Reaction of DNA with Alkylating Agents. Differential Alkylation of Poly[dA-dT] by Methylnitrosourea and Ethylnitrosourea†

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ABSTRACT: The poly[dA-dT] system developed to study the reaction of the alkylating species generated by ethylnitrosourea with nucleophilic sites on A-T base pairs in the helical β conformation of DNA (Jensen, D. E., & Reed, D. J. (1978) *Biochemistry 17* (preceding paper in this issue)) is extended to consider the methylation of this polymer resulting from incubation with methylnitrosourea. The assay for the kinds and degree of modification involves enzymic hydrolysis of the polymer and resolution of the digestion products utilizing anion-exchange high pressure liquid chromatographic methods. Modification of the phosphate group oxygen atoms using methylnitrosourea is observed to be 65–75% of the total assayed alkylation under a variety of reaction conditions. Some reaction preference for one of the available nucleotide phosphate oxygen sites relative to the other is indicated as determined by the differential yield of methyl dideoxynucleoside phosphotriester diastereomers. A high level of methylation at the 3-position nitrogen atom on the adenine moiety is observed, amounting to approximately 25% of the total alkylation. A low degree of methylation occurs at the 2-position and 4-position oxygen sites on thymine, the O^2-thymine modification accounting for only 3–5% of the total methylation products assayed. These results are in distinct contrast to our findings using ethylnitrosourea as the alkylating agent. A reaction mechanism to account for these differences is discussed.

Our study of the alkylation of the synthetic DNA copolymer poly[dA-dT] in the double-helical conformation by ethylnitrosourea has been extended to consider the methylation of the polymer by reaction with methylnitrosourea. Using the same reaction conditions and analytical high-pressure liquid chromatographic techniques, we have directly compared the degree of covalent modification at the phosphate groups, at the 2- and 4-position oxygen atoms on the thymine residues and the 3-position nitrogen on adenine as well as at a few minor reaction sites produced by these two alkylating carcinogens, relative to the total unmodified residues.

Previous comparisons of DNA alkylation by methylnitrosourea and ethylnitrosourea have indicated that the methylating compound is a more efficient DNA alkylating agent both in vivo and in vitro (Pegg & Nicoll, 1976; Frei et al., 1978). Relative to total alkylation, however, ethylnitrosourea appears to effect a higher degree of modification at phosphate group oxygen atoms. Alkylation at these sites has been observed to account for about 70% of the total in vitro DNA reaction using ethylnitrosourea (Sun & Singer, 1975) and about 18% of the total using methylnitrosourea (Lawley, 1973). Ethylnitrosourea has also been found to produce a high degree of alkylation at the 2-position oxygen on thymine residues in natural DNA (Singer, 1976) as well as in poly[dA-dT] (Jensen & Reed, 1978). Singer (1976) has estimated that the extent of ethylation at this site approaches that at the 6-position oxygen on guanine residues in natural DNA. Methylation of the O^2-thymine sites in helical DNA has not been previously considered.

Modification of the 4-position oxygen on the thymine residues of poly[dA-dT] appears to be a minor reaction using methylnitrosourea, the O^4-methylthymine to 3-methyladenine ratio being about 0.06 (Abbott & Saffhill, 1977).

Quantitation of the distinctly different distributions of site modification in the poly[dA-dT] reactions with methyl- and ethylnitrosourea is reported here. A reaction mechanism to account for these differences is considered as well as some of the possible biological implications of these findings.

Experimental Section

The materials and methods used in this study were identical with those described in the preceding paper (Jensen & Reed, 1978). Methylnitrosourea was synthesized by nitrosating methylurea (Aldrich Chemical Co.) in a manner analogous to that used in the synthesis of ethylnitrosourea (Jensen & Reed, 1978). The methylated compounds 3-MeA,[1] O^2-MedT, O^4-MedT, and 3-MedT were kindly provided by Dr. B. Singer for use as HPLC markers.

Extinction Coefficients. The extinction coefficients used for chromatography peak quantitation are listed in Table I. The coefficients for A, dA, T, dT, and dI are those used in the previous paper (Jensen & Reed, 1978). An estimate of the extinction coefficient for 3-MeA at 254 nm in the standard HPLC buffer (0.09 M NH_4OAC, 1 mM borax, pH 9.1) was made based on the assumption that the extinction coefficient at its wavelength of maximum absorption (273 nm) is the same as that determined for adenine at its absorption maximum (13 400 at 261 nm) in the standard buffer.

