The Specificity of Different Classes of Ethylating Agents toward Various Sites in RNA†

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ABSTRACT: The alkyl products of neutral *in vitro* ethylation of TMV-RNA by [^{14}C]diethyl sulfate, [^{14}C]ethyl methanesulfonate, and [^{14}C]ethylnitrosourea have been determined and found to differ significantly depending on the ethylating agent. Diethyl sulfate and ethyl methanesulfonate ethylate the bases of TMV-RNA in the following order: 7-ethylguanine > 1-ethyladenine, 3-ethylcytidine > 7-ethyladenine, 3-ethyladenine, O^6-ethylguanosine, 3-ethylguanine. Ethyl methanesulfonate was more specific for the 7 position of guanine, and other derivatives were found in lesser amounts than with diethyl sulfate. Neither reagent caused the formation of detectable amounts (<0.2%) of 1-ethylguanine, 1,7-diethylguanine, N^2-ethylguanine, N^6-ethyladenine, N^4-ethylcytidine, or 3-ethyluridine. Identified ethyl bases account for over 85% of the total radioactivity of [^{14}C]ethyl methanesulfonate and [^{14}C]diethyl sulfate treated TMV-RNA. Phosphate alkylation accounts for about 13 and 1%, respectively. In contrast, [^{14}C]ethylnitrosourea-treated TMV-RNA, while reacting to a similar extent (15–70 ethyl groups/6400 nucleotides), is found to cause considerably more phosphate alkylation. Upon either U_{4A} RNase or acid hydrolysis up to 60% of the radioactivity is found as volatile ethyl groups in the form of [^{14}C]ethanol, and a further 15% appears to be primarily ethyl phosphate and nucleosides with ethylated phosphate. Of the remaining radioactivity, half is found as O^6-ethylguanosine, the major identified ethyl nucleoside. Other ethyl bases found in ethylnitrosourea-treated TMV-RNA are 7-ethylguanine > 1-ethyladenine, 3-ethyladenine, 7-ethyladenine, 3-ethylcytidine, and 3-ethylguanine. It appears that ethylnitrosourea preferentially alkylates oxygens, and that formation of phosphotriesters is by far the predominant chemical event. Since the number of ethyl groups introduced into TMV-RNA by ethylnitrosourea is similar to the number of lethal events, one may conclude that phosphate alkylation leads to loss of infectivity. None of the three ethylating agents studied are strongly mutagenic on TMV-RNA or TMV. The role of phosphate alkylation in regard to *in vivo* mutagenesis and oncogenesis remains to be established. At present it appears possible that the extent of this reaction may correlate better with the oncogenic effectiveness of different ethylating agents, than the extent of any base reaction. Unfractionated HeLa cell RNA is ethylated primarily in acid labile manner even by diethyl sulfate and ethyl methanesulfonate, a fact that is attributed to its high content of low molecular weight RNA rich in terminal phosphates which alkylate readily.

Acylalkylnitrosamides are powerful carcinogens and highly mutagenic in a variety of systems. Since Loveless' (1969) finding that methylnitrosourea alkylates the O^6 position of guanine to a significant extent, other investigators have attempted, without clear success, to correlate the observed biological effects with the amount of O^6-alkylguanine formed in the DNA and RNA of animal and bacterial cells after treatment with methyl- and ethylnitrosoureas, or similarly effective carcinogens such as dimethylnitrosamine and nitrosoguanidine (den Engelse, 1974; Frei and Joshi, 1974; Goth and Rajewsky, 1974a,b; Kleihues and Magee, 1973). Neither ethyl methanesulfonate (of lesser but definite carcinogenicity and mutagenicity), nor diethyl sulfate (low in these respects) has been found to ethylate the O^6 of guanine to a significant extent (Singer, 1972; Lawley and Shah, 1972; Kleihues and Magee, 1973; Walker and Ewart, 1973). It thus appeared that a comparative study of all measurable alkylation products of these three different types of ethylating agents *in vivo* and *in vitro* might help in clarifying whether the formation of O^6-ethylguanine or other alkyl derivatives were likely to induce mutagenesis or carcinogenesis.

This portion of the work deals with the products of neutral ethylation of TMV-RNA and unfractionated HeLa cell RNA by diethyl sulfate, ethyl methanesulfonate, and ethylnitrosourea and makes some comparisons with the methyl products obtained when TMV-RNA is similarly treated with dimethyl sulfate and methyl methanesulfonate. The effects of the ethylating agents on HeLa cell DNA, *in vivo* and *in vitro*, represent the subject of a separate paper (Sun and Singer, 1975).

The biological effect of the three ethylating agents acting on TMV-RNA (and TMV) has been investigated and some conclusions are drawn on the correlation between chemical and biological events.

Experimental Section

Reaction of TMV-RNA with [^{14}C]Diethyl Sulfate, [^{14}C]Ethyl Methanesulfonate, and [^{14}C]Ethylnitrosourea. TMV-RNA (1 mg) in 0.7 ml of 0.75 M pH 7.3 cacodylate buffer was reacted with either (1) 20–60 μl of diethyl sulfate in 80 μl of ethanol (specific activity 4.09 Ci/mol) or (2) 20 μl of ethyl methanesulfonate (5.3 Ci/mol), or (3) 15 mg of ethylnitrosourea in 0.2 ml of ethanol (specific activity 35 μCi/mg) at 37°. The time of reaction varied from 15 min to 3 hr. In the case of reaction with diethyl sulfate for more than 1 hr the reagent was added in aliquots at hourly

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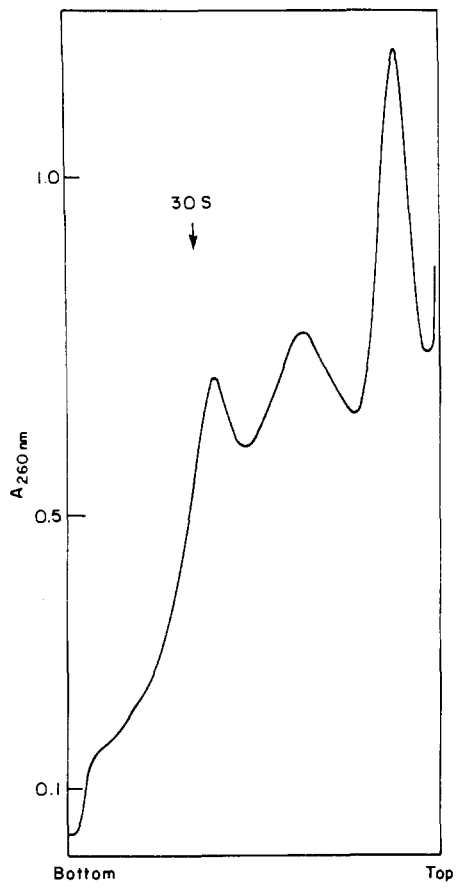


FIGURE 1: Sucrose density gradient of unfractionated HeLa-cell RNA; 100 μg of RNA was layered on a 10–25% sucrose gradient (containing 0.01 M pH 7.4 Tris, 0.1 M NaCl, 0.001 M EDTA, and 0.1% SDS) and centrifuged in an SW 50.1 rotor at 49,000 rpm 2 hr at 20°. The position of 30S TMV-RNA, in a parallel gradient, is indicated.

