Reaction of DNA with Alkylating Agents. Quantitation of Alkylation by Ethylnitrosourea of Oxygen and Nitrogen Sites on Poly[dA-dT] Including Phosphotriester Formation†

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ABSTRACT: A poly[dA-dT] system is being developed for a study of the in vitro reaction of alkylating agents with the oxygen and nitrogen sites on DNA and for an investigation of the effects of these modifications on the physical properties of the polymer and on its ability to serve as substrate for various biochemical processes. An assay for the kinds and degree of modification of the polymer is described, based on enzymic digestion and resolution of the products utilizing anion-exchange high pressure liquid chromatographic methods. The ethylation of poly[dA-dT] using ethylnitrosourea is considered. The degree of phosphate group ethylation using this compound was observed to be 60–65% of the total assayed alkylation under a variety of reaction conditions. The phosphate reaction appears to be independent of nucleotide base sequence. There is some reaction preference for one of the available oxygen sites in the phosphate group relative to the other as determined by the resolution of ethyl dideoxynucleoside phosphotriester diastereomers. Some properties of these phosphotriester diastereomers are described. Quantitation of base alkylation shows a very high level of ethylation at the 2-position oxygen on the thymine moiety, approximately 30% of the total alkylation. A low degree of ethylation occurs at the 4-position oxygen site on thymine and the 3-position nitrogen on adenine, each of these modifications accounting for 3–5% of the total ethylation products assayed. Attempts to modulate the ratio of base to phosphate group ethylation by the introduction of small DNA binding cations to the reaction mixture are described.

The positive correlation between the carcinogenic potential of alkylating compounds and their tendency to alkylate oxygen sites on DNA (reviewed by Lawley, 1976; Pegg, 1977) has generated considerable activity in the study of the in vivo and in vitro effects of oxygen alkylation on the DNA base moieties. Somewhat less attention has been given to the reaction at DNA phosphate group oxygens even though there is evidence that these sites are modified to a relatively high degree by several alkylating carcinogens (Bannon & Verly, 1972; Lawley, 1973; O'Connor et al., 1975). Indeed, in the reaction of ethylnitrosourea with DNA in vitro and in cell suspension, phosphate alkylation is the major reaction, the yield being approximately twofold greater than all other DNA nitrogen and oxygen alkylations combined (Sun & Singer, 1975; Singer, 1976). Other than indications that phosphate bound alkyl groups are only slowly removed from cellular DNA (Shooter & Slade, 1977; Shooter et al., 1977), the biological consequences of these lesions remain obscure. Apparently progress in this area has been slowed by the lack of a facile and quantitatively reliable assay for phosphate alkylation in natural DNA.

Potentially, considerable insight into the effects of phosphate alkylation on the physical properties of DNA and on the viability of modified DNA as a substrate for structural and catalytic proteins can be gained using alkylated synthetic DNA homopolymers and copolymers as model systems. As shown in this series of papers, the low base sequence complexity of synthetic polymers greatly facilitates the resolution and quantitation of phosphate group alkylation products as well as modifications at the base moiety. In addition, many synthetic DNA polymers have been fairly well characterized with respect to their structural properties and their ability to serve as substrates for certain DNA–protein interactions (Wells & Wartell, 1973; Jensen & von Hippel, 1976; Jensen et al., 1976). In this paper and the next we consider an assay for product yields in the reaction of the alternating copolymer poly[dA-dT] with ethylnitrosourea and methylnitrosourea, respectively. Under our reaction conditions this polymer assumes a double-helical conformation closely similar to the average helical conformation of natural DNA (reviewed by Wells & Wartell, 1973). Ethylnitrosourea and methylnitrosourea are potent laboratory carcinogens which are related both chemically and in biological effectiveness to the intensively studied laboratory and suspect human carcinogens diethylnitrosamine and dimethylnitrosamine (Pegg, 1977; Magee, 1977a).

When this work was initiated there was evidence that the treatment of natural DNA with both ethylnitrosourea and methylnitrosourea would result in phosphate alkylation (Lawley, 1973; Sun & Singer, 1975). Consideration of the data of Sun & Singer (1975) suggested that in the modification of poly[dA-dT] by ethylnitrosourea the major base reaction would occur at the 3-position of adenine but still to a minor extent relative to that occurring at the phosphate oxygens. Thus our experimental approach was to study poly[dA-dT] modified with ethylnitrosourea in parallel with polymers modified with diethyl sulfate which, in contrast, produces somewhat higher levels of 3-position adenine alkylation relative to that at phosphate sites (Sun & Singer, 1975). The effects of phosphate alkylation could in this way be determined. There were indications from diazoalkane reaction studies that the oxygen atoms in thymidine and thymine could be alkylated (Farmer et al., 1973; Kuśmierek & Singer, 1976), but the relative yield of these products in natural DNA was anticipated to be quite low (Lawley et al., 1973). The recent work of Singer (1976), however, shows that ethylnitrosourea is surprisingly effective at alkylating the thymine 2-position oxygen in natural DNA. Our results substantiate the high reactivity of this site. Since

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this modification will potentially complicate the assessment of the effects of phosphate alkylation on polymer properties, attempts were made to uncouple the ethylnitrosourea alkylation at thymine oxygen sites from the phosphate group reaction. Some of these experiments are described here. Subsequently it was found that methylnitrosourea is relatively poor at thymine–oxygen alkylation but is quite effective at alkylating phosphate group oxygens (Jensen, 1978). Thus the preparation of polymers for a study of the consequence of phosphate alkylation is more cleanly achieved using this compound.