Extinction coefficients for O^2-MedT and O^4-MedT in standard buffer, 70 °C, were determined by comparing the HPLC peak areas of these compounds with the peak areas of the products generated in corresponding aliquots which had undergone mild acid hydrolysis (pH 1 with HCl, 98 °C, 1 h). Kuśmierek & Singer (1976) have reported that heating of the analogous ethylated compounds in mild acid results in both dealkylation and hydrolysis of the *N*-glycosyl bond. In our

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[1] Abbreviations and notation used are those established in the preceding paper (Jensen & Reed, 1978) with the following additions: Me, methyl; dNp(Me)dN′ or NMN′, methyl phosphotriester of dideoxynucleoside (3′-5′)-monophosphate (methyl phosphotriesters are referred to as triesters in the text); MepdN, deoxynucleoside 5′-methyl phosphate; dNpMe, deoxynucleoside 3′-methyl phosphate; MNU methylnitrosourea (*N*-methyl-*N*-nitrosourea).

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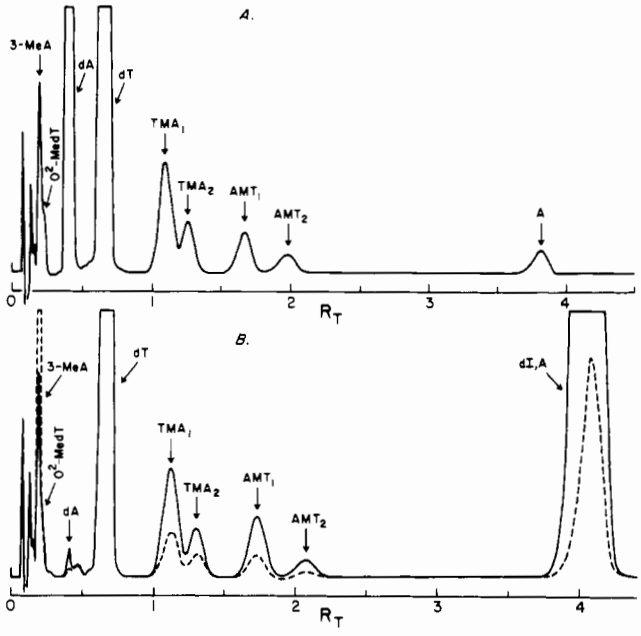

FIGURE 1: Chromatograms of enzymically digested poly[dA-dT] after reaction with methylnitrosourea. Reaction: poly[dA-dT], 8.95×10^{-4} M; MNU, 0.57 M; 0.01 M Na_2HPO_4, 0.1 mM Na_2EDTA, pH 7.7, 30 °C; initial volume, 1.20 mL; titrant, 2 M Na_2CO_3, pH 11; final volume, approximately 1.5 mL; reaction time, 2 h. Enzymic digestion (see Methods): (A) DNase I, venom exonuclease, alkaline phosphatase; (B) as in A plus spleen exonuclease. HPLC elution: 0.09 M NH_4OAc, 1 mM borax, pH 9.1, 70 °C, 0.16 mL/min; retention time was relative to thymine (R_T); thymine elution time, approximately 50 min; (—) 254 nm; (- - -) 280 nm.

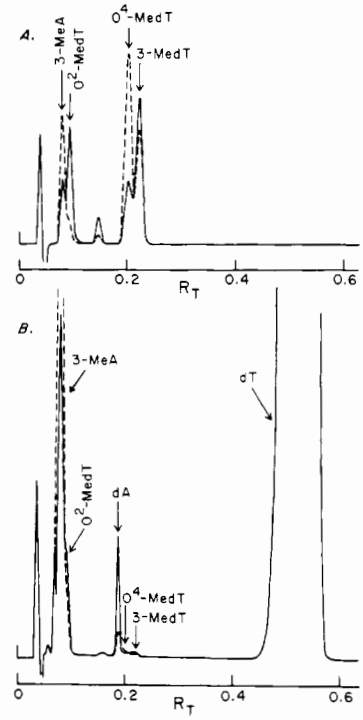

FIGURE 2: (A) Chromatogram of marker compounds. (B) Chromatogram of enzymically digested poly[dA-dT] after reaction with methylnitrosourea; same sample as is shown chromatographed in Figure 1B. HPLC elution: 0.07 M NH_4OAc, 1 mM borax, pH 9.5; all other conditions as described in Figure 1 legend.

experiments with the methylated derivatives we found that acid hydrolysis converted 90% of O^2-MedT to thymine and the remainder to deoxythymidine. The hydrolysis product yields for O^4-MedT were 27% thymine and 73% deoxythymidine. Using the extinction coefficients for T and dT (Table I), we calculated the O^2-MedT and O^4-MedT extinction coefficients.

