Influence of Hydrogen Bonding in DNA and Polynucleotides on Reaction of Nitrogens and Oxygens toward Ethylnitrosourea†

W. J. Bodell‡ and B. Singer*

ABSTRACT: The reactivity of ethylnitrosourea toward hydrogen-bonded sites in double-stranded DNA or poly(rA)·poly(rU) was compared with those sites in single-stranded DNA, RNA, or poly(rA). Alkylation of the N-1 of A in poly(rA)·poly(rU) was almost suppressed at 5 °C but could be markedly increased by raising the reaction temperature to 25 °C, well below the T_m of 56 °C. In contrast, the N-7 and N-6 of A, which are not hydrogen bonded, reacted to the same extent at temperatures ranging from 5 to 65 °C. The extent of reaction at the N-3 of A varied inversely with the reactivity of the N-1 of A, indicating that of these two nitrogens the N-1 of A is the most reactive. The proportion of reaction at the various nitrogens in poly(rA) was not affected by temperature. Hydrogen-bonded oxygens in double-stranded DNA are the O-6 of G, the O-4 of T, and the O-2 of C. All are equally reactive at 5, 25, and 51 °C. It is concluded that the observed temperature independence is due to these oxygens having an electron pair not involved in hydrogen bonding and, thus, available for reaction. In contrast, the electron pair of the N-1 of A (or the N-3 of C) is involved in hydrogen bonding, and the extent of their reactivity is dependent on thermal fluctuation providing transiently open base pairs at temperatures far below the T_m.

It has been a general concept that single-stranded and double-stranded nucleic acids differ greatly in their reactivity since those positions which are hydrogen bonded in a double strand are considered to be unavailable for reaction (Brown, 1974). To a certain extent this difference in reactivity as dependent on strandedness has been borne out by work with the simple alkylating agents, such as dimethyl sulfate, which react to a high extent with the N-3 of A[1] in a double-stranded DNA, while in a single-stranded RNA the N-1 of A is modified (reviewed by Singer, 1975). However, this specificity of reaction has not been absolute and even in the double-stranded form there is a low amount of reaction at the N-1 of A. The other position of reaction which generally differentiates single- from double-stranded polynucleotides is the N-3 of C. The explanation of the differing reactivities was clear, but in order to explain why there was incomplete suppression of reaction at a base-paired nitrogen, it was necessary to postulate that some single-stranded regions existed in the double-stranded nucleic acid.

When O^6-alkyl–G was found to be a major reaction product with certain types of alkylating agents (Loveless, 1969; Lawley & Thatcher, 1970), it also became apparent that, although the O-6 of G is hydrogen bonded to the N-4 of C in DNA, it reacts as well as in single-stranded RNA (Singer, 1976, 1979). Further work on alkylation of oxygens also indicated that the two other base-paired sites, the O-4 of T and the O-2 of C, were equally reactive in single-stranded and double-stranded nucleic acids (Singer et al., 1978b). Singer et al. (1978b) then proposed that the oxygens were reactive since they possessed an extra pair of electrons, free to react, even in a double strand. The purpose of the present experiments is to test this theory by using poly(rA)·poly(rU) and DNA as model polynucleotides.

Recent investigations from several laboratories have proposed and expanded the concept that double-stranded polynucleotides are in a dynamic state and fluctuational opening of the helix as measured by formaldehyde reaction does occur at temperatures far below the T_m (Lukashin et al., 1976; McGhee & von Hippel, 1977).

We were interested in investigating whether the reactivity of base-paired nitrogens and oxygens toward alkylation was influenced by the transient opening of the DNA helix as a function of temperature.