In our assay modified polymers are enzymatically hydrolyzed to the nucleoside level and the kinds and yields of alkylation products determined using anion-exchange high pressure liquid chromatography. Phosphate linkages which are alkylated prove to be resistant to nuclease activity; thus the digestion mixtures contain alkyldideoxynucleoside phosphotriesters which represent phosphate group alkylations. These compounds are readily resolved by our chromatography technique. Several characteristics of phosphotriesters and their formation are described.

Experimental Procedure

Materials

Deoxyadenosine, deoxythymidine, deoxyinosine, adenine, thymine, and the 3′- and 5′-mononucleotides of deoxyadenosine and deoxythymidine were purchased from Sigma Chemical Co. The dideoxynucleoside monophosphates, dApdT[1] and dTpdA, and the alternating copolymer, poly[dA-dT], were obtained from P-L Biochemicals, Inc., as were the protected nucleosides and nucleotides, *N*-benzoyl-5′-*O*-monomethoxytrityldeoxyadenosine, 5′-*O*-monomethoxytrityldeoxythymidine, *N*-benzoyl-3′-*O*-acetyldeoxyadenosine 5′-phosphate pyridinium, and 3′-*O*-acetyldeoxythymidine 5′-phosphate pyridinium.

Ethylnitrosourea was synthesized by nitrosating ethylurea (Aldrich Chemical Co.). Dry sodium nitrite (0.38 mol) was added in small portions over a period of 1 h to a stirred 0–5 °C solution of ethylurea (0.085 mol) in 3 N HCl (140 mL). The resulting solution and precipitate were extracted with five 35-mL portions of chloroform, the extracts were combined, dried over sodium sulfate, and the solvent was removed under reduced pressure. The dry compound was stored at −20 °C. Immediately before use the nitrosourea was redissolved in chloroform, the solution extracted several times with water, and the chloroform removed under reduced pressure. This procedure removed any decomposition products as determined by NMR.

DNase I (EC 3.1.4.5), DNase II (EC 3.1.4.6), alkaline phosphatase (EC 3.1.3.1), venom exonuclease (EC 3.1.4.1), and spleen exonuclease (EC 3.1.4.18) were purchased from Worthington Biochemical Corp. S1 nuclease (EC 3.1.4_) was obtained from Sigma Chemical Co.

Methods

Chromatography. Chromatograms were obtained using a Spectra-Physics Model 3500B high-pressure liquid chromatograph. A Spectra-Physics Model 748 forced-air oven was used to maintain the column at 70 °C. Samples were applied to the column using a Valco 7000 psi injection valve with a 200-μL sample loop and ultraviolet absorbing elution peaks detected using a Chromatronix Model 230 dual channel absorbance detector. A Spectra-Physics Autolab Minigrator was utilized for 254 nm for peak area integration before being recorded, along with the 280-nm channel output, with a dual pen strip-chart recorder.

Stainless steel 7 $mm^2 \times 250$ mm columns were used in this work, packed with about 2.5 g of Bio-Rad Aminex anion-exchange resin (8% cross-linked, 5–8 μm in diameter; analogous to Aminex A-29). In the packing procedure the column was fitted with a large volume precolumn, about 20 mL, and the pair filled with a slurry of the resin suspended in an isodense solution of cesium chloride (ρ = 1.17 g/mL). The tandem columns were attached to a Haskel air driven fluid pump and the resin was forced into the chromatography column at 6000 psi. The column was packed in less than a single cycle of the pump.

The anion-exchange elution salt was ammonium acetate at a concentration and pH depending on experimental requirements. Eluting solutions also contained 1 mM borax. Degassed double-distilled water was used in all buffer preparation and pH was adjusted with ammonium hydroxide.

Synthesis of Ethyl Nucleoside Phosphodiesters. Four phosphate ethylated mononucleotides, EtpdA, EtpdT, dApEt, and dTpEt, were synthesized from the corresponding mononucleotides using the method of Khorana (1959) without modification. The *N,N′*-Dicyclohexylcarbodiimide used in this synthesis was purchased from Aldrich Chemical Co. Each reaction mixture demonstrated a single, major UV absorbing peak upon anion-exchange high-pressure liquid chromatography. The structures of these products were verified using spleen and venom exonucleases. Samples of each ethyl derivatized nucleoside monophosphate were treated with enzyme and an aliquot of the mixture was applied to the anion-exchange column. It was found that spleen exonuclease did not affect the putative EtpdA or EtpdT but did cleave the ethyl groups from dApEt and dTpEt as determined by HPLC retentions identical with those of unmodified nucleotides. Correspondingly, venom exonuclease did not affect the supposed dApEt or dTpEt but did remove the ethyl group from EtpdA and EtpdT.