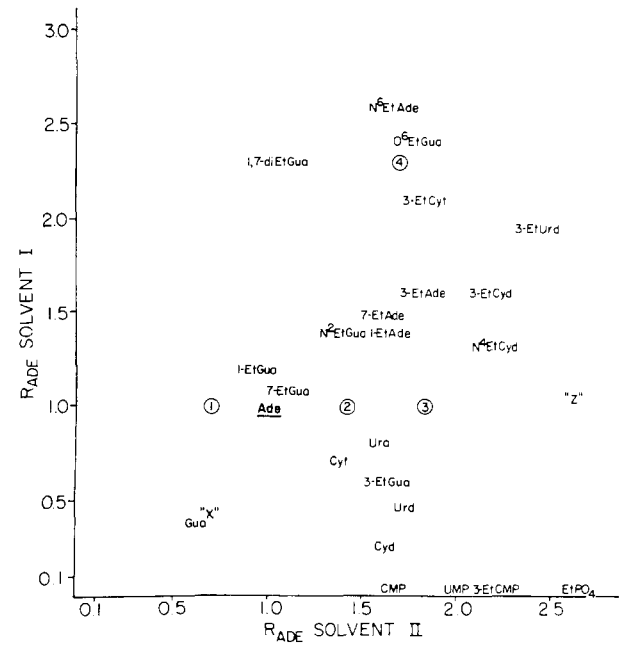


FIGURE 2: Diagram of R_{Ade} (chromatographic behavior relative to adenine) values in solvents I and II. See Experimental Section for the chromatographic methods and preparation of the derivatives. Circled positions 1 and 2 refer to imidazole ring-opened 7-ethylguanine and positions 3 and 4 refer to imidazole ring-opened 7-ethyladenine. Guanine and its alkyl derivatives exhibit a blue fluorescence under ultraviolet light and are easily distinguished from other derivatives. While there is a range of R_{Ade} values for each derivative in individual chromatograms, the relative positions remain constant.

intervals for 2–3 hr. The pH remained above 6 in each case. The RNA was freed from excess reagent by repeated ethanol precipitation (3 volumes of ethanol containing 0.1 ml of 3 M pH 5 acetate per ml, 0°) until constant specific activity was obtained. This generally took 5–6 reprecipitations. The specific activity of the several ethylated RNAs ranged from approximately 1×10^5 to 3×10^5 cpm/mg, or 15–70 ethyl bases per RNA chain containing about 6400 bases. The recovery of RNA was at least 90%.

Reaction of TMV-RNA with [^{14}C]Dimethyl Sulfate and [^{14}C]Methyl Methanesulfonate. TMV-RNA (0.5 mg) in 0.35 ml of 0.75 M pH 7.3 cacodylate buffer was reacted with (1) 10 μl of dimethyl sulfate (0.3 Ci/mol). After 1 hr at 37°, 10 μl of additional dimethyl sulfate and 0.2 ml of cacodylate buffer were added and the reaction was continued 2 hr at 37°; or (2) 10 μl of methyl methanesulfonate (0.5 Ci/mol) at 37° for 3 hr. Removal of unbound reagent was by the same technique of alcohol precipitation as in the previous section. The extent of methylation was 20–30 times higher than comparable ethylation. However, since the methylating agents were diluted with unlabeled reagent to lower specific activities, the radioactivity per mg of RNA was similar for methylated and ethylated nucleic acids.

Reaction of RNA from HeLa Cells with [^{14}C]Diethyl Sulfate and [^{14}C]Ethyl Methanesulfonate. Unfractionated RNA from HeLa cells was a gift of Dr. L. Sun. The sucrose density gradient centrifugation pattern indicated the presence of 28S, 18S, and 4–5S RNA (Figure 1); 0.5-mg samples were ethylated under the same conditions as used for ethylation of TMV-RNA, except that half the quantity of reagent was used. The extent of ethylation was similar to that for TMV-RNA.

Hydrolysis of Ethylated RNA. Due to the differing stabilities of the alkyl products (reviewed by Singer, 1975), two different methods were necessary to obtain quantitative data for the amount of each alkyl base formed in TMV-RNA.

Method I: 250–500 μg of RNA (2×10^4–5×10^4 cpm) was hydrolyzed in 1 N HCl at 100° for 1 hr. This yields purine bases and pyrimidine nucleotides.

Method II: 250–500 μg of RNA was enzyme digested at pH 5 to nucleosides by the following method. To 250 μg of RNA in 50 μl of H_2O were added 2 μl of 0.1 M pH 5 EDTA, 10 μl of 3 M pH 5 acetate buffer, 20 μl of U_{4A} RNase (200 units) (a generous gift of Dr. A. Blank) (Blank and Dekker, 1972), and 5 μl of a 5 mg/ml aqueous solution of *Escherichia coli* acid phosphatase. After 18 hr at 37°, the same amounts of U_{4A} RNase and acid phosphatase were added and the incubation was continued an additional 24 hr. Blank and Dekker (1972) indicate that, while this enzyme acts as an exoribonuclease, it does exhibit some phosphodiesterase activity, particularly at pH 5. Preliminary experiments with unmodified TMV-RNA indicated that all detectable products were nucleosides. By performing the enzyme digestion at pH 5, rearrangement of 1-ethyladenosine to N^6-ethyladenosine was prevented. It could also be demonstrated that imidazole ring opening of 7-ethylguanosine and 7-ethyladenosine was minimal, but there was a small amount of depurination as judged from the presence of up to 5% of the 7-ethylguanosine as 7-ethylguanine.

Methods for the Separation and Identification of Ethyl Derivatives. Three chromatographic systems and electrophoresis were used to separate the various alkyl derivatives