Materials and Methods

Treatment of DNA and Polymers. Six milligrams of purified calf thymus DNA was dissolved in 3 ml of 0.5 M pH 7.0 cacodylic buffer. Six milligrams of ethylnitrosourea (4.1 Ci/mM) dissolved in 0.15 mL of dimethyl sulfoxide was added, and the DNA samples were reacted at 5, 25, and 51 °C for 3 days, 45 min to 16 h, and 45 min, respectively. Twenty absorbancy units of poly(rA)·poly(rU) (Miles Laboratories) dissolved in 0.5 mL of 0.05 M pH 7.0 Tris-HCl, 0.1 M NaCl were mixed with 5 mg of [^{14}C]ethylnitrosourea in 0.05 mL of dimethyl sulfoxide and reacted at 5, 25, 56, and 65 °C for 3 days, 16–18 h, 45 min, and 30 min, respectively. Poly(rA) was treated similarly to poly(rA)·poly(rU). The alkylated DNA and polymers were freed from radioactive reagent by repeated ethanol precipitation until constant specific activity was achieved. The extent of reaction of DNA was 5–75 ethyls/10^5 DNA-P. Poly(rA)·poly(rU) and poly(rA) were reacted to 35–550 ethyls/10^5 P and about 100 ethyls/10^5 P, respectively.

Analysis of Alkylated DNA and Polymers. Aliquots of alkylated calf thymus DNA containing approximately 10 000 cpm were enzymatically digested, and the individual alkylation products were quantitated as previously described in detail (Singer, 1976). Under the conditions used for enzyme digestion and separation of *O*-ethyl derivatives, no detectable de-ethylation occurs (Singer et al., 1978a). *N*-Alkyl derivatives are stable (Singer, 1975).

Alkylated poly(rA)·poly(rU) and poly(rA) samples were hydrolyzed with 0.3 mL of 1 N NCl at 100 °C for 1 h, in order to depurinate adenine residues. [^{14}C]Ethanol, which was also

† From the Department of Molecular Biology and the Virus Laboratory, University of California, Berkeley, California 94720. *Received January 17, 1979.* This investigation was supported by National Institutes of Health Research Grant No. CA 12316 from the National Cancer Institute.

‡ Present address: Laboratory of Radiobiology, University of California, San Francisco, CA 94143.

[1] Abbreviations used: LC, high-pressure liquid chromatography; Et, ethyl; A, G, U, C, and T refer to the base moieties (adenine, guanine, uracil, cytosine, and thymine) in a polynucleotide or to an isolated base. Derivatives isolated from polynucleotides are abbreviated according to the system previously used (Singer et al., 1978a).

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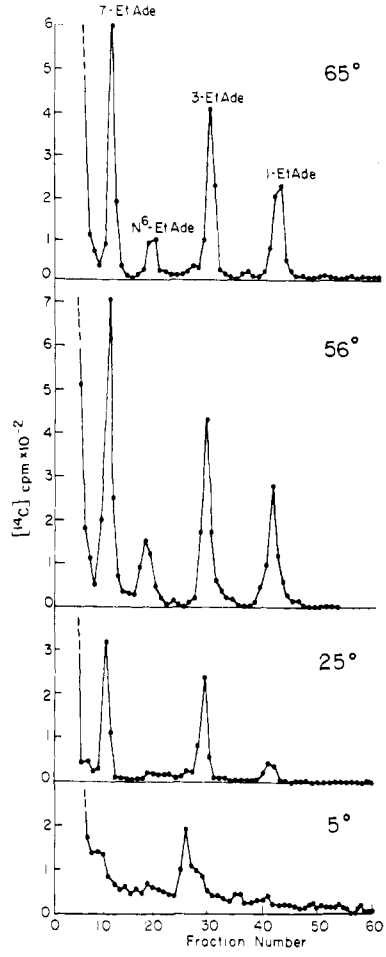

FIGURE 1: Radioactivity profiles of ethyladenines from [^{14}C]-ethylnitrosourea-treated poly(rA)·poly(rU) as analyzed by LC. See Materials and Methods for procedures. The temperature of reaction of polymers with ethylnitrosourea is shown in the figure. The positions of authentic unlabeled marker compounds is in the upper section. Fractions 1–16 are 1 mL each, while the remainder are 2 mL each.

present as a result of hydrolysis of *O*-alkyl bases and ethyl phosphotriesters (Singer & Fraenkel-Conrat, 1975; Singer et al., 1978a), was removed, after adding unlabeled ethanol as carrier, by evaporation of the ethanol in an air stream at room temperature. This procedure was repeated three times. Ammonium formate (0.4 M, pH 7.0) was added to give a final volume of approximately 90 μL. The hydrolyzed samples were chilled in an ice bath, and the pH was adjusted, by using test paper, to pH 5–7 by the stepwise addition of about 10 μL of 10 N KOH. Although 1-alkyladenine is known to rearrange to N^6-alkyladenine in alkali, this neutralization procedure did not cause detectable rearrangement, as determined by LC analysis. The other *N*-alkylpurines are stable under our conditions (Singer, 1975). The final volume of the samples was 90–100 μL.