The methyl triester extinction coefficients were assumed to be the same as those determined for the analogous ethyl triester diastereomers (Jensen & Reed, 1978). Correspondingly, extinction coefficients for the phosphate-methylated 3′- and 5′-nucleoside monophosphates were assumed to be the same as their ethylated analogues (Jensen & Reed, 1978).

TABLE I: Extinction Coefficients; 254 nm, 0.09 M NH_4OAc, 1 mM Borax, pH 9.1, 23 °C.

Compound	ϵ_{254}	280/254 ratio[b]
A	11 700	0.15
T	5 900	0.56
dA	13 600	0.14
dT	6 870	0.71
dI	11 700	0.16
3-MeA	6 500	2.08
O^2-MedT	9 700[a]	0.14
O^4-MedT	2 800[a]	2.87
TMA_1	19 100	0.37
TMA_2	17 700	0.37
AMT_1	19 700	0.37
AMT_2	17 100	0.37

[a] Extinction coefficient at 70 °C. [b] At 70 °C as determined from chromatograms.

Results

Identification of Methyl Phosphotriesters Derived from MNU-Treated Poly[dA-dT]. Our standard procedure was to incubate poly[dA-dT] and methylnitrosourea in buffered aqueous solution for 2 h at 30 (or 37) °C. The polymer was rapidly isolated from the reaction mixture using the Sephacryl-centrifugation technique previously described (Jensen & Reed, 1978) and enzymically hydrolyzed with nucleases and alkaline phosphatase to yield unmodified nucleosides, base-methylated nucleosides or free methylated bases and the nuclease resistant methyl phosphotriesters. The several digestion products were resolved and quantitated using anion-exchange high-pressure liquid chromatography. Chromatograms from a typical experiment are shown in Figures 1 and 2B. In this experiment the reaction buffer was 0.01 M Na_2HPO_4 and the pH controlled by pH-Stat titration with 2 M Na_2CO_3, pH 11. In other experiments the pH was held within the desired limits with a higher buffer concentration (0.1 M sodium cacodylate). As discussed below, we found that the relative product yields were approximately the same using either buffer system.

The results from our previous work on the ethylation of poly[dA-dT] using ethylnitrosourea (Jensen & Reed, 1978) led us to suspect that the four peaks eluting with retention times (relative to thymine) between 1 and 2.5, Figure 1, represented the anticipated two pairs of methyl triester diastereomers. Evidence that this was the case was obtained from identification of the several products, resolved by HPLC, which were generated from each supposed triester upon alkaline hydrolysis (see Jensen & Reed, 1978). A sample of each putative triester was isolated from an enzymic hydrolysis mixture which did not contain spleen exonuclease (see below) by collection of HPLC peak fractions and incubated in mild alkaline solution (pH 13 with KOH, 18 h, 37 °C). The four chromatograms obtained