Synthesis of Ethyl Dideoxynucleoside Phosphotriesters. The two types of dA- and dT-containing triesters, dAp(Et)dT and dTp(Et)dA, were each synthesized according to the method described by Miller et al. (1974) without modification. 2,4,5-Trimethylbenzenesulfonyl chloride (Aldrich Chemical Co.) and *p*-toluenesulfonyl chloride (Eastman Chemical Co.) used in these syntheses were recrystallized from pentane before use. Anhydrous pyridine, *N*-*N*-dimethylformate, ethanol, and 2,6-dimethylpyridine (Aldrich Chemical Co.) were prepared and stored as described by Miller et al. (1971). The two synthesis steps, condensation of a protected nucleotide with a protected nucleoside and ethylation of the phosphate group, were carried out without intermediate dinucleoside monophosphate purification. The compounds were deprotected following the method of Miller et al. (1974).

Before ethylating the putative dinucleoside monophosphate in each synthesis, an aliquot was removed from the reaction mix and analyzed to determine whether the condensation reaction was successful and to verify the base sequence. After the several deprotection steps, a portion of the sample was applied to the HPLC anion-exchange column. A major peak

[1] Abbreviations and notation used: dA, deoxyadenosine; dT, deoxythymidine; dI, deoxyinosine; A, adenine; T, thymine; Et, ethyl; dNpdN′, dideoxynucleoside (3′–5′)-monophosphate; dNp(Et)dN′ or NEN′, ethyl phosphotriester of dideoxynucleoside (3′–5′)-monophosphate (ethyl phosphotriesters will be referred to as triesters in the text); EtpdN, deoxynucleoside 5′-ethyl phosphate; dNpEt, deoxynucleoside 3′-ethyl phosphate; poly[dA-dT], alternating polymer containing dA and dT residues; ENU, ethylnitrosourea (*N*-ethyl-*N*-nitrosourea); Tris, tris(hydroxymethyl)aminomethane; TMACl, tetramethylammonium chloride; HPLC, high pressure liquid chromatography.

appeared in each case which showed a retention time identical with the corresponding authentic dinucleoside monophosphate. Other aliquots were treated with spleen exonuclease and with venom exonuclease. The putative dApdT phosphodiester was converted to deoxyadenosine 3′-monophosphate and dT by spleen exonuclease and to deoxythymidine 5′-monophosphate and dA by venom exonuclease. Correspondingly, the putative dTpdA phosphodiester was converted to deoxythymidine 3′-monophosphate and deoxyinosine (see below) by spleen exonuclease and to deoxyadenosine 5′-monophosphate and dT by venom exonuclease.

The compounds in the synthetic triester mixture which extracted into 1-butanol were used to obtain 100-MHz ^1H nuclear magnetic resonance (NMR) spectra; negatively charged dinucleoside monophosphates and nucleotides remained in the aqueous phase. The 1-butanol was removed under reduced pressure, the residual compounds were redissolved in deuterated dimethyl sulfoxide and their spectra compared with the corresponding spectra of the authentic phosphodiesters, dApdT and dTpdA. Other than the resonance peaks characteristic of the ethyl moiety, the phosphodiester and corresponding ethyl phosphotriester NMR spectra were found to be quite similar. A sharp triplet at 1.48 ppm downfield from the tetramethylsilane resonance, with a coupling constant of 3.6 Hz, was observed for both the dAp(Et)dT and dTp(Et)dA triesters. This triplet is probably due to the methyl proton resonances of the phosphate ethyl groups and displays a chemical shift similar to those observed by Miller et al. (1971) in their study of the triesters dTp(Et)dT (1.678 ppm) and dAp(Et)dA (1.563 ppm). A sharp quartet, probably due to the methylene proton resonances of the ethyl group, was observed at 4.64 ppm downfield from tetramethylsilane in both of the triester spectra. The coupling constant for this quartet was 3.6 Hz.

Nuclease Sensitivity of Phosphodiesters and Triesters. The nuclease sensitivities of dinucleoside monophosphates and triesters were tested in the following buffers: DNase I, 10 mM Tris, 8 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.5; DNase II, 30% glycerol, 1 mM $MgCl_2$, pH 7.0; micrococcal nuclease, 10 mM Tris, 10 mM $CaCl_2$, pH 7.5; S1 nuclease, 20 mM sodium acetate, 1 mM $ZnSO_4$, pH 4.6; spleen exonuclease and venom exonuclease, 10 mM Na_2HPO_4, pH 7.0. All incubations were at 37 °C for 5 h.

