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The Specificity of Different Classes of Ethylating Agents toward Various Sites of HeLa Cell DNA in Vitro and in Vivo†

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ABSTRACT: The sites and extent of ethyl products of neutral ethylation of HeLa cell DNA by [^{14}C]diethyl sulfate, [^{14}C]ethyl methanesulfonate, and [^{14}C]ethylnitrosourea have been determined in vitro and in vivo, and found to differ significantly depending on the ethylating agents. Diethyl sulfate and ethyl methanesulfonate ethylate the bases of HeLa cell DNA in the following order: 7-ethylguanine > 3-ethyladenine > 1-ethyladenine, 7-ethyladenine > 3-ethylguanine, 3-ethylcytosine, O^6-ethylguanine. Ethyl bases accounted for 84–87% of the total ethyl groups associated with HeLa cell DNA. Ethylnitrosourea, in contrast, has particular affinity for the O^6 position of guanine. It ethylates the bases of HeLa cell DNA in the following order: O^6-ethylguanine, 7-ethylguanine > 3-ethyladenine > 3-ethylguanine, 3-ethylthymine > 1-ethyladenine, 7-ethyladenine, 3-ethylcytosine. Ethylation of the bases only accounts for 30% of the total ethylation in the case of ethylnitrosourea. The remaining 70% of the [^{14}C]ethyl groups, introduced in vivo and in vitro, are in the form of phosphotriesters which after perchloric acid hydrolysis are found as [^{14}C]ethanol and [^{14}C]ethyl phosphate. In contrast, phosphotriesters amounted to only 8–20% of total ethylation in in vivo or in vitro diethyl sulfate and ethyl methanesulfonate treated HeLa cell DNA, and 25% of the total methylation in in vitro methylnitrosourea treated HeLa cell DNA. Alkylation at the N-7 and N-3 positions of purines in DNA destabilizes the glycosidic linkages. Part of 7-ethylguanine and 3-ethyladenine are found to be spontaneously released during the ethylation reaction. Incorporation of the ^{14}C of the alkylating agents into normal DNA bases of HeLa cells can be eliminated by performing the alkylations, in the presence of cytosine arabinoside, for 1 hr.

Different classes of alkylating agents differ in their affinity for a site on a nucleic acid. There are three types of alkylation that can occur in a nucleic acid, namely, base, ribose, and phosphate alkylation. However, in the case of DNA, ribose alkylation is not possible. Methylation of the bases in DNA has been studied extensively by using a variety of methylating agents both in vitro (Lawley and Brookes, 1963; Lawley et al., 1969; Lawley and Thatcher, 1970; Lawley et al., 1973; Lawley and Shah, 1973; Margison and O'Connor, 1973) and in vivo, either in animals (Lawley et al., 1968; Craddock, 1973; O'Connor et al., 1973; Kleihues and Magee, 1973) or in tissue culture (Lawley and Thatcher, 1970; Walker and Ewart, 1973). Phosphate alkylation was not studied in detail until Rhaese and Freese (1969) found that methyl methanesulfonate and ethyl methanesulfonate at neutrality rapidly alkylated the phosphate of dTMP and oligo(dT) forming phosphotriesters. Bannon and Verly (1972) later reported that not only were phosphotriesters quite stable in alkylated DNA, but also presented data that treatment of T7 phage or T7 phage DNA with ethyl methanesulfonate caused formation of 15 times more triesters than treatment with methyl methanesulfonate (Bannon and Verly, 1972; Verly et al., 1974).

Singer and Fraenkel-Conrat (1975) have treated TMV-RNA with diethyl sulfate, ethyl methanesulfonate, and ethylnitrosourea and found that these ethylating agents differed in their specificity. Diethyl sulfate and ethyl methanesulfonate predominantly ethylated the bases and produced relatively small amounts of O^6-ethylation on guanine and phosphate ethylation. Ethylnitrosourea, on the other hand, had a strong affinity for oxygen and O^6-ethylguanine was the major product of base ethylation. Moreover phosphotriesters represented up to 60% of the total ethylation. In this work we are dealing with the effects of these three types of ethylating agents acting on DNA. HeLa cell DNA has been treated with these reagents in vitro and in tissue culture. In both cases, the extent and sites of base ethylation and phosphate ethylation were determined and the reagent specificity was studied.

When Goth and Rajewsky (1974a,b) investigated the carcinogenic effect of ethylnitrosourea in animals they found that base ethylation amounted to only 20–30% of the total radioactivity, while the remainder (70–80%) which has not been described by other investigators, was not bound to the cation-exchange column and thus possibly represented phosphotriesters. The present study on ethylation of HeLa cell DNA in vitro and in vivo with ethylnitrosourea identifies such products as phosphotriesters.

We also present evidence for the first time that a significant part of the total 7-ethylguanine and 3-ethyladenine is released from DNA during the period of ethylation.

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Experimental Section

Materials. Dulbecco's modified Eagles (DME) medium and calf serum were obtained from the Grand Island Biological Co. Cytosine arabinoside hydrochloride was obtained from the Sigma Chemical Co. Ethyl phosphate was obtained from K & K. [^{14}C]Diethyl sulfate (4.81 Ci/mol) was obtained from ICN. [^{14}C]Ethyl methanesulfonate (5.3 Ci/mol) was obtained from Radiochemical Centre in Amersham. [^{14}C]Ethylnitrosourea (4.1 Ci/mol) was obtained from Farbwerke Hoechst AG, Germany. [^{3}H]Methylnitrosourea (17.5 Ci/mol) was obtained from New England Nuclear. Salmon sperm DNA was obtained from Sigma Chemical Co. All enzymes used were from Worthington Chemical Co. The ethylated purine and pyrimidine bases and nucleosides used as uv absorbing markers were made in this laboratory according to Singer (1972), Sun and Singer (1974), Singer et al. (1974), and Singer and Fraenkel-Conrat (1975). 3-Ethylthymidine was prepared using a modification of the method used by Farmer et al. (1973) to prepare 3-methylthymidine.