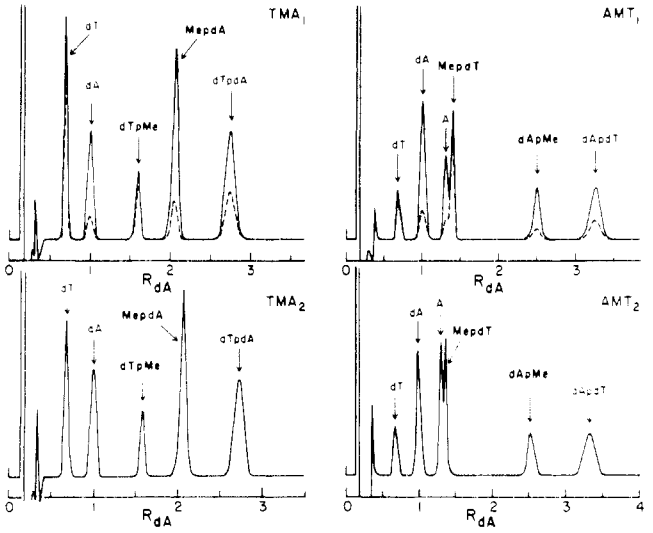

FIGURE 3: Chromatograms of base-hydrolyzed triester diastereomers. The isolation triester samples were adjusted to pH 13 with KOH, incubated at 37 °C for 18 h, and neutralized with HCl. Hydrolysates were applied directly to the HPLC column. Elution: 1 M NH_4OAc, 1 mM borax, pH 9.1, 70 °C, 0.16 mL/min; retention time is scaled relative to deoxyadenosine (R_{dA}); dA elution time, approximately 25 min; (—) 254 nm; (- - -) 280 nm.

from the neutralized hydrolysates are shown in Figure 3; most peak identifications were based on retention times relative to authentic standards and 280 nm/254 nm ratios. The methyl nucleoside phosphodiesters were identified by their susceptibility to exonucleases as previously described (Jensen & Reed, 1978). Yield quantitation was achieved by determining peak areas and application of the extinction coefficients listed in Table I. In each case a dideoxynucleoside monophosphate was found in the hydrolysate, either dTpdA or dApdT, due presumably to the loss of methanol from the triester. Base sequence assignments were based on the identification of these peaks. Ester hydrolysis which generated the dT observed in the chromatograms also produced either an equivalent quantity of 3′- or 5′-methyl deoxyadenosine phosphodiester[2] and correspondingly hydrolysis which produced dA also generated equimolar amounts of 3′- or 5′-methyldeoxythymidine phosphodiester. The quantitative results are listed in Table II. In all four of the triesters the 3′-ester linkage (see Figure 2, preceding paper [Jensen & Reed, 1978]) is most susceptible to alkaline hydrolysis, about 50% of the triesters degrading by the hydrolysis of this bond. The probability of hydrolysis at the 5′-oxygen is about equal to that observed for the oxygen carrying the methyl group. At the triester level, then, about 75% of the alkaline hydrolysis of the methylated phosphate group involves the oxygen atoms in the DNA backbone linkage.

Quantitation of Methyl Triester Yield. In our parallel study of the ethylation of poly[dA-dT] by ethylnitrosourea (Jensen & Reed, 1978), it was discovered that, in addition to DNase I, venom exonuclease, and alkaline phosphatase, spleen exonuclease had to be included in the digestion mixture to assure complete hydrolysis to nucleosides and triesters. As explained, this requirement is probably due to the nucleotide sequence specificity of DNase I and the inability of exonuclease to digest

TABLE II: Quantitation of the Alkaline Lability of Phosphoester Bonds in Methyl Triesters.[a]

	% of hydrolysis at the		
	3′ oxygen[b]	5′ oxygen	methyl group oxygen
TMA_1	49	24	27
TMA_2	48	27	25
AMT_1	49	27	24
AMT_2	48	28	24

[a] Estimated from the chromatograms shown in Figure 3. [b] See Figure 2 of Jensen & Reed (1978).

TABLE III: Typical Poly[dA-dT]-MNU Reaction Product Yields.[a]

compound	% yield[b]	yield rel to total alkylation
3-MeA	1.27	0.22
O^2-MedT	0.18	0.03
O^4-MedT	0.03	0.01
TMA_1	1.41	
TMA_2	1.04	
AMT_1	1.17	
AMT_2	0.64	
total triesters	4.26	0.74
total alkylation	5.74	

[a] This compilation is derived from the chromatograms shown in Figures 1 and 2. [b] As determined from the yields of the compounds listed and unmodified dA and dT.

through alkylated phosphate linkages. Using the complete complement of enzymes we found that the yield of ethyl triesters having the base sequence T-A was equal to the yield of triesters having the A-T base sequence. We took this as evidence that phosphate alkylation by ethylnitrosourea is random.