Alkylation Reactions. Two different methods were used to alkylate poly[dA-dT]. In one the pH of the reaction was held constant by the controlled addition of 2 M Na_2CO_3, pH 11, using a Radiometer pH-Stat. Reactions were carried out in 1.0–3.0 mL of solution contained in a water-jacketed reaction vessel fitted with a magnetic stirring device. Temperature was maintained with a Haake thermostated circulating water bath. The reaction pH was allowed to fall in the second method, the pH controlled, somewhat, by 0.1 M sodium cacodylate buffer. The reaction volumes were approximately 0.6 mL in tightly stoppered polypropylene test tubes and the reaction was carried out in a thermostated shaker bath. All reaction incubations were for 2 h. The pH-Stat experiments carried out at 30 °C indicated that 2 h was slightly less than twice the time required to complete the titration of the protons released as the nitroso compound decomposed.

Isolation of Reacted Polynucleotide. Polynucleotide was rapidly isolated from the reaction mixture using a modification of the desalting technique developed by Neal & Florini (1973). The alkylation mixture was applied to a basket of Sephacryl S-200 (Pharmacia) preequilibrated with the desired buffer and the polynucleotide eluted from the gel using centrifugal force. Using this method, isolation of the polynucleotide was achieved in less than 2 min. The gel basket was made from a 1 × 3.5 in. polyallomer centrifuge tube (Beckman no. 326823) holding 6 packed mL of Sephacryl S-200. This basket was fitted into a 50-mL capacity conical centrifuge tube (Dynalab no. 3104-0050). Sample size was somewhat less than 0.75 mL and centrifugation was at 350 rpm using a MSE-GT2 tabletop centrifuge, 1.5 min. The polynucleotide was recovered in the desired buffer in a volume close to that applied. The loss of polynucleotide due to adsorption to the gel was generally about 25% but the removal of small molecule reactants and by-products was over 99% effective.

Nuclease Digestion of Reacted Polynucleotide. Reacted polynucleotide was routinely transferred into 0.01 M Na_2HPO_4, pH 7.7 buffer using the Sephacryl centrifugation method and then subjected to enzymic digestion for HPLC product analysis. As discussed in Results, the digestion was carried out in two portions, with and without spleen exonuclease. The polynucleotide solution was adjusted to 2.25 mM $MgCl_2$, 0.6 mM $CaCl_2$ and the following quantities of enzyme were added per mL of solution: DNase I, 10 units; venom exonuclease, 0.5 unit; and alkaline phosphatase, 1.5 units. To a separate portion of this mixture, spleen exonuclease was added at a concentration of 0.5 unit per mL. Incubation was at 37 °C for 12 h. Aliquots of the digestion mixtures were applied directly to the anion-exchange HPLC column.

Isolation and Identification of Base-Modified HPLC Marker Compounds. Several compounds ethylated on the base moiety were isolated using HPLC from samples of deoxyadenosine and deoxythymidine reacted with ethylnitrosourea. As a final purification step peak fractions were concentrated and rechromatographed using a HPLC buffer which did not contain borax. Acid, neutral, and base spectra were obtained for each isolated compound and compared with published spectra and tabulations of spectral parameters (Singer, 1975). Identification of 1-EtdA, 3-EtA, and 3-EtdT markers were made in this manner. Chromatographic retention time of our 3-EtA marker was identical with that of a sample of 3-EtA kindly provided by Dr. B. Singer. Samples of O^2-EtdT and O^4-EtdT were also provided by Dr. Singer. Structural assignments for these oxygen-alkylated deoxythymidines have been made by Kuśmierek & Singer (1976).

Extinction Coefficients. The extinction coefficients used for chromatography peak quantitation are listed in Table I. The extinction coefficients for A, dA, T, dT, and dI in HPLC buffer at 254 nm, 23 °C, were determined by comparing the spectrum of an aliquot of the compound in 0.09 M NH_4OAc, 1 mM borax, pH 9.1 (standard HPLC buffer) with the spectrum of an identical aliquot in 0.5 M K_2HPO_4, pH 7.0, and using the published extinction coefficient for the compound in the latter buffer (*Specifications and Criteria for Biochemical Compounds,* 1972). An estimate of the extinction coefficient of 3-EtA at 254 nm in the standard buffer 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. In a similar manner the 254-nm extinction coefficients for 1-EtdA and 3-EtdT were estimated by comparison with the spectra of dA and dT, respectively, in the standard buffer. The extinction coefficients for phosphate-ethylated 3′- and 5′-nucleoside monophosphates were estimated from comparisons with the corresponding nonderivatized 3′- and 5′-nucleoside monophosphates, assuming the literature extinction coefficients (*Specifications and Criteria for Biochemical Compounds,* 1972) for these latter compounds.