Isolation of DNA from HeLa Cells. HeLa cells were grown in DME medium containing 2% calf serum. When confluent, the cells ($1-5 \times 10^8$ cells) were trypsinized and resuspended in 3–5 ml of 10 m*M* pH 7.4 Tris buffer containing 15 m*M* KCl, 3 m*M* $MgCl_2$, and 100 μg/ml of sodium heparin. Triton X-100 was added to 1% final concentration. After the mixture was vigorously shaken for 2 min, the nuclei were pelleted at 2000 rpm for 5 min. All the above steps were carried out at 4°. DNA was extracted from the nuclei by the following procedure. The nuclei were washed once in a small amount of pH 8.5 buffer containing 0.1 *M* NaCl, 0.05 *M* Tris-chloride, 0.05 *M* Na_2EDTA, and 1% (v/v) 2-mercaptoethanol, and resuspended in 2 ml of the same buffer per 10^7 nuclei. Ten percent sodium dodecyl sulfate was added to 0.3% final concentration and the mixture was stirred at room temperature until the nuclei had lyzed. Pronase which had been predigested at 45° 1 hr was added to the viscous lysate (to 0.5 mg/ml final concentration) which was then digested at 45° for 30 min. The mixture was cooled to room temperature and then extracted three times with an equal volume of phenol which had been equilibrated with the same buffer used for washing nuclei. Fibrous DNA was obtained from the aqueous layer by precipitation with two volumes of 95% ethanol, then washed in 70% ethanol containing 0.1 *N* sodium acetate, and redissolved in 10 ml of a tenfold dilution of standard sodium chloride and sodium citrate (SSC, 0.15 *M* NaCl–0.015 *M* sodium citrate). The DNA solution was incubated at 37° for 1 hr with 100 μg/ml of preheated pancreatic ribonuclease (80–100°, 10 min) and reextracted in SSC with an equal volume of phenol until there was very little interphase. DNA was finally obtained by precipitating the aqueous layer with two volumes of 95% ethanol, washed in 70% ethanol containing 0.1 *N* sodium acetate, and redissolved in a small amount of appropriate buffer.

In Vitro Ethylation and Methylation of DNA. HeLa cell DNA was isolated as described above. To the DNA (1 mg) dissolved in 1 ml of 0.5 *M* cacodylate buffer (pH 7.3) was added 20 μl of [^{14}C]diethyl sulfate, or 20 μl of [^{14}C]ethyl methanesulfonate, or 20 mg of [^{14}C]ethylnitrosourea, or 1.2 mg of [^{3}H]methylnitrosourea. The mixture was incubated at 37° for 3 hr, then freed of excess radioactive reagent by repeated precipitation with 95% ethanol containing 0.25 *M* sodium acetate. Constant specific activity of the alkylated HeLa cell DNA was achieved after four or five ethanol precipitations.

Ethylation of salmon sperm DNA with unlabeled reagents was as follows: 50 mg of salmon sperm DNA dissolved in 10 ml of pH 7.0 40 m*M* sodium acetate was treated at room temperature with 0.2 ml of diethyl sulfate for 4 hr. The pH of the reaction mixture was maintained at neutrality by addition of 1 *N* NaOH in a Radiometer pH-Stat. DNA was freed of reagent by precipitating twice with two volumes of 95% ethanol containing 0.25 *M* sodium acetate and redissolved in 10 ml of 5 m*M* potassium phosphate buffer (pH 7.0). Both supernatants were combined, desalted by charcoal (2 mg of charcoal/$A_{260\ nm}$ unit) according to Tsuboi and Price (1959) except that 0.1 *N* HCl was used for absorption. Elution from charcoal was in 50% ethanol containing 4 *N* NH_4OH.

In Vivo Ethylation of HeLa Cells. HeLa cells (at a density of 10^7 cells/ml) (20 ml) in DME medium containing 5% calf serum was treated with 100 μg/ml of cytosine arabinoside at 37° for 2 hr.

Radioactive labeling by ethylating agents was carried out with (a) 100 μl of [^{14}C]diethyl sulfate dissolved in 0.2 ml of dimethyl sulfoxide, or (b) 60 μl of [^{14}C]ethyl methanesulfonate dissolved in 0.2 ml of 95% ethanol, or (c) 50 mg of [^{14}C]ethylnitrosourea dissolved in 1 ml of citric phosphate buffer (pH 6.0). Each reagent was mixed with 2–3 ml of DME medium containing 5% calf serum and 100 μg/ml of cytosine arabinoside hydrochloride. This mixture was then added to the inhibitor treated cell suspension. After 1–4 hr at 37°, the cell suspension, in which the cells retained their normal appearance (without lysis occurring), was diluted with three volumes of cold Dulbecco's buffer (140 m*M* NaCl, 27 m*M* KCl, 8.1 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 0.9 m*M* $CaCl_2$, and 0.5 m*M* $MgCl_2$) and the cells collected by centrifuging. After washing the cell pellet with cold Dulbecco's buffer the DNA was immediately isolated as described above. In the case of labeling with ethylnitrosourea, the radioactivity in the cell lysate was used to calculate that the actual concentration of reagent in the cell was about 150 μg/ml containing 1 mg of DNA.