In Figure 1A it is seen that, when spleen exonuclease was not included in the digestion mixture of methylnitrosourea reacted poly[dA-dT], the yield of dAp(Me)dT triesters (AMT_1 and AMT_2) was apparently much less than the yield of dTp(Me)dA triesters (TMA_1 and TMA_2). This result is analogous to that observed in the ethylation experiments (Jensen & Reed, 1978). When spleen exonuclease was included in the digest,[3] the yield of dAp(Me)dT triesters increased somewhat (Figure 1B). However this increase was not sufficient to bring the dAp(Me)dT and dTp(Me)dA yields into parity. It is not known, as yet, whether this differential yield is an artifact of the analysis or if it is an indication that the reaction of methylnitrosourea with phosphate oxygens has some base sequence specificity. This effect was observed in all of our poly[dA-dT]–MNU experiments, but the degree of disparity was variable. Investigation continues on this point. We do know, however, that the difference is not due to differential stability of the triesters in the HPLC elution buffer; all the methyl triesters were stable in the standard buffer at 70 °C for at least 6 h. It is also evident that the apparently higher yield of dTp(Me)dA triesters was not due to coelution of these compounds with other ultraviolet absorbing compounds since (1) the 280 nm/254 nm ratio was constant across all of the triester elution peaks, and (2) HPLC elution under

[2] The yield of dApMe was low (Figure 3, right) and apparently due to the tendency of this compound to depurinate during alkaline hydrolysis producing the adenine observed in these chromatograms (see Jensen & Reed, 1978). The adenine yield was added to the yield of dApMe in our calculations.

[3] The effect of the deaminase activity present in commercial preparations of spleen exonuclease on the adenosine residues in these digests has been previously noted (footnote 4 in Jensen & Reed, 1978).

TABLE IV: Effect of Reaction Solvent Additives on Selected Poly[dA-dT]–MNU Product Yields.[a]

	poly[dA-dT] × 10^4 (M)	MNU (M)	% yield[b] 3-MeA	O^2-MedT	triester[c]
control	8.64	0.10	1.35 (0.29)[d]	0.16 (0.03)	4.66 (1.00)
	4.31	0.10	0.81 (0.29)	0.17 (0.06)	2.78 (1.00)
0.50 M TMACl	8.64	0.10	0.13 (0.15)	0.05 (0.06)	0.89 (1.00)
	4.31	0.10	0.09 (0.16)	0.05 (0.09)	0.57 (1.00)
0.05 M $MgCl_2$	8.64	0.10	0.18 (0.19)	0.05 (0.05)	0.94 (1.00)
	4.31	0.10	0.15 (0.22)	0.06 (0.09)	0.67 (1.00)

[a] Reaction: 0.10 M sodium cacodylate, 37 °C, 2 h, 0.6 mL, initial pH 8.2, final pH 7.2. [b] As determined from the yield of 3-MeA, O^2-MedT, triesters and unmodified dA and dT. [c] Sum of all triesters (two pairs of diastereomers). [d] In parentheses, yield relative to triester yield.

TABLE V: Comparison of Product Yields; Poly[dA-dT] Reaction with MNU and ENU.[a]

	poly[dA-dT] × 10^4 (M)	nitrosourea (M)	% yield[b] 3-AlkA	O^2-AlkdT	triester[c]
MNU	8.95	0.57	1.27 (0.30)[d]	0.18 (0.04)	4.26 (1.00)
MNU	4.48	0.57	1.08 (0.38)	0.14 (0.05)	2.87 (1.00)
ENU	8.95	0.57	0.09 (0.07)	0.66 (0.51)	1.30 (1.00)
ENU	4.48	0.57	0.08 (0.05)	0.66 (0.45)	1.47 (1.00)

[a] Reaction: 0.01 M Na_2HPO_4, 0.1 mM Na_2EDTA, pH 7.7, 30 °C; initial volume 1.20 mL; titrant, 2 M Na_2CO_3; pH 11, final volume 1.5 mL; reaction time, 2 h. [b] As determined from the yield of 3-AlkA, O^2-AlkdT, triesters, and unmodified dA and dT. [c] Sum of all triesters. [d] In parentheses, yield relative to triester yield.

conditions at which the resolution of compounds was somewhat better than indicated in Figure 1 did not reveal any new peaks in the triester area of the chromatogram.

Estimates of the triester yields in the digestion mixture shown chromatographed in Figure 1B are listed in Table III. Just as in the ethylation reaction (Jensen & Reed, 1978), the yield of one of the triester diastereomers in each pair (the second one eluting from the column) is consistently lower than the other. This is probably due to a difference in reactivity and/or steric accessibility of one of the available phosphate group oxygen atoms relative to the other.