Extinction coefficients for O^2-EtdT and O^4-EtdT in standard HPLC buffer, 70 °C, were determined by comparing the

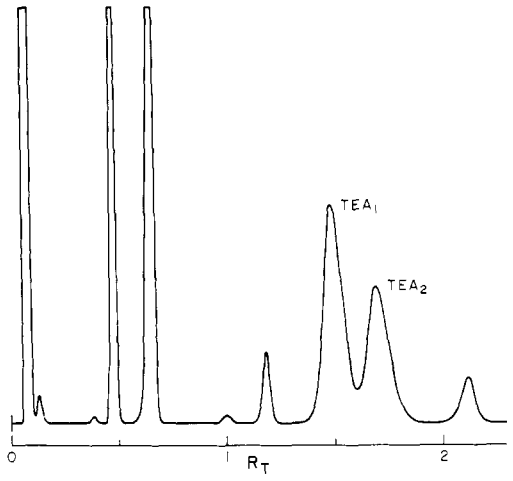

FIGURE 1: Chromatogram of the dAp(Et)dT synthesis mixture after the final extraction and deprotection steps. Elution: 0.09 M NH_4OAc, 1 mM borax, pH 9.1, 70 °C, 0.16 mL/min; 254 nm; retention time is scaled relative to a thymine marker (R_T); the thymine retention time was approximately 50 min.

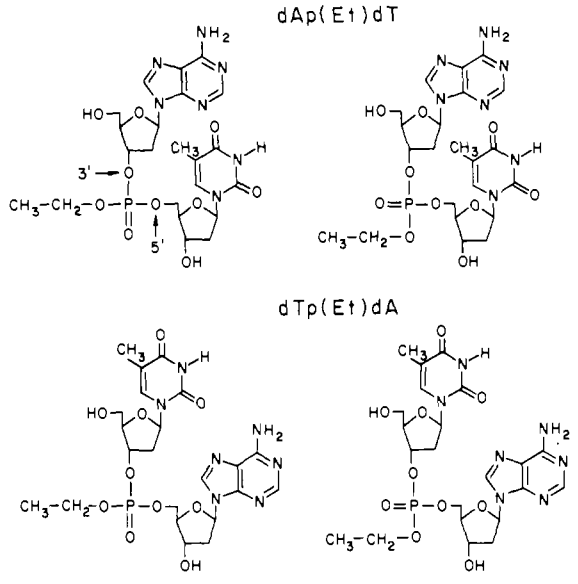

FIGURE 2: The diastereomers of dAp(Et)dT and dTp(Et)dA.

chromatogram peak areas of these compounds with the peak areas generated by corresponding aliquots which had undergone mild acid hydrolysis (pH 1 with HCl, 98 °C, 1 h). As reported by Kuśmierek & Singer (1976), mild acid hydrolysis of O^2-EtdT and O^4-EtdT results in both dealkylation and hydrolysis of the *N*-glycosyl bond. In our experiments 95% of O^2-EtdT was converted to thymine and 5% to deoxythymidine. The hydrolysis product yields for O^4-EtdT were 17% thymine and 83% deoxythymidine. These results are in accord with those obtained by Kuśmierek & Singer (1976). Using the assumed extinction coefficients for T and dT (Table I), we calculated the O^2-EtdT and O^4-EtdT extinction coefficients listed in Table I.

The triester extinction coefficients were determined by comparing the HPLC peak area of a sample of pure triester with the peak area of the adenine quantitatively liberated from an identical triester sample upon mild acid hydrolysis (pH 1 with HCl, 98 °C, 1 h). The assumed adenine extinction coefficient was that listed in Table I.

In some experiments the concentration of dA-containing triester or dinucleoside monophosphate in a sample was determined by the same mild acid hydrolysis method.

Integration of HPLC Peak Areas. The peak areas of all alkylated digestion products were determined by integration using a Hewlett-Packard calculator, digitizer (9821A/9864A), tracing the peak shapes by hand from the recorder chart. To determine the quantities of unreacted nucleoside in the sample which described peaks which were beyond the recorder full scale we relied on the Minigrator output and correlated Minigrator values with digitizer values by analyzing the on-scale peaks that eluted early during a chromatographic run.

Spectral Methods. NMR spectra were determined using a 100-MHz Varian Model HA-100 nuclear magnetic resonance spectrometer. Spectra were obtained at 30 °C from solutions of sample in deuterated dimethyl sulfoxide (Stohler, 2–5 mg of sample per 0.4 mL) using tetramethylsilane as an internal or external standard.

Ultraviolet (UV) spectra were obtained at room temperature using a Cary 14 recording spectrophotometer or a Gilford spectrophotometer. In determining the acid, neutral and base spectra of isolated alkylated products, an aliquot of the sample was taken first to pH 7 and then to pH 1 with 6 N HCl and a second aliquot adjusted to pH 13 with 6 N NaOH.

Circular dichroic (CD) spectra were obtained at room temperature using a Durrum-Jasco circular dichroism spectrophotometer J-10. Circular dichroism is expressed as $\Delta\epsilon$ ($=\theta/33lc$, where θ is the observed ellipicity in degrees, l is the pathlength in centimeters, and c is the molar concentration).