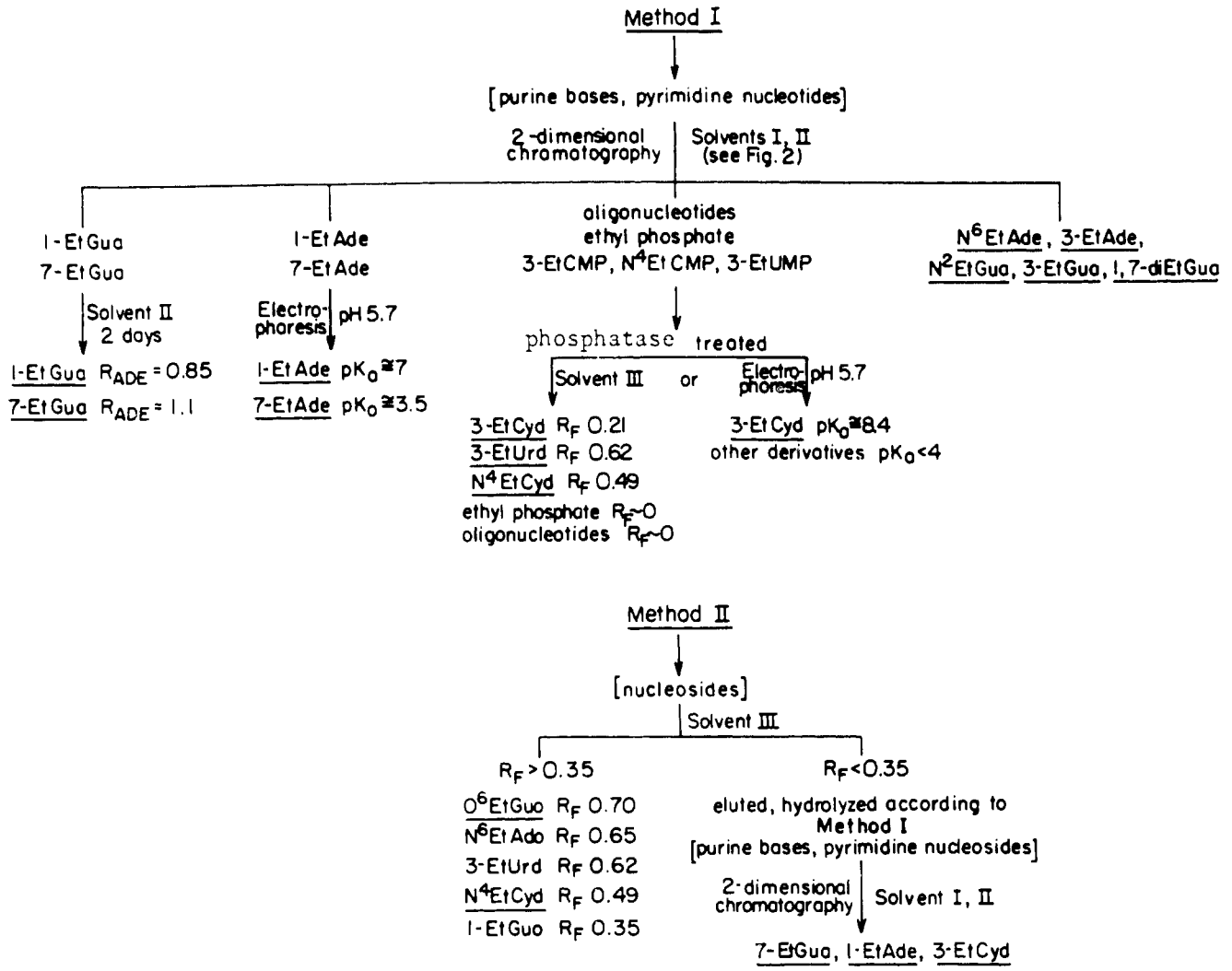


FIGURE 3: The general scheme for hydrolysis of alkylated RNA and subsequent separation of alkylated nucleotides, nucleosides, and bases. Hydrolysis methods and chromatographic and electrophoretic techniques are given in the Experimental Section. Figure 2 presents some additional chromatographic separations of ring-opened 7-ethylguanine (1 and 2) and 7-ethyladenine (3 and 4) as well as unknown products ("X" and "Z"). Ethyl phosphate was separated from undigested alkyl oligonucleotides in solvent I and further characterized by its hydrolysis to [^{14}C]ethanol upon digestion with 70% $HClO_4$ (100°, 1 hr). Underlining denotes preferred methods of determining the amount of derivatives. The rationale in each case is based on the stability of each derivative and the degree of separation from all other derivatives.

after acid hydrolysis or enzyme digestion. A diagram of the positions of many ethyl bases and ethylpyrimidine nucleotides and nucleosides in a two-dimensional paper chromatographic system (Singer and Fraenkel-Conrat, 1969) is shown in Figure 2. It should be noted that the diagram is not a chromatographic map but rather presents R_{Ade} values. Solvent I is 1-butanol–concentrated NH_4OH–H_2O (85:2:12 v/v) (descending, 20 hr) and solvent II is methanol–concentrated HCl–H_2O (70:20:10 v/v) (descending, 8–10 hr).

A third chromatographic system (solvent III) was used to separate O^6-ethylguanosine from most other nucleosides in the enzyme digest. Solvent III is 1-butanol–ethanol–H_2O (80:10:25) (descending, 20 hr). R_F values for ethyl nucleosides in this solvent are given in previous papers from this laboratory (Singer, 1972; Singer *et al.*, 1974; Sun and Singer, 1974).

Electrophoresis was in 0.05 M pH 5.7 formate as previously described (Singer *et al.*, 1974).

The general scheme for the various separations used to obtain quantitative data is shown in Figure 3. The scheme indicates, by underlining, the procedures which give the best separations and least degradation of each derivative. Up to several per cent of the 7-alkylpurines was found in ring-opened form regardless of the hydrolysis method. The analytical data for the 7-ethylpurines is reported as the total of ring-opened and intact derivative.

All ethyl derivatives were prepared in this laboratory (see next section for details) and various ones were used as ultraviolet absorbing markers added to hydrolyzed RNA samples prior to separation procedures. The usual markers added were, for HCl hydrolysates: 1-ethyladenine, 3-ethyladenine, 7-ethyladenine, N^6-ethyladenine; and for enzyme digests: O^6-ethylguanosine, 1-ethylguanosine, N^6-ethyladenosine. As shown in Figure 3, a portion of the chromatogram of enzyme digests, containing all unmodified nucleosides and the major ethyl nucleosides, was eluted by capillarity with 10^{-4} N HCl and then hydrolyzed in 1 N HCl, 100°, 1 hr (Method 1). This hydrolysate had as added markers: 3-ethylcytidine, 1-ethyladenine, 7-ethylguanine. At times other added markers used were 1-ethylguanine, 1,7-diethylguanine, N^2-ethylguanine, N^4-ethylcytidine, 3-ethyluridine, and imidazole ring-opened 7-ethyladenine and 7-ethylguanine. 3-Ethylguanine was prepared only at the

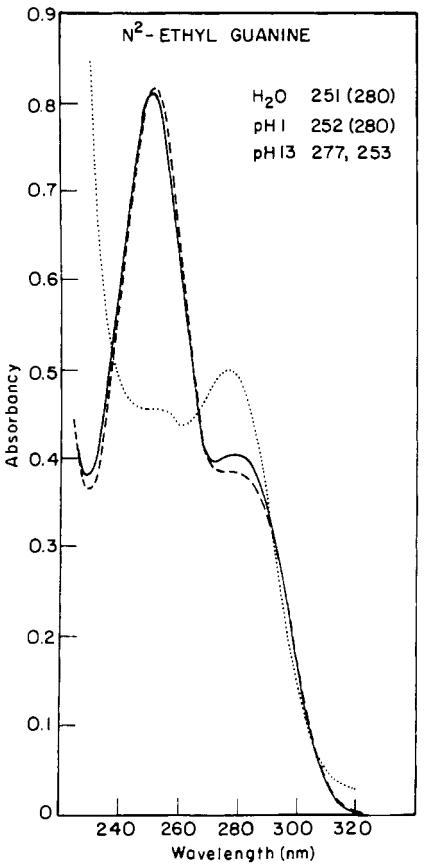


FIGURE 4: Ultraviolet absorption spectra of N^2-ethylguanine in H_2O (—), 0.1 N HCl (- -), and 0.1 N KOH (• • •). The absorption maxima at each pH is shown in the figure. Figures in parentheses indicate inflections or shoulders.

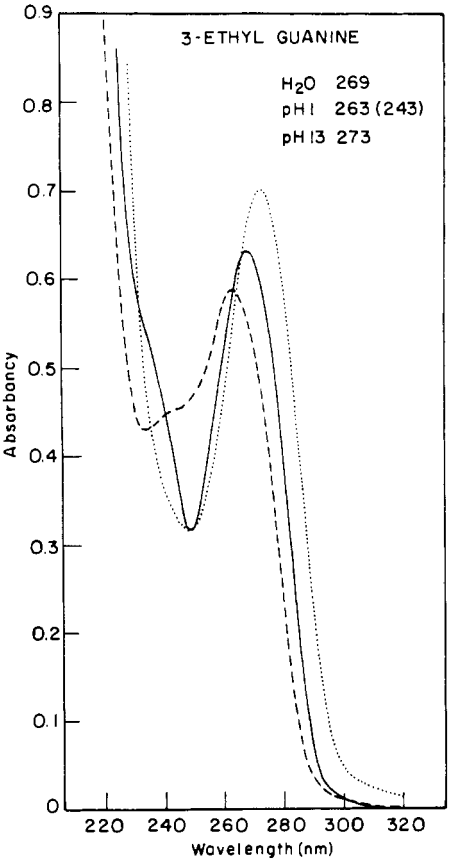


FIGURE 5: Ultraviolet absorption spectra of 3-ethylguanine in H_2O (—), 0.1 N HCl (- -), and 0.1 N KOH (• • •). The absorption maxima at each pH is shown in the figure. Figures in parentheses indicate inflections or shoulders.

end of this work. It was therefore not used as a uv marker in hydrolysates but was so used when rechromatographing an unknown area of radioactivity, the R_{Ade} of which corresponded to authentic 3-ethylguanine.

After observing and marking uv absorbing areas on two-dimensional chromatograms, the entire paper was cut into sections of approximately 0.5 × 1 cm to 1 × 2.5 cm and each section was placed in a scintillation vial containing 5 ml of toluene containing 14.3 g of Omnifluor/3 l. and counted in a Beckman scintillation counter. One-dimensional chromatograms and electrophoresis papers were similarly strip-counted.

Those strips which contained derivatives which were to be further separated (Figure 3) were washed several times in toluene, dried, and eluted in H_2O, or in the case of 7-alkylpurine nucleosides, eluted in 10^{-4} N HCl to prevent ring opening which occurs even at neutral pH.

The amount of each derivative was calculated from the total radioactivity associated with an authentic marker or, in some cases, with an area not corresponding to a known derivative.