Base-Methylated Products in the Poly[dA-dT]–MNU Reaction. The two major base-alkylated products found in the enzymic digestion mixture of poly[dA-dT] which had been incubated with methylnitrosourea, 3-MeA and O^2-MedT, were weakly retained on the anion-exchange column, eluted early and were only partially resolved (Figure 1). We routinely assayed for the yields of these compounds by measuring the areas under the chromatogram peaks and applying the extinction coefficients listed in Table I. An effort was made to over-estimate the area of the shoulder due to O^2-MedT which rides on the large 3-MeA peak; thus our yield estimates for O^2-MedT probably tend to be high.[4] In addition, loss of 3-MeA due to spontaneous depurination during the reaction incubation is unavoidable (Margison & O'Connor, 1973; Lawley, 1976a). An attempt was made to limit this loss by keeping the reaction pH slightly basic, limiting the reaction time to 2 h and using a very rapid technique for isolating the polymer from the reaction mixture. Nevertheless the estimate of 3-MeA yield is probably low. Base-methylated product yield estimates based on the chromatograms shown in Figure 1 are listed in Table III.

Slightly better resolution of the base-alkylated products was achieved by using a different elution salt concentration and pH. Under these conditions the sample used to generate the chromatogram shown in Figure 1B displayed the elution profile shown in Figure 2B. (Figure 2A demonstrates the elution time [relative to thymine] of several marker compounds.) The yields of O^4-MedT and 3-MedT are seen to be quite low. Generally the yields of O^4-MedT was about 10% of the O^2-MedT yield.

Effect of Solvent Additives on the Poly[dA-dT]–MNU Reaction Product Yield. The experiments of Table IV were directed at determining whether the ratio of base to phosphate alkylation could be manipulated by addition of DNA-binding cations to the reaction mixture. Nearly saturating levels (Jensen & Reed, 1978) of the binding cations tetramethylammonium ion and magnesium ion resulted in a marked decrease in the absolute alkylation product yield. This was probably due, at least partially, to the general salt effect discussed in the preceding paper (Jensen & Reed, 1978). The occlusion of potential reaction sites by specific ion binding no doubt also plays a role in this drop in yield. It is seen in Table IV that the extent of reaction producing 3-MeA can be decreased relative to the phosphate group alkylation by including tetramethylammonium chloride or magnesium chloride in the reaction mixture.

Comparison of the MNU and ENU Reactions with Poly[dA-dT]. An indication of the similarities and differences in poly[dA-dT] alkylation by MNU and ENU using the sodium cacodylate buffer system can be obtained by comparing the MNU results listed in Table IV with those using ENU listed in Table IV of the preceding paper (Jensen & Reed, 1978). Qualitatively these results are the same as those obtained using the titration method of controlling reaction pH. In Table V we have tabulated the product yields for MNU and ENU reactions run in parallel using the titration method and identical solute concentrations. With regard to total poly[dA-dT] alkylation it was found that methylnitrosourea is approximately twofold more effective than ethylnitrosourea. This difference is reflected in the triester yield where it is seen that MNU is somewhat more efficient at phosphate alkylation than is ENU. With either compound the observed phosphate

[4] 3-MeA, O^2-MedT, and O^4-MedT were stable in our standard HPLC buffer for up to 6 h at 70 °C.

group alkylation product is in the range of 65–75% of the total major product yield.

The most pronounced and perhaps important differences between MNU and ENU are found in their ability to alkylate sites in the base moiety. Using the level of alkylation at the phosphate group as a normalizing factor it is seen that the alkylation of the 3-position of adenine in poly[dA-dT] by MNU is 30–40% of that at the phosphate groups, while O^2-thymine alkylation is somewhat less than 10%. In contrast, ENU is much more effective at O^2-thymidine alkylation, relative to phosphate alkylation amounting to 40–50%, while the alkylation of the 3-position nitrogen in deoxyadenosine is less than 10%.