The acid-hydrolyzed poly(rA)·poly(rU) or poly(rA) samples (70–90 μL) were analyzed on an Aminex HR-C (250 × 4 mm) LC cation exchange column (NH_4^+ form) (Bio-Rad Laboratories) at 55 °C by using 0.4 M pH 7 ammonium formate, as solvent. The column was operated at a flow rate of 0.64 mL/min and 1-mL or 2-mL fractions were collected directly into liquid scintillation vials. Fifteen milliliters of Aquasol-2 (New England Nuclear) was added to each vial, and the samples were counted in a Beckman liquid scintillation counter. The elution volume of each of the derivatives was determined by injecting authentic 1-ethyladenine, 3-ethyladenine, 7-ethyladenine, and N^6-ethyladenine (Singer et al., 1974).

Table I: Effect of Hydrogen Bonding on Reactivity of Nitrogens in Polynucleotides

polynucleotide	reaction temp (°C)	proportion of total adenosine alkylation after reaction with ethylnitrosourea[a] N-1 A	N-3 A	N-7 A	N^6 A[b]
double stranded					
salmon sperm DNA[c]	37	6	88	6	ND
poly(rU)·poly(rA)	5	<4	42	36	~17
	25	10	40	43	7
	56	23	31	37	9
	65	22	27	38	13
single stranded					
TMV RNA[d]	37	58	25	17	ND
poly(rA)	25	51	22	21	5
	56	52	23	19	6

[a] Reaction conditions and analytical methods are given under Materials and Methods. Nitrogen alkylation represented 10–25% of the total, the remainder being bound to oxygens (Singer, 1976). ND indicates that this derivative was not detected. Analyses of poly(rU)·poly(rA) and poly(A) were by LC (Figures 1 and 2). Analyses of salmon sperm DNA and TMV-RNA used paper chromatography for separation of ethylated adenines. [b] The N^6-ethyladenine was a direct product of reaction and not due to rearrangement of 1-ethyladenine. [c] Data taken from Singer (1977). [d] Data taken from Singer & Fraenkel-Conrat (1975).

Results

Reactivity of Nitrogens in Single- and Double-Stranded Polynucleotides. As a model for a double-stranded polymer, we used poly(rA)·poly(rU) which has a relatively low T_m, so that changes in reactivity as a function of temperature can easily be studied. Alkylation of this polymer with ethylnitrosourea was carried out at 5, 25, 56, and 65 °C. Since alkylation was faster at the higher temperatures, the times of reaction were adjusted in an attempt to obtain comparable ethylation levels over the wide temperature range. However, at 5°C the reaction was extremely slow and, even after 3 days of reaction, the number of bound alkyl groups did not exceed about 20% of that at the higher temperatures.

After depurination of the ethylated polymer, the released ethyladenines represented only about 10% of the total ethylation products since the primary sites of ethylation were the phosphodiesters and 2′-O of ribose as well as the O-2 and O-4 of uridine (Singer & Fraenkel-Conrat, 1975; Kušmierek & Singer, 1976; Singer & Kušmierek, 1976). The acid hydrolysis conditions used for depurination also dealkylated all of the *O*-alkyl products except the alkylated ribose which did not interfere in the subsequent analysis of *N*-ethyladenines.

In the LC system used, the first peak of radioactivity was not associated with any ethylated adenine derivative and is probably due to ribose ethylation (Figure 1). Figure 1 shows the radioactivity profiles of alkyladenines separated by using a LC cation-exchange column. In the chromatogram of poly(rA)·poly(rU) reacted at 65 °C, it is clear that the four nitrogens of adenine (N-1, N-3, N-7, N-6) are all capable of reaction and can be easily separated and quantitated (Figure 1, top panel). At the T_m of the polymer (56 °C), the radioactivity profile is very similar to that of the 65 °C reaction. However, when the temperature of reaction is decreased to 25 °C, the peak of 1-ethyladenine is markedly decreased and when reaction is at 5 °C, it is difficult to detect reaction at this position.