Results

Isolation of Triester Diastereomers. It has been shown that methylation or ethylation of an internal phosphate group in DNA renders the alkylated linkage resistant to the hydrolytic activity of DNase I and venom exonuclease (Miller et al., 1971; Bannon & Verly, 1972; Lawley, 1973). Exhaustive enzymic hydrolysis of alkylated DNA with these enzymes and alkaline phosphatase will thus generate unique phosphate alkylated products, alkyldideoxynucleoside phosphotriesters, as well as unmodified and base-modified nucleosides (or free bases). In this study of the ethylation of poly[dA-dT], anion-exchange HPLC was used to resolve and quantitate the yield of the several enzymic digestion products. The poly[dA-dT] phosphate alkylation products which we expected to find in the enzymic digest mixture, the triesters dAp(Et)dT, and dTp(Et)dA were synthesized as described in Methods for use as HPLC marker compounds. An anion-exchange chromatogram of an aliquot of the dTp(Et)dA synthetic mixture after the final extraction and deprotection steps is shown in Figure 1. In addition to several reaction by-product peaks and a peak which has a retention time identical with that of the parent dideoxynucleoside monophosphate, dTpdA, there were two major peaks which, as discussed below, we identify as being due to triesters. The chromatogram of an aliquot of the dAp(Et)dT synthetic mixture (not shown) is quite similar to the chromatogram shown in Figure 1 except that the pair of putative triester peaks are retained longer by the anion-exchange column as is the parent dideoxynucleoside monophosphate, dApdT.

As indicated in Figure 2, the covalent attachment of the ethyl moiety to the phosphate group of the dideoxynucleoside monophosphate can occur at either of the two available oxygen atoms, thus generating a diastereomeric pair. The experiments described below indicate that the pair of peaks which we identify as triesters (as in the dTp(Et)dA case, Figure 1) are the resolved diastereomers. These diastereomers are designated

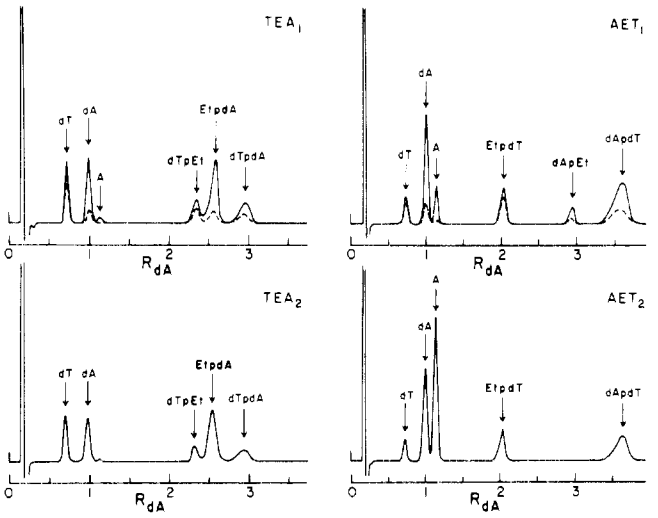

FIGURE 3: Chromatograms of base-hydrolyzed triester diastereomers. The isolated triester samples were adjusted to pH 13 with KOH, incubated at 37 °C for 18 h, and neutralized with HCl. Hydrolysates were applied directly to the HPLC column. Elution: 1 M NH_4OAc, 1 mM borax, pH 9.1, 70 °C, 0.16 mL/min;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

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

compound	ϵ_{254}[a]	280/254 ratio[c]
A	11 700	0.15
T	5 900	0.56
dA	13 600	0.14
dI	11 700	0.16
dT	6 870	0.71
3-EtA	7 800	2.05
1-EtdA	14 100	0.18
3-EtdT	7 400	0.77
O^2-EtdT	10 300[b]	0.15
O^4-EtdT	2 500[b]	2.96
TEA_1	19 100[b]	0.37
TEA_2	17 700[b]	0.37
AET_1	19 700[b]	0.37
AET_2	17 100[b]	0.37
dApEt	13 000	0.17
dTpEt	6 200	0.67
EtpdA	13 000	0.16
EtpdT	6 200	0.72

[a] Determined as described in Methods. [b] Extinction coefficient at 70 °C. [c] At 70 °C as determined from chromatograms.

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

	% of hydrolysis at the		
compound	3′ oxygen[b]	5′ oxygen	ethyl group oxygen
TEA_1	54	30	16
TEA_2	59	28	13
AET_1	40	20	40
AET_2	34	27	39

[a] Estimated from the chromatograms shown in Figure 3. [b] See Figure 2.

according to their elution order from the anion-exchange column which is found to be invariant over a range of elution buffer compositions. In both of the triester synthesis mixtures the second triester eluting had a peak area equal to approximately 75% of that observed for the first eluting triester. Since the extinction coefficients for the ethyl triesters are about the same (see below), this result suggests that the ethylation of one of the two oxygen atoms is slightly favored.

The rather good resolution of the putative triesters using HPLC permitted us to isolate pure samples of these compounds for further analysis.