Discussion

A linear free-energy relationship for nucleophilic substitution reactions described by Swain & Scott (1953) correlates the relative nucleophilic strength of a reaction site with the ratio of the alkylation reaction rate with the site to that with water. (Nucleophilic strength can be considered to be a measure of the propensity of electrons at the reaction site to participate in covalent bond forming activities.) The coefficient *s*, generated from an analysis of the alkylation reaction with several kinds of reaction sites covering a range of nucleophilic strengths, is an indication of the sensitivity of the alkylating agent toward differences in nucleophilic strength. In the ideal case of complete insensitivity this coefficient is zero. The *s* coefficients for ENU and MNU have been determined by Veleminský et al. (1970) and are equal to 0.26 and 0.42, respectively. These values are quite low; for comparison, the *s* coefficients for ethyl and methyl methanesulfonate are 0.67 and 0.86, respectively (Osterman-Golkar et al., 1970); the value for methyl bromide being set equal to one (Swain & Scott, 1953; reviewed by Lawley, 1976b). In the present context these results indicate that even though ENU and MNU are relatively insensitive to nucleophilic strength and thus will tend to more readily alkylate weakly reactive sites on DNA than, for example, ethyl or methyl methanesulfonate, these agents will still demonstrate some reaction site selectivity, MNU more so that ENU. The striking differences in poly[dA-dT] alkylation patterns reported here are probably a reflection of this characteristic.

Perhaps the source of the difference in MNU and ENU reactivity can be conceptualized within the framework of the classical S_N2 reaction hypothesis (Ingold, 1953) which assumes a three-centered reaction transition state complex composed of the nucleophilic reaction site, the alkyl group being transferred and the leaving group. The reactive alkylating intermediates generated by ENU and MNU are likely to be the corresponding alkyl diazohydroxides or alkyldiazonium ions (see Jensen & Reed, 1978, and references therein) which are expected to have the same, very stable, leaving groups, N_2 and perhaps hydroxide ion. The factor which may determine the nucleophile sensitivity of the reactive intermediates could be the relative ability of the alkyl groups to assume a partial positive charge in the transition state complex (Ingold, 1953). A degree of stabilization of this partial charge will not only make it easier for the leaving group to depart but will also make the success of the transfer less dependent on the electron-donating capabilities of the reaction site. The methyl substituent on the reactive carbon in the ethyl moiety being transferred may contribute to the reaction by effecting a distribution of the partial positive charge; in the case of methylation, the carbon being transferred has no such advantage. Thus the methylation reaction may require a higher degree of nucleophile electron involvement in the transition state resulting in a greater sensitivity to nucleophilic strength.

In our experiments the degree of O^2-thymine alkylation in helical poly[dA-dT] was about 30% of the total using ENU and 5% or less using MNU. Perhaps this 2-position oxygen atom has a nucleophilic strength which is too low to effect a high probability of successful bond forming collisions with the methylating intermediate but sufficient for the ethylation reaction. Modification at the 4-position oxygen of thymine was observed to be a very minor reaction using both ENU and MNU. It is possible that this site, involved in hydrogen bonding in the helical structure, has an extremely low nucleophilic strength.

A further consequence of this differential sensitivity to reaction site nucleophilic strength is the degree to which the reactive MNU or ENU intermediates are consumed by nucleophiles other than those on the DNA molecule. We observe a two- to three-fold greater overall level of poly[dA-dT] alkylation in the MNU reaction relative to the ENU reaction. This difference may be an indication that fewer of the collisions with solvent nucleophiles result in successful alkylation in the methylation case, thus permitting a greater effective concentration of the methylating intermediate for the polymer reaction.[5] The magnitude of this effect becomes more apparent when considering the alkylation reaction at the 3-position nitrogen site on adenine in poly[dA-dT]. This site is considered to be the most nucleophilic in the A-T base pair. We find that MNU is at least 14-fold more efficient at alkylating this site than ENU and take this as an indication that the effective concentration of the MNU reactive intermediate is at least 14-fold greater. That somewhat less than a 14-fold difference in the overall degree of polymer alkylation is found when comparing the MNU and ENU reactions is consistent with the notion that MNU is considerably less efficient at alkylating sites with low nucleophilic strength, the phosphate oxygens and the 2-position oxygen on thymine residues.