Methods for Degradation of Ethylated DNA. Several methods of hydrolysis, in various combinations, were used in order to obtain quantitative data for all the presently known ethyl bases in DNA. For each method 0.5–1.5 mg of ^{14}C-ethylated DNA ($0.5-1 \times 10^5$ cpm) or 50 mg of ethylated salmon sperm DNA was used. The hydrolysis procedures were as follows.

Method I was neutral heating (0.005 *M* pH 7 phosphate buffer, 100°, 20 min) to release N^3- and N^7-ethylpurines.

Method II was mild acid heating (0.1 *N* HCl, 70°, 30–40 min) which completely depurinates DNA.

Method III was enzyme digestion of the residual DNA from Method I followed by hydrolysis in 1 *N* HCl. The DNA was dissolved in 0.25 *M* pH 8 ammonium bicarbonate containing 0.01 *M* $MgCl_2$, and digested with deoxyribonuclease (1:20 by weight) at 37° for 30 min, then further digested to nucleotides by two additions of snake venom phosphodiesterase (1:20 by weight) over an 18 hr, 37° incubation period. The digest was evaporated to dryness, redissolved in 35–50 μl of 1 *N* HCl, and heated at 100°, 5 min. This method yields purine bases and pyrimidine deoxynucleotides.

Method IV was the same enzyme digestion as used in method III, followed by alkaline phosphomonoesterase digestion (1:20 by weight) at 37° for 18 hr. The products are

purine and pyrimidine deoxynucleosides.

Method V was hydrolysis with 70% perchloric acid (25 μl, 100°, 1 hr). Under these conditions purines and pyrimidines are completely released as bases. The only ethyl base which cannot be determined with this method is O^6-ethylguanine which is deethylated by strong acid (Lawley and Thatcher, 1970). Most of the ethyl groups bound to the oxygens of phosphodiesters are also under these conditions released in the form of ethanol (Singer and Fraenkel-Conrat, 1975).

Methods for the Separation and Identification of Ethyl Derivatives. Either one- or two-dimensional descending paper chromatography on Whatman 3 MM paper was carried out with the following solvent systems: (I) 1-butanol–NH_3 (specific gravity 0.88)–H_2O (85:2:13, v/v), (II) methanol–concentrated HCl–H_2O (70:20:10, v/v), (III) 1-butanol–ethanol–H_2O (80:10:25, v/v), (IV) 1-butanol–glacial acetic acid–H_2O (50:20:30, v/v), and (V) 2-propanol–NH_3 (specific gravity 0.88)–H_2O (70:20:10, v/v); or on Whatman paper No. 1 with (VI) ethyl acetate–formic acid (90%)–H_2O (70:20:10, v/v). Paper electrophoresis was performed on Whatman 3MM paper in 0.05 *M* pH 5.7 ammonium formate at 1000 V for 3 hr/30 cm.

A diagram representing R_{Ade} values of many ethyl bases and ethyl pyrimidine nucleotides and nucleosides in a two-dimensional paper chromatographic system using solvent system I followed by solvent system II has been shown by Singer and Fraenkel-Conrat (1975).[1] With the additional R_{Ade} values in the two dimensions respectively of thymine (1.5, 2.0) and 3-ethylthymine (2.9, 2.6), this diagram was used to identify the ethylated products from DNA.

The general scheme for the various separations used to obtain quantitative data is shown in Figure 1. The scheme indicates, by underlining, the procedures which give the best separations and least degradation of each derivative.

Various nonradioactive ethyl derivatives were used as ultraviolet absorbing markers added to hydrolyzed DNA samples prior to separation procedures. The usual markers added were, for supernatants from neutral heating, 3-ethylguanine, 7-ethylguanine, 1-ethyladenine, 3-ethyladenine, and 7-ethyladenine; for HCl hydrolysates with or without prior enzyme digestion, all the above markers plus N^6-ethyladenine and O^6-ethylguanine; for $HClO_4$ hydrolysates, all the above markers plus 3-ethylcytosine and 3-ethylthymine; and for enzyme digests, O^6-ethyldeoxyguanosine, N^6-ethyldeoxyadenosine, and 3-ethylthymidine.

After observing and marking uv absorbing areas on a two-dimensional chromatogram, the entire chromatogram was cut into strips (0.5 × 1 cm to 1 × 3 cm), and counted in 5 ml of toluene containing 4 g of Omnifluor/l, using a Beckman liquid scintillation counter. One-dimensional chromatograms and electrophoresis paper were similarly strip-counted. The coincidence of the radioactivity with a uv absorbing marker indicates the nature of the derivative. The amount of each derivative was calculated from the total radioactivity associated with an authentic marker. Those paper strips containing radioactive materials, which were to be further analyzed, were washed three times in toluene, dried, and eluted in H_2O.

Detection of Ethyl Phosphate. The two-dimensional chromatographic system described by Bannon and Verly (1972) (solvents IV and V) was used to determine [^{14}C]ethyl phosphate in perchloric acid hydrolysates. Nonradioactive ethyl phosphate was cochromatographed as internal marker. Its position on the two-dimensional chromatogram was detected according to Bandurski and Axelrod (1951).

Nitrous Acid Deamination. Samples to be deaminated were heated in 0.5 ml of 1 *N* HCl containing 12.5 mg of sodium nitrite for 3 hr at 80–90°. The products of deamination were resolved on Whatman No. 1 paper in solvent VI.

Results

Spontaneous Release of Ethylated Purines. Since alkylation at the N-7 and N-3 positions of purines in DNA is known to destabilize the glycosidic linkages (Lawley et al., 1973) we attempted to isolate any 7-ethylguanine or 3-ethyladenine released from DNA during the actual period of ethylation. The ethanol supernatant after precipitating 50 mg of salmon sperm DNA treated with diethyl sulfate at pH 7.0 for 4 hr at room temperature was found to contain 4 to 6 A_{260nm} units of uv absorbing material. After desalting (see Experimental Section), over 75% of the absorbancy was recovered and, upon chromatography, 7-ethylguanine and 3-ethyladenine, as well as small amounts of unidentified materials, were found.