Hydrolysis of Triesters in Alkaline Solution. As has been noted previously (Rhaese & Freese, 1969; Walker & Ewart, 1973; Shooter, 1976; Swenson et al., 1976), deoxyribose phosphotriesters are labile in alkaline solution. It is found that, when one of the three phosphoester bonds in the compound is hydrolyzed, a phosphodiester is formed which proves to be quite resistant to further hydrolysis. The anticipated triester alkaline hydrolysis products in the present study were (with reference to Figure 2) ethanol and a dideoxynucleoside monophosphate, deoxyadenosine and ethyl deoxythymidine phosphodiester, and deoxythymidine and ethyl deoxyadenosine phosphodiester. Chromatograms of the alkaline hydrolysate of each of the four isolated putative triester compounds are shown in Figure 3; hydrolysis products were identified by retention times and 280 nm/254 nm ratios relative to marker compounds. It is seen that the alkaline hydrolysis of the compounds designated TEA_1 and TEA_2 produced the expected and same products (Figure 3, left), thus establishing them as diastereomers of dTp(Et)dA. Correspondingly, the dAp(Et)dT diastereomers were verified on the basis of the chromatograms shown in Figure 3, right.

Quantitation of the relative yields of the several alkaline hydrolysis products derived from each triester diastereomer (by measuring the areas under the peaks and assuming the extinction coefficients listed in Table I) permitted us to determine the fraction of hydrolysis which occurs at each of the three phosphoester bonds. These results are listed in Table II. (It is noted that EtpdA tends to depurinate under the conditions used for base hydrolysis producing the adenine peak seen in dAp(Et)dT hydrolysates, Figure 3.) The base sequence in these ethyl triesters appears to influence the lability of the phosphoester bonds. The bond on the 3′ side of the phosphate group (see Figure 2) is the most readily hydrolyzed in the dTp(Et)dA triesters, while the 3′-phosphoester bond and the ethyl phosphoester bond are about equally labile in the dAp(Et)dT triesters. In these isolated ethyl triesters the fraction of hydrolysis which involves the DNA backbone ester linkages ranges from 60 to 87% with a calculated average for the four triesters of 73%.

Spectral Properties of Isolated Triesters. Spectra of the four isolated triesters (two pairs of diastereomers) in the ultraviolet region 230–340 nm in neutral solution have the same general shape and the same wavelengths of maximum and minimum absorption, 261 and 232 nm, respectively, as observed for an equimolar mixture of dA and dT and for solutions of the dideoxynucleoside monophosphates, dApdT and dTpdA. The extinction coefficients at 254 nm estimated for these triesters in standard HPLC buffer (Table I) are very simlar to those determined for the dideoxynucleoside monophosphates dTpdA and dApdT, 18 700 and 17 800, respectively, using the same technique.

The CD spectra of the isolated triesters are compared with the spectra of the analogous dideoxynucleoside monophosphates in Figure 4. These spectra are normalized with respect to concentration; the diester and triester concentrations were determined by assaying with HPLC the quantity of adenine liberated by these compounds upon mild acid hydrolysis. It is

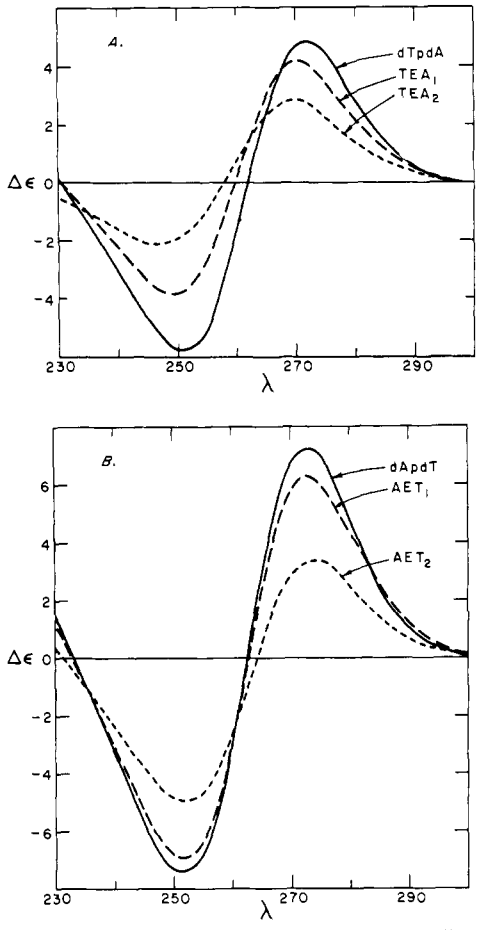

FIGURE 4: CD spectra of (A) dTpdA and the dTp(Et)dA diastereomers and (B) dApdT and the dAp(Et)dT diastereomers. Wavelength (λ) is in nanometers and Δε is in terms of dideoxynucleoside monophosphate or triester concentration.

seen that the dideoxynucleoside monophosphates and the triesters have similar CD spectra. Notable is the decrease in band intensity of the triesters relative to the dinucleoside monophosphates (the second diastereomer eluting from the column being the more strongly perturbed) indicating, perhaps, some uncoupling of the chromophore transition moments as a consequence of a small amount of base unstacking in the triesters.