Preparation of Ethyl Nucleosides and Bases. 1-Ethylguanosine, 7-ethylguanosine, 1,7-diethylguanosine, and O^6-ethylguanosine were prepared according to Singer (1972). 7-Ethylguanosine was treated with 0.01 N NaOH for 18 hr at 37° to open the imidazole ring, then hydrolyzed with 1 N HCl at 100° for 1 hr to obtain the purine products. Upon chromatography in solvents I and II, two major and one minor derivatives were found. The R_{Ade} values of the major products of ring opening are shown in Figure 2. 1-

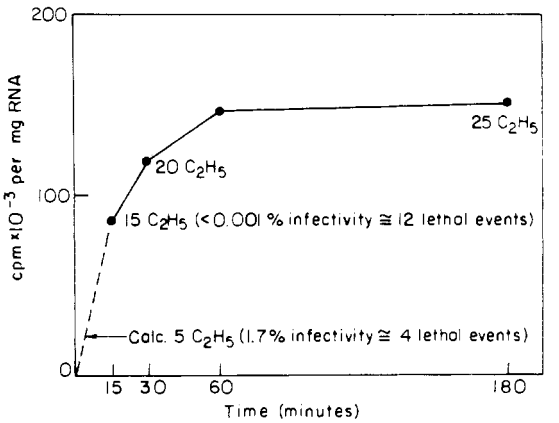


FIGURE 6: The rate of ethylation of TMV-RNA with [^{14}C]ethylnitrosourea. Reaction conditions are described in the Experimental Section. For each time period the figure indicates the number of ethyl groups introduced per TMV-RNA molecule of approximately 6400 nucleotides. After 5- and 15-min reactions, aliquots were taken and reconstituted with TMV protein, and the residual infectivity was determined (see Experimental Section for methods). The number of lethal events was calculated from the first-order equation $N/N_0 = e^{-x}$ (N_0 = initial infectivity, N = infectivity after alkylation, x = average number of lethal events).

Ethylguanine, 7-ethylguanine, and 1,7-diethylguanine were obtained as products after hydrolysis of the respective nucleosides in 1 N HCl at 100° for 1 hr. O^6-Ethylguanine was prepared by hydrolyzing the nucleoside in 0.1 N HCl at 37° for 18 hr.

N^2-Ethylguanine was prepared according to Elion *et al.*

Table I: Distribution and Recovery of Ethyl Products from Hydrolysates of TMV-RNA Reacted with [^{14}C]Diethyl Sulfate, [^{14}C]Ethyl Methanesulfonate, or [^{14}C]Ethylnitrosourea.[a]

	Reagent					
	Diethyl Sulfate		Ethyl Methanesulfonate		Ethylnitrosourea	
	Hydrolysis Method					
	I cpm	II cpm	I cpm	II cpm	I cpm	II cpm
Identified ethyl bases[b]						
7-EtG	8,335 (67)	12,955 (69)	26,365 (81)	22,543 (82)	1,895 (69)	1,376 (39)
1-EtA	1,326 (11)		2,505 (7.7)		465 (17)	282 (7)
7-EtA	438 (3.5)		985 (3)		81 (3)	130 (4)
1-EtA + 7-EtA		3,145 (17)		3,112 (11.2)		
3-EtA	378 (3.1)	n.d.	372 (1.1)	n.d.	226 (8.5)	
3-EtC	1,446 (12)	1,747 (9)	1,960 (6)	1,341 (4.8)		109 (3)
O^6-EtG		732 (2)		424 (1)		2,182 (47)
3-EtG	427 (3.4)	186 (1)	526 (1.6)	309 (1.1)	60 (2.2)	31 (0.8)
Other ethyl products						
"X" (see Figure 2)	n.d.	n.d.	542	565		
Origin	109	n.d.	116	166	440	n.d.
"$EtPO_4$"[c]	n.d.	n.d.	1240	1,506	2,065	1,753
Other unidentified	882	864	137		1,040	426
Summation						
Before hydrolysis	24,500	31,500	49,500	49,500	28,500	28,500
Total recovered	13,341	19,669	34,748	29,966	6,212	6,258
Identified bases	12,350, 92%	18,805, 96%	32,713, 94%	27,736, 93%	2,727, 44%	4,110, 66%
Other products	991, 8%	864, 4%	2,035, 6%	2,237, 7%	3,485, 56%	2,148, 34%
% recovery[d]	55	62	70	61	22	22

[a] Reaction conditions and ratio of reagent to RNA were the same for all three reagents. Hydrolysis methods I and II are described in detail in the Experimental Section. The level of alkylation of TMV-RNA in this experiment was: diethyl sulfate, 20 ethyl groups per TMV-RNA molecule of 6400 nucleotides; ethyl methanesulfonate, 64 ethyl groups per 6400 nucleotides; and ethylnitrosourea, 25 ethyl groups per 6400 nucleotides. All radioactivity was measured on dry paper strips immersed in 5 ml of scintillation fluid (14.3 g of Omnifluor/3 l. of toluene). The figures in parentheses represent the distribution of radioactivity in authentic ethyl bases, expressed in per cent. n.d. indicates that the derivative could not be detected. [b] For purposes of simplicity, all data are presented for bases although, depending on the method used, derivatives were at times isolated as bases, nucleosides, or nucleotides. See Experimental Section for the methods of hydrolysis and chromatography. In the case of O^6-EtGua, this product was always determined as the nucleoside using one-dimensional chromatography (solvent III). Thus the per cent figure is separately derived from the recovery of radioactivity in this system. [c] The term "$EtPO_4$" is used to indicate a product (or products) of 1 N HCl hydrolysis which does not move in solvent I (suggesting that it bears a phosphate), and moves far, with some trailing in solvent II, as does authentic ethyl phosphate (see Figure 2). [d] No corrections for losses in handling have been made in the data in this table. In similar model experiments, testing the recovery of radioactivity in these procedures, the recoveries have been found to be approximately 65%. Thus recoveries of 55–70% found for TMV-RNA treated with diethyl sulfate and ethyl methanesulfonate are within the expected range. The much lower recovery of radioactivity found for ethylnitrosourea-treated RNA is due to the loss of [^{14}C]ethanol released from the phosphate groups upon either enzyme or acid hydrolysis. See Results section and Tables IV and V.

(1956) and purified by chromatography in solvent II followed by solvent III. 3-Ethylguanine was prepared in low yield by a modification of the method of Litwack and Weissmann (1966) for the preparation of methylguanines. Ethyl chloride was the alkylating agent and 3-ethylguanine was separated from all other ethylguanines (including 7, 9, and 1,7 derivatives) by chromatography in solvents I and II. Figures 4 and 5 show the previously unpublished spectra of N^2-ethylguanine and 3-ethylguanine. Both compounds are easily distinguished from other alkylguanines by their unique spectra.

1-Ethyladenosine, N^6-ethyladenosine, and 7-ethyladenosine were prepared according to Singer *et al.* (1974). 7-Ethyladenosine was ring-opened and converted to the base in the same manner as 7-ethylguanosine. It, like ring-opened 7-ethylguanine, was found to consist of at least two products upon chromatography in solvents I and II. The R_{Ade} values of the values of the major products are shown in Figure 2. 3-Ethyladenine was obtained upon neutral heating of ethylated DNA as previously described (Singer *et al.*, 1974).

3-Ethylcytidine, N^4-ethylcytidine, and 3-ethyluridine were prepared according to Sun and Singer (1974).

Infectivity and Mutagenicity of Methylnitrosourea and Ethylnitrosourea Treated TMV-RNA and TMV. Aliquots of TMV-RNA reacted for various times with the reagent were reconstituted with TMV protein (Fraenkel-Conrat and Singer, 1964) and tested for infectivity and mutation as previously described (Singer and Fraenkel-Conrat, 1969a) (Figure 6).

Intact TMV virus, diluted with 1 M pH 7 cacodylate buffer to 10 mg/ml, was saturated with reagent or treated with 5 mg of reagent/mg of virus (the reagent was freshly prepared by dissolving 100 mg in 1 ml of ethanol), held at 37° for 4–18 hr, then diluted with 0.1 M pH 7 phosphate buffer to measure infectivity and mutation frequency (Singer and Fraenkel-Conrat, 1969a).