Comparing the amount of 7-ethylguanine and 3-ethyladenine released from completely depurinated DNA with the amount of these derivatives spontaneously released, it was found that 16% of the total 7-ethylguanine and 29–45% of the total 3-ethyladenine were in the fraction spontaneously released during the ethylation process. Consequently the amounts of these derivatives found in hydrolysates of alkylated DNA are erroneously low. No corrections for such losses have been made in the data presented in Tables I and II.

Although it was not possible to measure the loss of these ethylpurines when HeLa cell DNA was ethylated in vivo or in vitro with radioactive agents at the level of 1–3 mCi, the evidence found with nonradioactive diethyl sulfate acting on salmon sperm DNA suggested that in either case HeLa cell DNA had probably lost much of the 3-ethyladenine and some 7-ethylguanine. Due to additional specific enzyme excision demonstrated both in vitro (Kirtikar and Goldthwait, 1974) and that can occur in cells (Lawley and Orr, 1970; Margison and O'Connor, 1973; Craddock, 1973), the loss of these labile ethylpurines, as well as of O^6-ethylguanine, may be even higher in vivo than in vitro.

Products of in Vitro Ethylation of HeLa Cell DNA by [^{14}C]Diethyl Sulfate, [^{14}C]Ethyl Methanesulfonate, [^{14}C]Ethylnitrosourea, and [^{3}H]Methylnitrosourea. The extent of ethylation of HeLa cell DNA in vitro by the three ethylating reagents under the same conditions is shown in Table I. The reactivity was consistently in the order of diethyl sulfate < ethyl methanesulfonate < ethylnitrosourea. The sites of ethylation were determined using the schemes in Figure 1 as well as other described procedures. The ethyl products were separated and quantitated using the chromatographic methods described in the Experimental Section. Table I also shows the distribution of these ethyl derivatives from different hydrolysates of the ethylated HeLa cell DNA. Ideally the total amount of each derivative found with methods I, III, and IV should be the same as that for method V, with the exception of O^6-ethylguanine.

Heating a neutral solution of ethylated HeLa cell DNA (method I) released variable amounts of N^7- and N^3-ethylpurines. Quantitatively, only 3-ethyladenine was completely depurinated. Of the others, 87–91% of the total 7-ethylguanine, 83–85% of the total 7-ethyladenine, and 50–78% of

[1] Figure 2 is reproduced from Singer and Fraenkel-Conrat (1975).

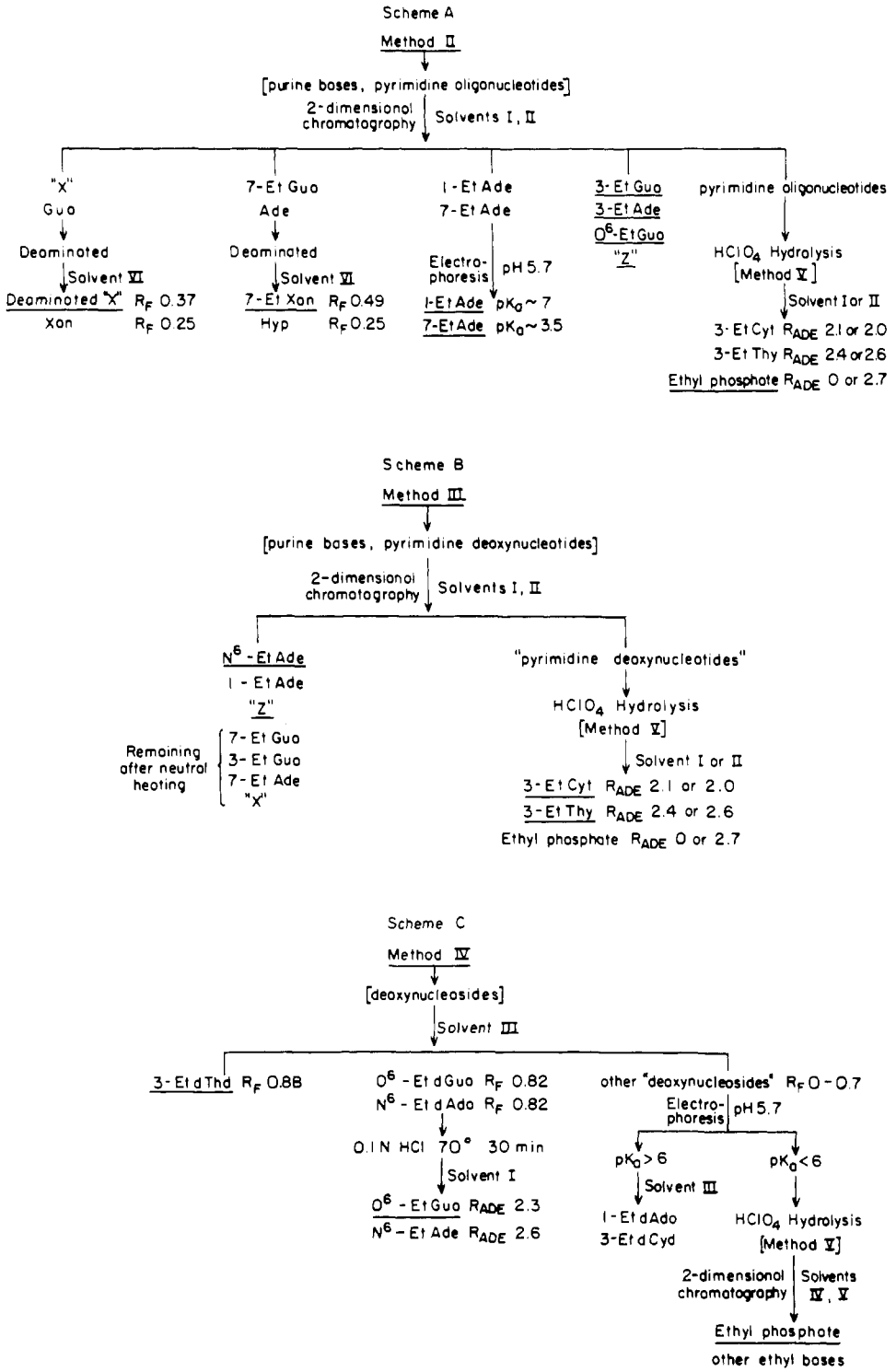

FIGURE 1: The general schemes for hydrolysis of ethylated DNA and subsequent separation of ethylated deoxynucleotides, deoxynucleosides, and bases. Hydrolysis methods and chromatographic and electrophoretic techniques are given in the Experimental Section. Before proceeding to Schemes B and C, the DNA is heated in neutral solution (method I, Experimental Section) which depurinates most of the N-3 and N-7 purines, as well as the unknown derivative "X". Solvents I and II separate these purine derivatives (Singer and Fraenkel-Conrat, 1975). Ethyl phosphate was separated from ethyl bases in solvent I and further characterized by a two-dimensional chromatographic system in solvents IV and V. Underlining denotes preferred methods for determining the amount of derivatives.

the total 3-ethylguanine were depurinated. More than 90% of the unknown derivative "X", found only in ethyl methanesulfonate treated DNA, was also released upon neutral heating. Complete quantitation of these derivatives was obtained by further depurination in 0.1 *N* HCl (method II).

Complete degradation of the ethylated DNA was achieved only by perchloric acid hydrolysis (method V). However, appreciable amounts of radioactivity were found as [^{14}C]ethanol (and [^{3}H]methanol in the case of methylnitrosourea), the major source of which will be discussed later in this section. A small portion of the recovered alcohol originated from O^6-alkylguanine which is dealkylated by strong acid. Therefore this derivative could only be determined in enzyme digests (method IV) or after mild acid

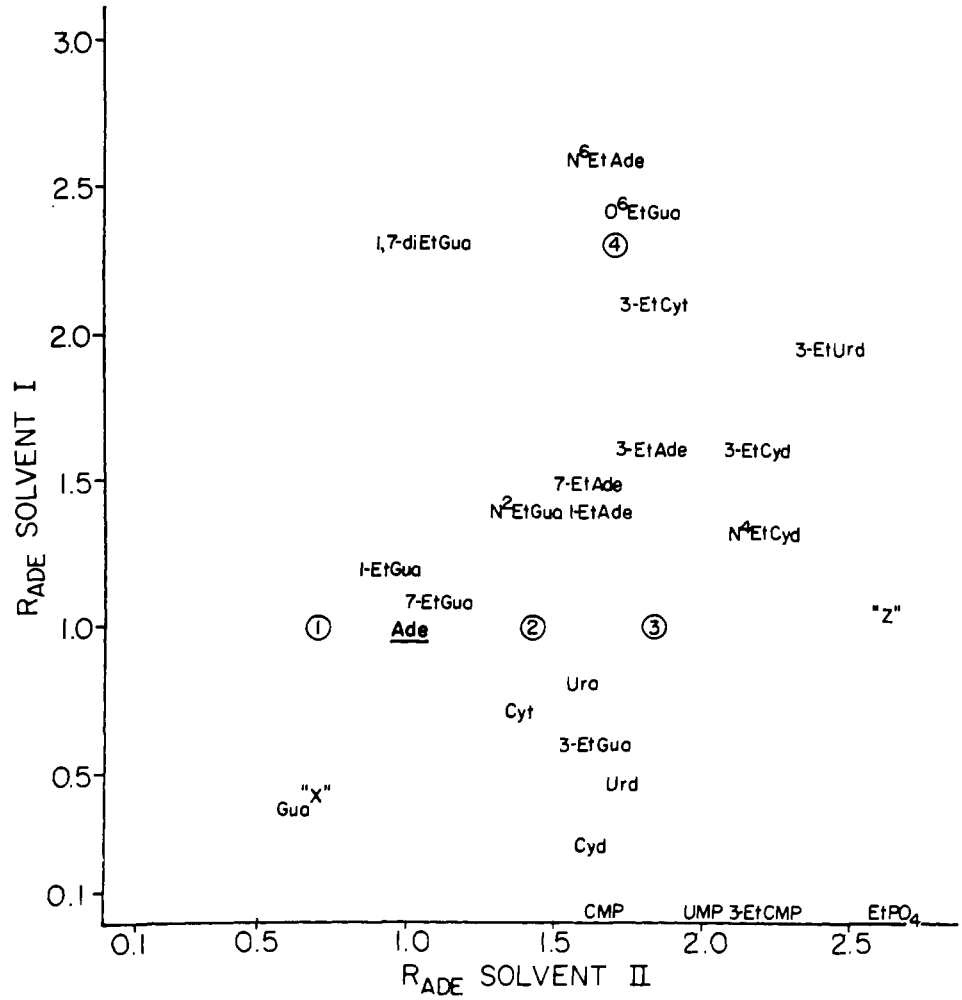

FIGURE 2 (Singer and Frankel-Conrat, 1975): Diagram of R_{Ade} (chromatographic behavior relative to adenine) values in solvents I and II. See Experimental Section for the chromatographic methods and preparation of the derivatives. Circled positions 1 and 2 refer to imidazole ring-opened 7-ethylguanine 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.

depurination (method II). Heating alkylated DNA in perchloric acid completely releases stable alkyl bases, deoxyribose and phosphate. It not only allows a direct quantitation of some alkyl pyrimidines, but also differentiates the types of alkylation formed in DNA; 5–18% of the total ethyl groups were found in the $HClO_4$ hydrolysate as [^{14}C]ethyl phosphate (Table I) indicating that some of the phosphodiester groups in the DNA were ethylated.