Triester Sensitivity to Nuclease Activity. The isolated synthetic triesters were tested for their sensitivity to the hydrolytic action of several nucleases by treating with nuclease for 5 h at 37 °C in the appropriate buffer. HPLC anion-exchange chromatography of the digestion mixtures revealed that the phosphoester bonds in the four triesters were totally resistant to the action of DNase I, DNase II, micrococcal nuclease, S1 nuclease, venom exonuclease, and spleen exonuclease. In control experiments the total concentration of dApdT and dTpdA diesters was decreased about 34% by DNase I, 20% by DNase II, 13% by micrococcal nuclease, and 8% by S1 nuclease. The control diesters were completely hydrolyzed by spleen exonuclease and venom exonuclease.

Quantitation of Phosphate Alkylation in the Poly[dA-dT]-ENU Reaction. Chromatograms of the enzymic digest of a poly[dA-dT] sample reacted with ethylnitrosourea are shown in Figures 5 and 6. In this typical run the reaction pH was controlled using the titration method and, as in all of the reactions considered here, the solvent composition and temperature adjusted to favor the poly[dA-dT] helical conformation.[2] Chromatography peaks representing the base-modified compounds 3-EtA, 1-EtdA, 3-EtdT, O^2-EtdT, and O^4-EtdT were identified by their retention times relative to marker compounds, their unique 280 nm/254 nm ratios (see Table I) and their sensitivity to mild acid treatment. The triester peaks were identified by their retention times relative to markers, their 280/254 ratios, and the several compounds liberated from each upon mild base hydrolysis as detected by HPLC (see above). The relative yields of the several enzymic hydrolysis products were determined by chromatogram peak integration as described in Methods and application of the extinction coefficients listed in Table I. Table III lists the results obtained upon analysis of the chromatograms shown in Figures 5 and 6.

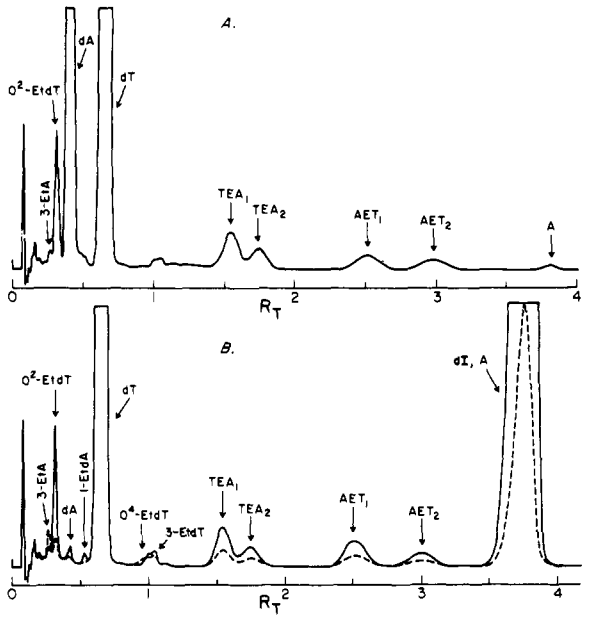

FIGURE 5: Chromatograms of enzymically digested poly[dA-dT] after reaction with ethylnitrosourea. Reaction: poly[dA-dT], 8.95×10^{-4} M; ENU, 0.57 M; 0.01 M Na_2HPO_4, 0.1 mM Na_2EDTA, pH 7.7, 30 °C; initial volume, 1.20 mL; titrant, 2 M Na_2CO_3, pH 11; final volume, approximately 1.5 mL; reaction time, 2 h. Enzymic digestion (see Methods): (A) DNase I, venom exonuclease, alkaline phosphatase; (b) as 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 relative to thymine (R_T); thymine elution time was approximately 50 min; (—) 254 nm; (- - -) 280 nm.

Determination of the triester yields in the chromatogram shown in Figure 5A of a digest mixture containing DNase I, venom exonuclease, and alkaline phosphatase indicates that the apparent yield of the pair of dTp(Et)dA triesters is somewhat greater than the apparent yield of the dAp(Et)dT triesters. In a study involving the nuclease digestion of poly[dA-dT], Scheffler et al. (1968) found that DNase I has a strong preference for cleaving the polymer at dApdT bonds. The differential yield of dTp(Et)dA triesters relative to dAp(Et)dT triesters in the present investigation is probably due to this specificity. Since it has been shown that venom exonuclease will not hydrolyze a phosphate linkage which has been ethyl-

[2] Thermal denaturation of poly[dA-dT] in the buffer used for the pH-Stat controlled reactions (0.01 M Na_2HPO_4, 0.1 mM Na_2EDTA, pH 7.7) indicates that the midpoint of the helix-coil transition occurs at approximately 48 °C. