfonate, and ethyl methanesulfonate. The products of ethylation of guanosine and cytidine have been discussed in the previous two papers (Singer, 1972; Sun and Singer, 1974). This paper deals with the isolation and characterization of various methyl- and ethyladenosines after reacting adenosine with ethyl iodide, diethyl sulfate (Et_2SO_4), dimethyl sulfate (Me_2SO_4), methyl methanesulfonate ($MeMeSO_3$), and ethyl methanesulfonate ($EtMeSO_3$).

No ethyladenosines have thus far been characterized, although some ethyladenines have been described, either obtained directly from the reaction of $EtMeSO_3$ with adenine (Pal, 1962), or from neutral hydrolysis of diethyl sulfate treated DNA (Lawley and Brookes, 1964), or ethyl methanesulfonate treated deoxyadenosine, yeast RNA, salmon sperm DNA, T2 phage DNA (Lawley and Brookes, 1963), and poly(A) (Ludlum, 1969). These include 1-ethyl-, 3-ethyl-, 7-ethyl-, and 9-ethyladenine. In addition N^6-ethyl- and N^6,N^6-diethyladenine have been synthesized by Elion *et al.* (1952).

The literature on methylation of adenine, adenosine and nucleic acids is extensive and many mono-, di-, and trimethyl derivatives have been either isolated from direct methylation or synthesized. There are two expected derivatives which have been described only as the base, not as the nucleoside. These are the 3- and 7-substituted adenines, predicted to be labile as the nucleoside (Leonard *et al.*, 1965).

The present paper presents data on the isolation and characterization of 1-ethyladenosine, N^6-ethyladenosine, 7-ethyladenosine, and N^6,7-dialkyladenosine and makes some comparisons of the relative reactivities of the 1, 3, N^6, and 7 positions of adenosine toward dimethyl sulfate, diethyl sulfate, and ethyl methanesulfonate.

Experimental Section

Reaction of Adenosine with Dimethyl Sulfate, Diethyl Sulfate, Methyl Methanesulfonate, and Ethyl Methanesulfonate. Several sets of conditions were used, depending on the purpose of the experiment. To obtain markers for experiments using ^{14}C-labeled reagents, 500 mg of adenosine in 6 ml of 0.4 M ammonium bicarbonate or pH 7.2 cacodylate buffer were reacted with 0.2 ml of Et_2SO_4 or $EtMeSO_3$ at 30°. After 8-hr stirring, 2.5 ml of ammonium bicarbonate or pH 7.2 cacodylate buffer and 0.1 ml of Et_2SO_4 or $EtMeSO_3$ were added and stirring was continued 18 hr. The final pH of these reaction mixtures was pH 3–4.

To compare the reaction products of the neutral reaction of Me_2SO_4 and Et_2SO_4 with adenosine at room temperature, 50 mg of adenosine in 2 ml of H_2O was adjusted to pH 7.0 in a Radiometer pH-Stat; 20 μl of Me_2SO_4 or Et_2SO_4 was added and the pH was maintained at 7.0 with 1 N NaOH. After 5 hr

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