Examining only the ethylation of the bases, the extent of the nitrogen and oxygen ethylation was found to vary according to the types of ethylating agents used; 83–87% of the total ethylation was on the purine bases when diethyl sulfate and ethyl methanesulfonate acted on HeLa cell DNA. Of this, 67–75% was on the N-7 of guanine. O^6-Ethylguanine amounted to a barely detectable level (0.2%) using the techniques described. On the other hand, only 30% of the total ethylation was on the purine bases when ethylnitrosourea acted on HeLa cell DNA, with the O^6-ethylation (12%) being equivalent to the N-7 ethylation (11%) on guanine.

Methylnitrosourea was considerably less effective in alkylating oxygens and 25% of the total methyl groups were on phosphates. The other major products were 4% O^6-methylguanine, 60% 7-methylguanine, and 8% 3-methyladenine.

Ethylpyrimidines, determined by method II followed by III and IV, only amounted to <1% of the total ethylation. The residual radioactivity was likely to be present as ethyl groups bound to the deoxyribose chain. When ethylnitrosourea acted on HeLa cell DNA, 71% of the total radioactivity was found bound to the apurinic deoxyoligonucleotides, all of which was recovered after phosphodiesterase and phosphomonoesterase digestion. However, 1 *N* HCl hydrolysis of these oligonucleotides released three-quarters of the radioactivity as [^{14}C]ethanol. Upon perchloric acid hydrolysis, more [^{14}C]ethanol was released, and the residual radioactivity was almost entirely [^{14}C]ethyl phosphate, indicating that the original oligonucleotides contained phosphotriesters. Additional evidence for the phosphotriesters was obtained by further enzymatic degradation of the ethylated DNA, which had already been depurinated by neutral heating. No bound ethyl groups were found to be destabilized since [^{14}C]ethanol was not detected. After separating O^6-ethyldeoxyguanosine, N^6-ethyldeoxyguanine, and 3-ethylthymidine (by chromatography in solvent III), the bulk of the radioactivity, which represented about 73% of the total radioactivity associated with ethylnitrosourea treated HeLa cell DNA, behaved like phosphotriesters chromatographically (Singer and Fraenkel-Conrat, 1975). When these phosphoalkyl dinucleosides were further de-

Table I: Distribution of Ethyl Products from Hydrolysates of HeLa Cell DNA Reacted in Vitro with [^{14}C]Diethyl Sulfate, [^{14}C]Ethyl Methanesulfonate, and [^{14}C]Ethylnitrosourea.[a]

	Reagent							
	Diethyl Sulfate			Ethyl Methanesulfonate			Ethylnitrosourea	
Hydrolysis Method	I	III, IV	V	I	III, IV	V	II	V
Identified ethyl bases[b]								
1-EtAde		2.3[c]	2.4[c]		2.1[c]	2.3[c]	0.2	0.3
3-EtAde	11		9.1	4.7		4.1	3.7	4.7
7-EtAde	1.1	0.2	1.7	1.0	0.2	1.6	0.2	0.3
3-EtGua	0.3	0.3	1.7	0.7	0.2	0.6	0.6	0.6
O^6-EtGua		0.2	0		0.2	0	12	
7-EtGua	62	6	66	64	10	76	9.7	12
3-EtCyt		0.6	0.9		0.8	0.7	0.2	0.3
3-EtThy		n.d.	n.d.		n.d.	n.d.	1	0.6
Other ethyl products								
Ethyl phosphate		11	10		9	8	18	5
[^{14}C]Ethanol		4	7[d]		4	5[d]	53	75[d]
Unidentified								
"X"				1.9	0.2	1.5		
"Z"		1.2	0.9		1.2	0.5	2.2	0.8

[a] Reaction conditions and hydrolysis methods I–V are described in the Experimental Section. The level of ethylation for each reagent in this experiment was: diethyl sulfate 0.95 M/100 M P; ethyl methanesulfonate 1.1 M/100 M P; and ethylnitrosourea 1.7 M/100 M P. Data are presented as percent of total ethylation. [b] For purposes of simplicity, all data are presented for bases although, depending on the method used, derivatives were at times isolated as deoxynucleotides or deoxynucleosides. See Experimental Section for chromatographic methods. n. d. indicates that the derivative could not be detected. [c] The yield, higher than expected for a double-stranded DNA, was probably due to the degree of denaturation. Any N^6-ethyladenine, which is formed by rearrangement of 1-ethyladenine under the relatively alkaline enzyme digestion conditions used, was included in this figure. [d] This figure includes the deethylation product from O^6-ethylguanine.

Table II: Summary of Distribution of Ethyl Products Formed in HeLa Cell DNA Treated with [^{14}C]Diethyl Sulfate, [^{14}C]Ethyl Methanesulfonate, and [^{14}C]Ethylnitrosourea in Vitro and in Vivo.[a]

	% of Total Ethylation					
	in Vitro			in Vivo		
Derivative	Diethyl Sulfate	Ethyl Methanesulfonate	Ethylnitrosourea	Diethyl Sulfate	Ethyl Methanesulfonate	Ethylnitrosourea
1-EtAde	2	2	0.2	0.3	0.1	0.1
3-EtAde	10	4.4	4.2	4	2.2	4.6
7-EtAde	1.5	1.4	0.2	0.5	0.7	1.3
3-EtGua	0.9	0.7	0.6	0.2	0.4	1.5
O^6-EtGua	0.2	0.2	12	1.6	0.3	7.5
7-EtGua	67	75	11	71	81	17
3-EtCyt	0.7	0.8	0.2	0.5	0.1	0.3
3-EtThy	n.d.[b]	n.d.[b]	0.8	0.2	0.1	0.4
"Ethyl phosphate"[c]	16	13	70	20	8	66
Unidentified derivatives	1	2.5	1.5	2	7	1

[a] This summary for in vitro ethylation is based on the data in Table I. In vivo ethylated DNA was analyzed using the same methods and additional analyses necessary when metabolic labeling occurred (Figure 1, Scheme A). The analysis for each product judged to be most accurate was used (Figure 1), as well as averages in many cases. [b] n. d. indicates the derivative could not be detected. [c] This figure represents the total ethylation on phosphates. Any [^{14}C]ethanol released, except that originating from O^6-ethylguanine, is included.

graded by perchloric acid hydrolysis, more than three-quarters of the radioactivity was found as [^{14}C]ethanol and almost all of the rest as [^{14}C]ethyl phosphate. In contrast, when diethyl sulfate and ethyl methanesulfonate acted on HeLa cell DNA, only 13–17% of the total ethylation was on phosphate (Table I).

Products of in Vivo Ethylation of HeLa Cell DNA by [^{14}C]Diethyl Sulfate, [^{14}C]Ethyl Methanesulfonate, and [^{14}C]Ethylnitrosourea. The extent of ethylation of HeLa cell DNA in vivo was much less than in vitro and ranged from 0.03 to 0.14 mM C_2H_5/100 mM P whereas in vitro 1–2 C_2H_5/100 mM P were introduced. However sufficient radioactivity was introduced so that the same techniques of hydrolysis and separation of products could be applied as were used for the analysis of ethyl products from in vitro treated DNA. The accuracy of determining the minor products is, however, less. The data obtained are summarized in Table II. The general pattern of products follows that obtained in in vitro experiments. The proportion of alkylation on the phosphates, as contrasted to the bases, is very similar. Again only ethylnitrosourea preferentially alkylates the phosphates as well as forming relatively much O^6-ethylguanine.

Incorporation of the Radioactivity of Alkylating Agents by Normal Biosynthetic Pathways. In addition to direct chemical alkylation of cellular constituent, the ^{14}C label of alkylating agents can appear in one-carbon and other pools as a result of ordinary metabolic reaction (Whittle, 1969). Radioactivity is then also incorporated into newly synthesized nucleic acids. Efforts were made to diminish metabol-

ic incorporation into HeLa cell DNA by means of cytosine arabinoside as a DNA synthesis inhibitor. When cytosine arabinoside was added to 100 μg/ml of growth medium, the metabolic labeling in DNA from HeLa cells, either in suspension or in monolayers, treated with [^{14}C]diethyl sulfate was nondetectable after 1 hr but after 4 hr of treatment about 20% of the label was in the form of unalkylated purines.

Similarly when HeLa cells were treated with [^{14}C]ethyl methanesulfonate for 1 hr in the presence of cytosine arabinoside, metabolic labeling was completely absent, but after 4 hr represented over 50% of the radioactivity. No metabolic labeling of DNA was found after 1 hr treatment with [^{14}C]ethylnitrosourea. In general the extent of alkylation of DNA was almost maximal after 1 hr.

Since our attempts to inhibit RNA synthesis, by means of actinomycin D, during the alkylations of HeLa cells were not successful (probably because the inhibitor became alkylated), the labeling of normal RNA purines was very high (up to 90% of the total incorporated radioactivity) even with 1 hr of labeling. For this reason, the RNA from alkylated HeLa cells was not analyzed for alkyl bases.

When there was radioactivity in adenine and guanine of HeLa cell DNA, it was chromatographically coincident with 7-ethylguanine and the unidentified derivative "X", respectively. Separation of ethyl bases could, however, be achieved after deamination by heating the pairs of unresolved purines (adenine and 7-ethylguanine, and guanine and "X") in HNO_2 80–90° for 3–4 hr. Chromatography on Whatman No. 1 paper in solvent VI resolved these two pairs of deaminated derivatives: hypoxanthine (R_f 0.25) from 7-ethylxanthine (R_f 0.49), and xanthine (R_f 0.25) from the presumed deaminated derivative of "X" (R_f 0.37). The radioactivity found in hypoxanthine and xanthine thus gave an estimate of the extent of metabolic labeling.

Discussion

The three ethylating agents used in this work were chosen because they (and the analogous methylating agents) have been reported to differ greatly in their mutagenicity (Freese and Freese, 1966; Singer and Fraenkel-Conrat, 1969; Veleminsky et al., 1970; Ehrenberg, 1971; Bresler et al., 1972) and carcinogenicity (Swann and Magee, 1968, 1969, 1971; Druckrey et al., 1970; Searle and Jones, 1972). It was hoped that there would be analytically significant differences in their specificity toward various possible sites in DNA which could be correlated with their biological effects.

Parallel work of this laboratory (Singer and Fraenkel-Conrat, 1975) on the ethylation of RNA indicated that with diethyl sulfate or ethyl methanesulfonate, the pattern of alkylation was quite similar to that obtained under the same conditions with the analogous methylating agents, dimethyl sulfate and methyl methanesulfonate, except that ethylation on the O^6 of guanine occurred to a low extent, and, with ethyl methanesulfonate, a moderately effective carcinogen, about 10% of the ethyl groups were in the form of phosphotriesters. Much more striking was the fact that ethylnitrosourea, a very potent carcinogen, ethylated the O^6 of guanine more than the N-7 of guanine and that about 60% of the ethyl groups were on phosphates. Methylnitrosourea had much less affinity for oxygens and of the total methyl groups introduced into HeLa cell DNA, 25% were found as phosphotriesters.

When HeLa cells were treated with the same three ethylating agents, either in monolayers or in suspension, the distribution of ethyl derivatives in the DNA was very similar to that found upon in vitro treatment of HeLa cell DNA, as well as of TMV-RNA. That is, while about 8–20% of the ethyl groups were on phosphates after diethyl sulfate or ethyl methanesulfonate treatment, only ethylnitrosourea had a particularly strong affinity for alkylating oxygens; 8–12% of the total ethylation was on the O^6 of guanine and 65–70% on the phosphates, forming stable alkyl phosphotriesters. The fact that the pattern of in vivo and in vitro ethylation of DNA is so similar (Table II) suggests that the ethylating agents enter the cell and act in their original form. The hypothesis that alkyl nitrosoureas act through intermediate products of metabolism (Kleihues and Magee, 1973) is not supported by our data which show that in vivo and in vitro ethylnitrosourea ethylates DNA with the same specificity (Table II). This is in line with the in vivo work of Lijinsky et al. (1972) and the in vitro work of Lawley and Shah (1973) who arrived at the conclusion, on the basis of mass spectra and radioactivity measurements, that the methyl group of methylnitrosourea is transferred intact and not through diazomethane. Furthermore, while TMV-RNA and double-stranded DNA offer some different sites for alkylation on the bases (N-1 of adenine and N-3 of cytosine are unreactive in native DNA), the affinity of ethylnitrosourea for the O^6 of guanine and the oxygen of the phosphodiester in both RNA and DNA appears to be unaffected by hydrogen bonding or secondary structure.

Our finding that most of the ethylation by ethylnitrosourea is on phosphates is new inasmuch as we present direct and quantitative chemical evidence for phosphotriester formation. However, in recent years, a number of investigators have observed that in [^{14}C]methylnitrosourea and [^{14}C]ethylnitrosourea treated DNA, both in vitro and in vivo, various proportions of the radioactivity are not recovered as alkyl bases and expressed the possibility that this "pyrimidine-oligonucleotide-like" fraction may contain phosphotriesters (Walker and Ewart, 1973; Goth and Rajewsky, 1974b; Lawley et al., 1973). And indeed, phosphotriesters have been quantitated in DNA isolated from T7 DNA and T7 phage treated with methyl methanesulfonate and ethyl methanesulfonate (Bannon and Verly, 1972; Verly et al., 1974) and amount to about 1 and 15%, respectively.

Goth and Rajewsky's (1974b) "early peak" from the DNA of ethylnitrosourea treated rats (or from in vitro treated DNA) amounts to 70–80% of all bound radioactivity (Dr. M. F. Rajewsky, private communication) and agrees with our figures of 66–80% phosphotriester formations in HeLa cell DNA, strongly suggesting that this material corresponds to our phosphotriesters.

After it was generally agreed that the amount or presence of 7-alkylguanine did not correlate with mutagenesis and/or carcinogenesis, interest focused on 3-alkylcytosine and O^6-alkylguanine, both of which, in polynucleotides, led to mispairing (reviewed by Singer, 1975) and might be of biological importance. However, there is little alkylation of cytosine in DNA and the amount of O^6-alkylguanine formed upon reaction with carcinogens did not vary in target tissues vs. nontarget tissues (Kleihues and Magee, 1973; Goth and Rajewsky, 1974a; den Engelse, 1974; Frei and Joshi, 1974). Additional hypotheses were proposed which postulated that O^6-alkylguanine is enzymatically released

at a lower rate in target tissues and thus remains to act by mispairing (Goth and Rajewsky, 1974a).

We find that the formation of phosphotriesters in DNA can, in the case of the ethylating agents, be correlated with the available data on their relative carcinogenicity. Although it is difficult to assess carcinogenicity quantitatively due to the differing methods of introducing carcinogens, it is clear that ethylnitrosourea administered orally or intraperitoneally is extremely effective in causing tumors in rats (Swann and Magee, 1971; Goth and Rajewsky, 1974a). Diethyl sulfate and ethyl methanesulfonate, administered under similar conditions, caused very few tumors (Druckrey et al., 1970; Swann and Magee, 1971). The same relative carcinogenicity was also observed with the analogous methylating agents (summarized by Swann and Magee, 1968). Our studies of in vitro alkylation of RNA indicate that the amount of phosphotriesters appears to correlate with the reported mutagenicity, in several systems, of the three ethylating agents as well as that of dimethyl sulfate and methyl methanesulfonate. In TMV-RNA, however, nitrosoureas are not mutagenic (Singer and Fraenkel-Conrat, 1975) and the reason for this apparent disagreement with the data of others who study mutagenesis of DNA is likely to be due to the relative lability of ribophosphotriesters, in contrast to deoxyribophosphotriesters which are stable.

We propose and are investigating the premise that the major chemical event occurring when DNA or RNA are reacted with ethylnitrosourea, namely alkylation of the phosphodiesters, is a major though not necessarily the only biologically important event. There are some indications from studies of dideoxynucleoside methyl and ethyl phosphotriesters that phosphate esterification leads to loss of susceptibility to enzyme hydrolysis, changes in interaction with complementary polynucleotides and conformation (Miller et al., 1971, 1974; Kan et al., 1973). The importance of the internal structure of the phosphate group is also shown by its correlation with the conformation about the ester P–O bonds of the backbone of dinucleoside phosphates (Perahia et al., 1974).

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