Materials. [^{14}C]Diethyl sulfate (4.09 Ci/mol) was obtained from ICN. [^{14}C]Ethyl methanesulfonate (5.3 Ci/mol) and [^{14}C]methyl methanesulfonate (56 Ci/mol) were obtained from Amersham/Searle. [^{14}C]Ethylnitrosourea (5.72 Ci/mol) was obtained from Farbwerke Hoechst. [^{14}C]Dimethyl sulfate (8.32 Ci/mol) was obtained from Schwarz/Mann.

Results

Reaction of TMV-RNA with [^{14}C]Diethyl Sulfate, [^{14}C]Ethyl Methanesulfonate, and [^{14}C]Ethylnitrosourea. The extent of ethylation by these three reagents under the same conditions was not markedly different, although diethyl sulfate was consistently less reactive than the other two reagents. Since ethylnitrosourea is reported to decompose at a high rate at and above pH 7 (Goth and Rajewsky, 1974a), the rate of reaction of this reagent with TMV-RNA at pH 6–7.3 was determined (Figure 5). It is evident that under the conditions used, ethylation reaches a maximum in about 1 hr. This extent of ethylation is similar to that found for the more classical ethylating agents.

Table I presents representative detailed analytical data used to determine the products of ethylation. Table II gives a summary of several such experiments. Diethyl sulfate and ethyl methanesulfonate ethylate TMV-RNA predominantly on the bases with some small consistent differences in the patterns of ethylation (Table II). The proportion of various ethyl products differed somewhat from that previously found for methyl derivatives in TMV-RNA reacted with dimethyl sulfate and methyl methanesulfonate (Singer and Fraenkel-Conrat, 1969b), but since different techniques and methods of calculation were used at that time, these analyses were repeated with TMV-RNA reacted with dimethyl sulfate and methyl methanesulfonate, using the present techniques. Data from these methylation experiments are shown in parentheses in Table II and differ from ethylation in several respects. Neither 3-MeG nor O^6-MeG is detected while the corresponding ethyl derivatives are. The higher affinity of ethylating agents for the O^6 of guanine was previously reported for guanosine (Singer, 1972). The present work indicates that such higher affinity is also true for the 3 position of guanine. More methylation, compared to ethylation, is found on the N-1 of adenine; 2–3% of the methylation was found as 1,7-dimethylguanine but no 1,7-diethylguanine was detected. The higher level of methylation in these experiments probably leads to 7-methylguanine being additionally methylated on the N-1 (Singer, 1972). Another important difference, to be discussed in a later section, is the high level of ethylation of the internal phosphate groups, in contrast to a very much smaller extent of methylation of these groups.

Ethylnitrosourea, a different class of alkylating agent than those discussed above, has a strong affinity for alkylating oxygens as is shown by the fact that, of the base ethylations, approximately half is found as O^6-ethylguanosine (Tables I and II); and that, of the total ethylation, about 65% represents ethyl groups liberated by 1 N HCl and presumed to be on the phosphodiesters (see section on Ethylation of Phosphate Groups). Those ethyl groups which are on the nitrogens of bases, representing only about 16% of the ethyl groups introduced by ethylnitrosourea, are distributed in a rather similar pattern as the ethyl groups substituted on the ring nitrogens by diethyl sulfate and ethyl methanesulfonate (~65% 7-EtG, ~13% 1-EtA, ~10% 3-EtC, ~7% 3-EtA, ~4% 7-EtA, ~3% EtG).

Table II: Summary of Distribution of Ethyl Products Formed in TMV-RNA Reacted with [^{14}C]Diethyl Sulfate, [^{14}C]Ethyl Methanesulfonate, and [^{14}C]Ethylnitrosourea.[a]

	% of Total Ethylation		
Derivative	Diethyl Sulfate	Ethyl Methane-sulfonate	Ethyl-nitrosourea
7-EtG	62 (56)	70 (67)	10
1-EtA	11 (21)	6 (13)	2
3-EtA	3 (3)	1 (2)	1.2
7-EtA	3 (6)	3 (4)	0.6
3-EtC	11 (9)	5 (7)	1.7
O^6-EtG	2 (n.d.)	1 (n.d.)	12
3-EtG	2 (n.d.)	1 (n.d.)	0.5
"$EtPO_4$"	n.d. (n.d.)	3 (n.d.)	10
Unidentified	6 (2)	2 (3)	6
[^{14}C]Ethanol[b]	~1	10 (2)	55

[a] This summary is based on the data in Table I and additional analyses. The analysis for each product judged to be most accurate (see Figure 3) was used as well as averages in many cases; 2–4 separate alkylations were performed with each reagent and each alkylated RNA was analyzed 2–4 times under varying conditions. Data in parentheses are for the analogous methylating agent. In addition to the derivatives listed, 2–3% 1,7-dimethylguanine was found. n.d. indicates that the derivative could not be detected. [b] See Table IV, footnote *a* for the method of determining [^{14}C]ethanol (or methanol).

In addition to known or presumed oxygen and nitrogen ring alkylation products, all three ethylating agents formed unidentified radioactive products, one of which ("X") was reproducibly found at the same position on chromatograms. Very little RNA remained as oligonucleotides (0–2%) after 1 N HCl hydrolysis or enzyme hydrolysis followed by 1 N HCl hydrolysis.

Counting of the entire one- or two-dimensional chromatograms, in small segments, has made it possible to assign all radioactivity to one of the categories listed in Table I.

Reaction of HeLa Cell-RNA with [^{14}C]Diethyl Sulfate and [^{14}C]Ethyl Methanesulfonate. In conjunction with current unpublished experiments on *in vivo* ethylation of HeLa cells, HeLa-cell RNA was ethylated *in vitro* under the same conditions as used for TMV-RNA with these two classical ethylating agents. Table III presents detailed analytical data on the products of ethylation. Only a small fraction of the ethyl groups was recovered as ethyl bases and these were in the order 7-EtG ≫ 1-EtA > 3-EtC, 7-EtA > 3-EtA, O^6-EtG, 3-EtG. There were also unidentified radioactive areas on the chromatograms, one of which was the same as found in hydrolysates of ethylated TMV-RNA, and one of which, "Z," was found only as a product of HeLa-RNA ethylation. The rest of the radioactivity (which is ac-

Table III: Distribution and Recovery of Ethyl Products from Hydrolysates of Unfractionated HeLa Cell RNA Reacted with [^{14}C]Diethyl Sulfate and [^{14}C]Ethyl Methanesulfonate.[a]

	Reagent			
	Diethyl Sulfate		Ethyl Methanesulfonate	
	Hydrolysis Method			
	I cpm	II cpm	I cpm	II cpm
Identified ethyl bases[b]				
7-EtG	3,404 (85)	7,820 (82)	4,842 (87)	8,804 (89)
1-EtA		1,010 (11)		
7-EtA		159 (1.7)		
1-EtA + 7-EtA	329 (8)		387 (7)	609 (6)
3-EtA	39 (1)	n.d.	n.d. (< 1)	n.d.
3-EtC		449 (4.7)		97 (1)
O^6-EtG		80 (0.8)		84 (0.9)
3-EtG	244 (6)		309 (6)	277 (2.8)
Other ethyl products				
"X" (see Figure 2)			275	155
"Z" (see Figure 2)	708	1,280	389	207
Origin	160	82	142	95
"$EtPO_4$"[c]	3,562	5,649	3,309	4,336
Other unidentified	400	1,400		482
Summation				
Before hydrolysis	33,000	66,000	32,000	64,000
Total recovered	8,846	17,929	9,653	15,146
Identified bases	4,016; 45	9,518; 53	5,538; 57	9,871; 65
Other products	4,830; 55	8,411; 47	4,115; 43	5,275; 35
% recovery[d]	27	27	30	24

[a] Reaction conditions and ratio of reagent to RNA were the same for both reagents. The level of alkylation of HeLa-cell RNA in this experiment was: diethyl sulfate, 0.4%, and ethyl methanesulfonate, 1.5%. All radioactivity was measured on dry paper strips immersed in 5 ml of scintillation fluid. The figures in parentheses represent the distribution of radioactivity in authentic ethyl bases, expressed in per cent. [b] See footnote *b*, Table I. [c] See footnote *c*, Table I. [d] No corrections for losses in handling have been made in the data in this table. The recoveries of 24–30% appear low but do not include approximately 50% of the radioactivity as acid-labile [^{14}C]ethanol which is evaporated during hydrolysis. In other experiments, discussed in the Results section, it was found that [^{14}C]ethanol was not liberated when the RNA was digested to nucleosides using U_{4A} ribonuclease and acid phosphatase, but was found upon acid hydrolysis.