Several of our observations using the poly[dA-dT] system find their parallel in studies of the in vivo alkylation of DNA and cellular response due to treatment with ENU and MNU or the closely related, analogous dialkylnitrosamines. It has been reported that about fivefold less MNU than ENU is required to produce the same degree of tumor induction in laboratory animals (Frei et al., 1978). If we assume that the level of DNA alkylation at the 7-position of guanine (considered to be the most nucleophilic site in natural DNA) is a fairly good measure of the relative availability of the alkylating species for the DNA reaction, it becomes apparent that the effective cellular concentration of the methylating intermediate is much greater. Comparing MNU and ENU in vivo DNA alkylation, the yield of 7-alkylguanine is at least an order-of-magnitude greater using the methylating agent (Pegg & Nicoll, 1976; Frei et al., 1978).

There are many lines of evidence which indicate that the ethylating compounds are, on the basis of percent of total yield, more efficient at alkylating the oxygen atoms in cellular DNA (Sun & Singer, 1975; Pegg & Nicoll, 1976; Lawley, 1976a; Frei et al., 1978). However, the greater effectiveness of the methylating compounds as DNA alkylating agents would appear to compensate, to some degree, for their lower efficiency as oxygen alkylators as shown in the case of phosphate alkylation here and in recent in vivo studies (Frei et al., 1978). An exception could be the O^2-thymine alkylation considered in this investigation. Methylation at this site is so inefficient that,

[5] In both the ENU and MNU reactions, the fraction of input nitroso compound actually reacting with the polynucleotide was quite small, the greatest amount in our experiments being 0.043% and 0.014% of input MNU and ENU, respectively.

on a molar basis, ENU was found to be 3.5-fold more effective at producing this modification. If indeed DNA chemical modification is responsible at least in part for the observed biological effects of these compounds we would expect, to a first approximation, that the degree of biological response be proportional to the degree of one or a few kinds of critical DNA lesions. The ENU-MNU comparison suggests that MNU is more efficient, on a molar basis, at inducing a biological effect and at cellular DNA alkylation, while ENU is more effective at O^2-thymine site modification. This, then, may be the first indication that O^2-thymine alkylation is not, in itself, a critical DNA lesion.

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Homology of Single Copy and Repeated Sequences in Chicken, Duck, Japanese Quail, and Ostrich DNA†

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ABSTRACT: The extent of reassociation of ^{3}H-labeled repetitive or single copy DNA sequences from the chicken with excess unlabeled DNA from the duck, the Japanese quail, and the ostrich, respectively, was measured by hydroxylapatite chromatography. Chicken repetitive DNA reassociated to an equal or greater extent than chicken single copy DNA with the DNA of each of the other birds. Using an isolated subfraction of chicken repetitive DNA representing those DNA sequences common to the chicken and ostrich genomes, we determined that many repetitive DNA sequences that occur at high repetition frequency in the chicken genome have a much lower repetition frequency in ostrich DNA. The data indicate that there has been a striking change in the number of copies of many repetitive DNA sequences during avian evolution.

The repeated DNA of eukaryotes contains families of related sequences that are not identical but are sufficiently similar to reassociate with one another under standard conditions of salt and temperature (Britten et al., 1974). Repeated DNA usually contains families of related sequences with widely varied repetition frequencies, ranging from a few copies to many thousands of copies per genome (Britten & Kohne, 1968; Britten & Davidson, 1971; Davidson et al., 1975). It is not yet clear whether these repetition frequencies reflect a functional requirement for different numbers of copies of different sequences, or whether the number of copies present is the result of other processes related to the origin and evolution of repeated DNA (Kohne, 1970; Britten & Davidson, 1971).

Studies of DNA homology between species can contribute greatly to our understanding of the process of evolution as it relates to the structural organization of the genome. It should be especially informative to compare the rates of nucleotide substitution within different frequency classes of repetitive DNA, and to compare these rates with the rate of divergence of single copy DNA. It is also important to determine whether the repetition frequency of a sequence in DNA remains constant in the DNA of related species, or whether the repetition frequency of DNA sequences changes during evolution.

It has recently been determined that the chicken genome contains an unusually small fraction of repetitive DNA (Eden, in press). However, the repetitive DNA does conform to the typical pattern of containing more than one frequency class; two-thirds of the chicken repeated DNA consists of sequences repeated about 1500-fold, and one-third of the repeated DNA has a repetition frequency of about 15-fold. Thus, studies that compare the extent of homology of chicken repeated and single copy DNA fractions with the DNA of other birds should be

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