Table I presents the data derived from Figure 1 and other experiments. Within the experimental error, there is no effect of temperature on the reactivity of the N-7 and N-6 of A. As

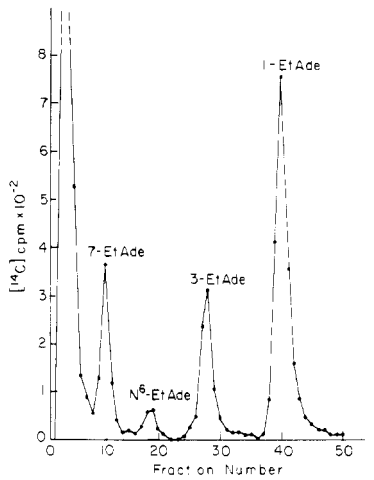

FIGURE 2: Radioactivity profile of ethylated adenines from [^{14}C]-ethylnitrosourea-treated poly(rA) as analyzed by LC. The temperature of reaction was 25 °C. The same general profile was obtained after 56 °C reaction. The methods and collection of fractions are as in Figure 1.

the temperature of reaction is increased from 5 to 65 °C, however, the formation of 3-ethyladenine increases fivefold. Over the same temperature range there is some reduction in the relative amount of 3-ethyladenine.

Poly(rA) was also alkylated with ethylnitrosourea and the proportion of the *N*-alkyladenines determined after reaction at 25 and 56 °C. The radioactivity profile from a LC analysis is shown in Figure 2. In contrast to poly(rA)·poly(rU), no difference in the relative alkylation of the N-1 and N-3 of A was found at the two reaction temperatures. Data are given in Table I. It should be noted that the relative amounts of the four ethyladenines are quite different in poly(rA) and in the fully melted out poly(rA)·poly(rU) (65 °C reaction). However, poly(rA)·poly(rU) has many reactive pyrimidines which may be competitive, and absolute comparisons are difficult.

Reactivity of Oxygens in Double-Stranded DNA. Calf thymus DNA was reacted with ethylnitrosourea at 5, 25, and 51 °C. The alkyl products previously quantitated were determined in all samples. This included ethyl phosphotriesters, 7-EtGua, 3-EtAde, O^6-EtdGuo, O^2-EtThd, O^4-EtThd, and O^2-EtCyt (Singer et al., 1978b). There appeared to be little or no difference in the proportion of any of these products as a function of temperature, notwithstanding the fact that three of the alkylated sites (O^6-G, O^4-T, O^2-C) are base-paired in double-stranded DNA. From the analytical data shown in Table II, it can be seen that the percent of *O*-alkylation is the same over a tenfold temperature change in double-stranded DNA and is the same as found for a single-stranded DNA.

Discussion

Our experimental observations can be summarized as follows: (*1*) base-paired nitrogens are less reactive than other nitrogens not involved in base pairing; (*2*) base-paired nitrogens are capable of reacting at temperatures far from the T_m, to an extent which is determined by the degree of thermal fluctuation; (*3*) base-paired oxygens react independently of strandedness; (*4*) although the overall extent of reaction depends of temperature, the relative reactivity of oxygens remains constant.

The first point is well known from the literature. The data in Table I comparing alkylation of poly(rA)·poly(rU) and poly(rA) at 25 °C focus on the specific reaction of the N-1 of A. The results show that the reactivity of the N-1 of A is

Table II: Effect of Hydrogen Bonding on Reactivity of Oxygens in Polynucleotides

polynucleotide	reaction temp (°C)	proportion of total ring oxygen alkylation after reaction with ethylnitrosourea[a] O^6-G	O^2-T	O^4-T	O^2-C
double stranded calf thymus DNA[b]	5	45	22	14	18
	25	33	28	17	22
	51	43	29	14	14
poly(dG)·poly(dC)[c]	25	87			13
single stranded M13 DNA[d]	25	35	25	25	15

[a] Reaction conditions and analytical methods are given under Materials and Methods. [b] These values are similar to those obtained after 25 °C ethylnitrosourea reaction of DNA from fibroblasts, HeLa cells, salmon sperm, etc. Ring oxygen alkylation represented 18–22% of the total alkylation. [c] Data taken from Singer et al. (1978b). No reaction at the N-3 of C was detected. Reaction at the N-3 of C represents about 30% of the total alkylation of poly(dC) (Singer, 1977). [d] Data taken from Singer (1976).

suppressed in the double-stranded polymer. This was also the conclusion of Ludlum (1966), Pochon & Michelson (1967), and Singer & Fraenkel-Conrat (1969) who reacted poly(rA)·poly(rU) with methyl methanesulfonate or dimethyl sulfate. Similarly there is a suppression of this reaction in double-stranded DNA as compared with a single-stranded RNA or polynucleotide (Table I). Earlier work from this laboratory on alkylation of poly(dG)·poly(dC) with ethylnitrosourea showed that at 25 °C the reactivity of the N-3 of C was too low to be detected (Singer et al., 1978b).

The results in the tables clearly show that base pairing greatly reduces reactivity of the nitrogen in the 1 position in the adenine ring. There is, however, a continuum of increasing or decreasing reactivity as the reaction temperature is raised or lowered. Our lowest reaction temperature with poly(rA)·poly(rU) was 50 °C below the T_m, and yet the reaction of the N-1 of A was still detectable. These results suggest that, as the amount of "breathing" in the double-stranded polymer increases, the availability of the N-1 of A for alkylation increases.

In contrast, the four exocyclic base oxygens can be reacted in double-stranded DNA to a similar extent as in single-stranded nucleic acids (Singer et al., 1978b), and varying the reaction temperature of double-stranded DNA from 5 to 51 °C does not change the distribution of *O*-ethyl bases. These results suggest that for alkylation of oxygens disruption of Watson–Crick base pairing prior to reaction is not necessary.

The prevailing concept is that base-paired positions in nucleic acids are unreactive. This is essentially correct for nitrogens but not for oxygens. The important difference between the two, and the explanation for these observations, is that the base-paired oxygens (O-2 of C, O-6 of G, O-4 of T) have an extra electron pair exposed to solvent and not involved in hydrogen bonding, which the base-paired ring nitrogen in adenine lacks (Singer et al., 1978b). As illustrated in Figure 3, the exocyclic amino groups, whether or not hydrogen bonded, are always capable of reacting. The N-6 of A, as expected, behaves like the base-paired oxygens since its reactivity is independent of temperature (Figure 1). The NH groups of G, U, and T do not react significantly, even in single-stranded form, with alkylating agents at neutrality (Figure 3).

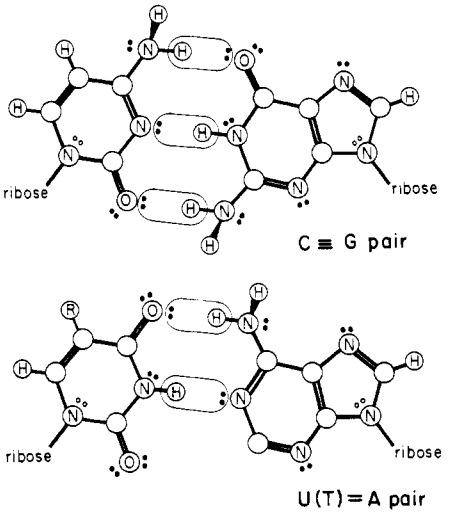

FIGURE 3: Participation of electron pairs in base pairing. The ovals define hydrogen bonds. Solid pairs of dots inside of ovals represent electron pairs involved in hydrogen bonding. Other pairs of dots at the O-2 of C, the O-4 of U(T) and the O-6 of G are potential sites for reaction and can be alkylated at neutrality (see Table II). Similarly it might be predicted that the N-3 of U(T) and the N-1 of G possess a potential for reacting. However, even in a single-stranded polymer, these positions are quite unreactive except in alkaline solution (Kriek & Emmelot, 1963; Ludlum, 1966; Pochon & Michelson, 1967; Shapiro, 1968; Lawley & Shah, 1972; Kuśmierek & Singer, 1976). In the case of the U(T)–A pair, the N-1 of A, as shown, has no free electron pair. All other nitrogens on the adenine moiety are free to react (see Table I).

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