Table IV: Quantitation and Identification of ^{14}C-Labeled Alcohol Released from Acid Digests of Alkylated TMV-RNA.[a]

Reagent	Distillate cpm	Distillate %	Residue cpm	Residue %
Methyl methanesulfonate	180	1.8	10,000	98.2
Ethyl methanesulfonate	940	11	7800	89
Ethylnitrosourea	5400[b]	71	2200	29

[a] 18–35 μg of alkylated RNA (see Experimental Section) was hydrolyzed with 50 μl of 1 N HCl at 100° 1 hr in a sealed tube; 1 ml of either methanol or ethanol, depending on the expected alkyl group, was added and the sample was distilled at 78–80° for ethanol and 65–68° for methanol. Aliquots of the distillate and of the residue were counted in 5 ml of Brays scintillation fluid. [b] This figure includes about 900 cpm calculated to be derived from O^6-ethylguanosine since its alkyl group is found to be released under these hydrolysis conditions.

tually most of it) was either [^{14}C]ethanol (originating from phosphate esters, as discussed in the next section), or termed "$EtPO_4$" from its chromatographic behavior (Figure 2) as well as other criteria discussed in the next section.

Ethylation of Phosphate Groups. As briefly outlined in the previous two sections, there were indications that ethylnitrosourea alkylated the phosphodiester groups of TMV-RNA more readily than either ring oxygens or nitrogens. Diethyl sulfate and ethyl methanesulfonate acting on TMV-RNA formed very little of such presumed phosphotriesters, but when acting on unfractionated HeLa-cell RNA most of the radioactivity seemed to be associated with phosphate groups.

The conclusion that ethylnitrosourea led to appreciable alkylation of the tertiary phosphate groups of the TMV-RNA chain is based on the following considerations. The ethyl groups not accounted for as modified bases (70%) (Table II) were largely labile to 1 N HCl (Table IV), while they were partly released under the conditions of enzyme treatment (pH 5, 37°) (Table V). Those ethyl groups remaining bound after such digestion were further characterized by chromatography in solvent III. They remained in

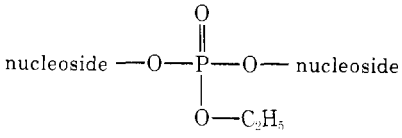
Table V: Release of [^{14}C]Ethanol from ^{14}C-Ethylated RNA under Various Conditions of Hydrolysis.

Reagent	RNA	Hydrolysis Conditions[a]	[^{14}C]Ethanol Distilled % of total cpm
Ethyl methanesulfonate	HeLa	U_{4A} RNase, phosphatase, pH 5, 37°, 18 hr	4
		1 N HCl, 100°, 1 hr	51
		70% $HClO_4$, 100°, 1 hr	67
Ethylnitrosourea	TMV	U_{4A} RNase, phosphatase, pH 5, 37°, 18 hr	42
		1 N HCl, 100°, 1 hr	71
		70% $HClO_4$, 100°, 1 hr	85

[a] Conditions of hydrolysis with enzymes or 1 N HCl are given in the Experimental Section; 50 μl of 70% $HClO_4$ was added to RNA which had been evaporated to dryness in the hydrolysis tube. Hydrolysis in a sealed tube was at 100° 1 hr. See Table IV, footnote *a* for the method of determining [^{14}C]ethanol.

small part at the origin but mainly formed two peaks near the origin (Figure 7, peaks I and II) from which upon 1 N HCl hydrolysis (1 hr, 100°) almost all radioactivity could be recovered by distillation as ethanol. This was in contrast to the material under peak III which contained mostly 7-ethylguanosine and 1-ethyladenosine. The material in peak IV could be identified as O^6-ethylguanosine, its proportion to that under peak III again illustrating that the extent of ethylation of the O^6 of guanine by ethylnitrosourea exceeds that of the N-7 position.

The chromatographic behavior of the materials in peaks I and II, and the lability of its ethyl groups is in accord with their representing dinucleoside-ethyl phosphates[1]



In contrast to the belief that in such triesters the ribose-phosphate bonds would be labilized, our data indicate that the most labile is the ethyl ester bond. Since these analyses were performed with RNA alkylated for 15, 30, 60, and 180 min (Figure 7), it is clear that the preference of ethylnitrosourea for the oxygens on phosphate and on guanine residues is the same over this wide time span.

The finding that the ethylation of HeLa-cell RNA with diethyl sulfate and ethyl methanesulfonate yielded largely acid-labile ethyl groups (Tables II and V) is attributed to the fact that the HeLa-cell RNA preparation used contained a high percentage of 4-5S RNA (Figure 1) indicating chain lengths of 70-120 nucleotides and thus relatively rich in terminal secondary phosphates which, in contrast to the internal tertiary phosphates, are known to be as reactive toward typical alkylating agents as the most reactive sites on the bases (Windmueller and Kaplan, 1961; Griffin and Reese, 1963; Haines *et al.*, 1964; Abell *et al.*, 1965). These phosphodiesters would on the other hand not be labile at pH 5, which is borne out by only 4% of the radioactivity being released during enzyme digestion (Table V). Chromatography in solvent III of such an enzyme digest is shown in Figure 8. The differences between this chromatogram and

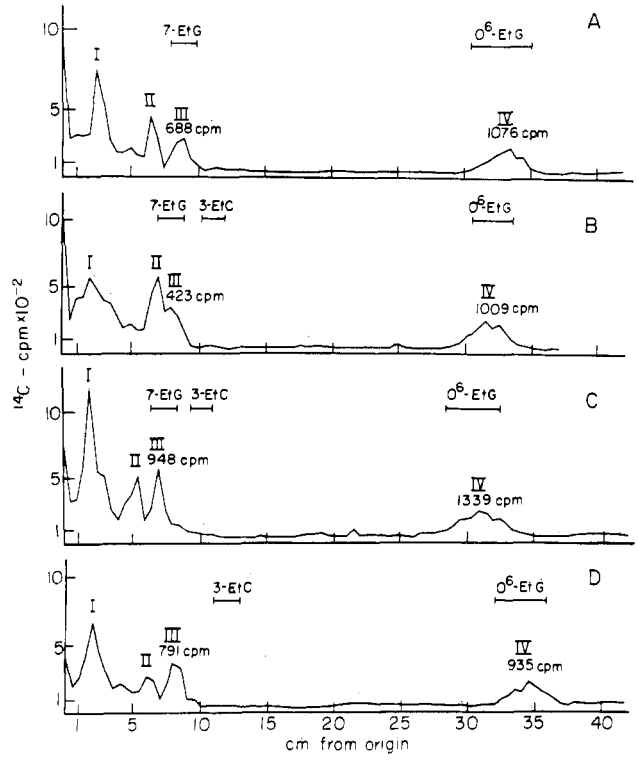


FIGURE 7: Radioactivity profiles of chromatograms of enzyme digests of TMV-RNA reacted with [^{14}C]ethylnitrosourea at 37° for various times. (Background of 30 cpm has not been subtracted in the plot.) See Experimental Section for reaction conditions and method of enzyme digestion. Ethylated RNA was digested at pH 5 with ribonuclease U_{4A} and acid phosphatase. Digests were chromatographed in solvent III with both internal and external uv absorbing reference compounds. The positions of O^6-ethylguanosine, 7-ethylguanosine, and 3-ethylcytidine are indicated by lines. Chromatograms were cut into 0.5-cm strips for counting. A is reacted for 15 min, B for 30 min, C for 60 min, and D for 180 min. Peaks I and II are believed on the basis of evidence given in the Results section to be dinucleoside ethyl phosphates. Peak III contains not only 7-ethylguanosine, but also 1-ethyladenosine and 7-ethyladenosine. 3-Ethylcytidine is a small unnumbered peak or shoulder moving slightly further than peak III. Peak IV is O^6-ethylguanosine. The amount of radioactivity associated with peaks III and IV is shown below the peak number. The total recovery of radioactivity, not corrected for any losses in handling, is approximately 50% for each sample. Of the recovered radioactivity 51-61% is in peaks I and II.

those in Figure 7 are (1) no O^6-ethylguanosine is detected, (2) 68% of the radioactivity is associated with material at or near the origin, indicating that it is either oligonucleotides or nucleotide-like, and (3) peaks I and II are absent. The

[1] This was further confirmed by finding ^{32}P in peaks I and II, and only in these, when ^{32}P TMV-RNA was analyzed that had been treated with unlabeled ethylnitrosourea.

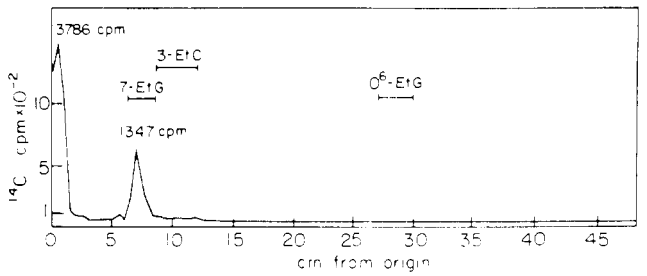


FIGURE 8: Radioactivity profile of a chromatogram of enzyme digested HeLa cell RNA reacted with [^{14}C]ethyl methanesulfonate for 3 hr at 37°. (Background of 30 cpm is not subtracted in the plot.) See Experimental Section for reaction conditions and method of enzyme digestion. Ethylated RNA was digested at pH 5 with ribonuclease U_{4A} and acid phosphatase, then chromatographed in solvent III and strip counted in the same manner as in Figure 7. The only significant radioactive peaks detected were those at the origin (3786 cpm) and in the area of 7-ethylguanosine (1347 cpm). The total recovery of radioactivity, not corrected for any losses in handling, is approximately 86%. Of the recovered radioactivity 68% is at the origin.

radioactivity at or near the origin was eluted, treated with snake venom phosphodiesterase, then distilled with added ethanol. About half the radioactivity was recovered as [^{14}C]ethanol, the remainder not further digestible with snake venom phosphodiesterase. Thus at least one-third of the original ethylation of HeLa cell RNA appears to be on terminal phosphate groups.

The corresponding material at the origins of chromatograms of enzyme digests of ethylnitrosourea-treated TMV-RNA in Figure 7 (2–10% of the total radioactivity) was similarly treated with snake venom phosphodiesterase. No radioactive ethanol was found and therefore this material did not contain appreciable numbers of terminal phosphodiesters. Subsequent hydrolysis in 1 N HCl at 100° for 1 hr did not release ethanol. It seems likely that this undigested fraction contains oligonucleotides alkylated on the bases and/or ethyl phosphate.

Tables IV and V summarize part of the data in this section with additional data showing that methyl methanesulfonate alkylates only ring nitrogens while about 10% of the alkylation by ethyl methanesulfonate is on phosphates, also indicated in Table I.

In addition to the amount of phosphate ethylation shown to be labile, releasing ethanol upon HCl hydrolysis, some ethyl groups were still bound to phosphate in the form of ethyl phosphate, ribose ethyl phosphate, or pyrimidine ribose ethyl phosphate. This material was found, to varying extents, in HCl hydrolysates of all ethylated RNAs (Tables I and III) except for diethyl sulfate treated TMV-RNA where it was not detectable (Table I). $HClO_4$ hydrolysis (70% $HClO_4$, 100°, 1 hr) of the material on chromatograms termed "$EtPO_4$" caused most of the radioactivity in this fraction to become volatile, and the remainder cochromatographed with authentic ethyl phosphate in the chromatographic systems used by Bannon and Verly (1972). When ethyl methanesulfonate treated HeLa cell RNA or ethylnitrosourea treated TMV-RNA were similarly hydrolyzed with $HClO_4$ without prior degradation, most of the ethyl groups were liberated as ethanol (Table V). The total volatile ethyl groups (67 and 85%, respectively) were in good agreement with the number calculated from the difference between radioactivity in the acid stable ethyl bases and the expected recovery of radioactivity (75 and 85%, respectively) (Tables II and III).

It has long been presumed that ribosephosphotriesters are too labile to be isolated from alkyl RNA (reviewed by Singer, 1975), and direct proof of their formation, based on their isolation, has been lacking (Shooter *et al.*, 1974a). The experimental data in this section identifies the major product of ethylnitrosourea treated TMV-RNA as ethyl phosphotriesters which are relatively stable, releasing ethanol to varying extents only upon hydrolysis (Table V).

The major product of diethyl sulfate or ethyl methanesulfonate treatment of HeLa cell RNA (much of which is of low molecular weight) is terminal ribose-ethylphosphodiesters. Model experiments on the reaction of nucleotides with dimethyl and diethyl sulfate (to be published separately) indicate that the secondary phosphates are esterified at almost equal rates by the methylating and ethylating reagents and that in the case of AMP, the rate of formation of the ethyl phosphate ester is greater than that of ethylation of the ring nitrogens, thus accounting for the results observed when HeLa-cell RNA is ethylated (Table III).

Biological Effect of Methyl- and Ethylnitrosourea acting on TMV-RNA and TMV. Since the TMV-RNA and TMV used were highly infective it was possible to measure loss of infectivity as a function of reagent concentration and time, and to determine whether many of the remaining infective particles had been mutated.

At neutrality, methylnitrosourea inactivated TMV-RNA about ten times faster than did ethylnitrosourea. With 2 mol of methylnitrosourea per nucleotide (37°, 5 min) 4% infectivity remained, while with a four-fold excess of ethylnitrosourea 33% infectivity remained after 5 min and 5% after 15 min. When the ratio of ethylnitrosourea to nucleotide was increased to 15, the infectivity after 5 min at 37° was 1.7%. Therefore the loss of infectivity of TMV-RNA upon reaction with ethylnitrosourea is proportional, at least for 15 min, to time and reagent concentration, as calculated from the number of lethal events indicated by the infectivity loss (*e.g.*, 37% remaining infectivity = 1 lethal event, 14% = 2, 5% = 3, 1.9% = 4).

TMV is less reactive than TMV-RNA and was only slowly inactivated by a considerable excess of reagent (5 mg of nitrosourea/mg of virus). After 4 hr at 37°, 5% infectivity remained in the methylnitrosourea-treated virus and 15% in the ethylnitrosourea-treated virus. Saturation of the TMV solution with ethylnitrosourea, or longer reaction times, did not cause substantially greater losses in infectivity of TMV.

Neither RNA nor virus was mutated to a high extent by either methyl- or ethylnitrosourea. All samples tested were similar and had a mutation frequency of 2–6 in the test system used (Singer and Fraenkel-Conrat, 1969a). For purposes of comparison, the mutation frequencies for nitrous acid and nitrosoguanidine-treated TMV are 110 and 68, respectively.

In Figure 6 is shown a calculation of lethal events, as measured by infectivity, *vs.* the number of ethyl groups per RNA molecule, introduced by ethylnitrosourea. While only two points can be used for infectivity assay (5 min and 15 min reaction time), the number of ethyl groups appears very similar to the number of lethal events. Since in the previous section it was demonstrated that over half the ethyl groups were on phosphodiesters it appears very probable that esterification of the tertiary phosphate groups is a biologically lethal event. Whether lethality is due to chain breaks as a result of the lability of triesters or whether to the alkylation *per se* is under study.

Discussion

The original objective of this work was to compare the sites and extent of base ethylation obtained when RNA was treated with diethyl sulfate, a poor carcinogen or mutagen; ethyl methanesulfonate, a moderately effective carcinogen or mutagen; and ethylnitrosourea, one of the most potent carcinogens presently known. The analytical data obtained (Tables I and II) represent the first complete analysis of ethylated RNA accounting for all bound alkyl groups, although there are included values for as yet unidentified minor derivatives.

The most striking fact regarding ethylation of the base moiety is that, in contrast to all classical alkylating agents, ethylnitrosourea reacted more with the O^6 than the 7 position of guanine. A small amount of O^6-EtG (1–2%) was also found in TMV-RNA treated with diethyl sulfate or ethyl methanesulfonate, but not when treated with the analogous methylating agents, dimethyl sulfate, and methyl methanesulfonate. Thus ethylating agents reacted to a higher extent with ring oxygens than did methylating agents. Similar comparative data were found for DNA treated with methyl- and ethylnitrosourea *in vitro* (Lawley and Shah, 1973; Sun and Singer, unpublished) and *in vivo* (Kleihues and Magee, 1973; Goth and Rajewsky, 1974a). Examining only the alkylation of the bases, the differences found with different reagents represent genuine chemical affinities, but they are probably, with the possible exception of the formation of O^6-EtG, not responsible for the range of biological effects observed.

In general, the ethylating agents acting on RNA follow the pattern of methylation. For example, both dimethyl and diethyl sulfate alkylate the N-1 of A and the N-3 of C more than methyl and ethyl methanesulfonates, while the reverse is found for the N-7 of guanine. Ethylnitrosourea, like methylnitrosourea (Lawley and Shah, 1972) alkylated the N-3 of C to a lower extent than the other alkylating agents.

In addition to the alkyl derivatives quantitated in Tables I and II, several other possible alkyl bases were looked for and none of these was detected (<0.2% of the total alkylation). These derivatives were N^6-EtA, N^4-EtC, 1-EtG, N^2-EtG, 1,7-Et_2G, and 3-EtU, all of which have been found as products of ethylation of nucleotides or nucleic acids (Singer, 1975). 1,7-Dimethylguanine was a minor product of methylation and is probably formed by N-1 methylation of 7-methylguanine (Singer, 1972).

Although 3-alkylcytidine (incorporated into poly(C)) causes mispairing when used as a template (Singer and Fraenkel-Conrat, 1970) and this alkylation has been shown to correlate with mutagenesis in TMV (Singer and Fraenkel-Conrat, 1969), the amounts of 3-EtC shown in Table II are very low when compared to the amount of 3-MeC formed in intact TMV (Singer and Fraenkel-Conrat, 1969a,c) and thus it is not surprising that there is no correlation in this case with mutagenesis. On the other hand, the amount of O^6-EtG, relative to 7-EtG, formed in ethylnitrosourea treated RNA is high. Alkylation of the O^6 of guanine is postulated as at least a contributing factor in carcinogenesis or mutagenesis since O^6-MeG, incorporated into polymers, also mispairs when used as template (Gerchman and Ludlum, 1973). However, since neither TMV nor TMV-RNA is mutated to a significant extent by methyl- or ethylnitrosourea, the formation of O^6-alkyl-G does not appear in this case to be a mutagenic event. A possible explanation could be that, in contrast to 3-ethylcytidine which is very stable, O^6-ethylguanosine in RNA is either enzymatically depurinated or dealkylated in tobacco cells. Enzymatic depurination of O^6-alkyl-G has been reported to be very high in endonuclease II treated methylnitrosourea alkylated DNA (Kirtikar and Goldthwait, 1974) and the lack of such depurination in the DNA of cells most susceptible to carcinogenesis is believed to be an important factor in neoplastic transformation by ethylnitrosourea (Goth and Rajewsky, 1974a).

Alkylation of the bases accounted for 87–98% of the alkyl groups introduced into TMV-RNA by dimethyl sulfate, diethyl sulfate, methyl methanesulfonate, and ethyl methanesulfonate. In distinct contrast, at least 50% of the ethyl groups of ethyl nitrosourea treated TMV-RNA (or diethyl sulfate and ethyl methanesulfonate treated HeLa-cell RNA) were not recovered as alkylated bases. The possibility that alkylation occurred on the ribose had to be considered. However, Broom and Robins (1965) find that under our conditions of hydrolysis in 1 N HCl at 100° for 1 hr the alkyl group is not released from 2′-*O*-methylribose, nor are the alkylation conditions used by us favorable for their formation.

However, analysis for labile ethyl groups indicated that ethylnitrosourea caused over 60% of the introduced ethyl groups in TMV-RNA to be on oxygen atoms, partly on the O^6 of guanine, but mostly in the form of phosphotriesters. The same was true to a much lesser extent (10%) with ethyl methanesulfonate, and not detectably with diethyl sulfate. The labile ethyl groups introduced into HeLa cell RNA were also esters, but in this case diesters resulting from esterification of terminal secondary phosphate groups.

A comparison of the extent of phosphate alkylation by different alkylating agents acting on TMV-RNA is shown in Table IV. The data for methyl methanesulfonate and ethyl methanesulfonate are very similar to data reported for phosphate alkylation of DNA with the same reagents (Bannon and Verly, 1972; Verly *et al.*, 1974). Our finding that ethylnitrosourea forms a much higher per cent of phosphotriesters than other classes of alkylating agents is also in agreement with earlier data. Thus methylnitrosourea-treated DNA contained about 18–20% of alkylated material which Lawley (1973) and Walker and Ewart (1973) believed to be phosphotriesters. Earlier, Lawley and Shah (1972) postulated that about 10% unidentified material from methylnitrosourea and nitrosoguanidine-treated RNA was due to reaction with the ribose–phosphate chain. Further indirect evidence comes from Shooter *et al.* (1974b) who find 10–12% unidentified methyl groups in RNA of methylnitrosourea treated bacteriophage R17.

The work reported here presents evidence that not only are phosphotriesters formed in ethylnitrosourea treated RNA, but such triesters are sufficiently stable to be isolated intact and are the major product of ethylation, representing up to 75% of the total ethyl groups. A subsequent paper will demonstrate that this is true also for DNA, *in vivo* and *in vitro* (Sun and Singer, 1975).

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DNA–Protein Interactions of the Rat Liver Non-Histone Chromosomal Protein†

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ABSTRACT: Native rat liver NHC protein–DNA interactions have been investigated by use of a nitrocellulose filter assay sensitive in detection of protein–DNA complexes. Optimal conditions for DNA–protein interactions occurs at low ionic strength conditions (110 mM–0.04 M phosphate buffer). A fraction of NHC proteins was enriched 25-fold by their affinity for rat DNA immobilized on cellulose columns under these conditions. At higher ionic strength (260 mM–0.04 M phosphate buffer and 0.15 M sodium chloride), this fraction binds approximately sevenfold less to rat DNA but with a substantial increase in stability of the complexes. Equilibrium competition experiments indicate that at the higher ionic strength there is a considerable DNA sequence specificity of the rat DNA binding NHC protein. Since rat DNA contains three components as defined by their reassociation kinetics: single copy DNA ($C_0t_{1/2}^{pure} = 1.6 \times 10^3$); middle repetitive DNA ($C_0t_{1/2}^{pure} = 1.1$); and highly repetitive ($C_0t_{1/2}^{pure} < 0.02$). The two former were isolated and employed in the DNA binding assays. At the high ionic strength criterion, the rat DNA binding NHC proteins showed a substantial preference for a subset of middle repetitive DNA sequences. This suggests a preferential interaction between a class of NHC proteins and a class of middle repetitive DNA sequences.

Rat liver non-histone chromosomal proteins (NHC proteins) can be fractionated into three groups by tandem DNA-cellulose column chromatography (van den Brock *et al.,* 1973). The column series consisted of an *Escherichia coli* DNA-cellulose column connected to a rat DNA-cellulose column. The groups consist of NHC proteins which do not bind to either DNA-cellulose column, proteins which bind to the *E. coli* DNA column, and a third group which bind to the rat DNA-cellulose column. The present paper analyzes in more detail the specificity of the rat DNA binding NHC proteins by use of the nitrocellulose filter assay

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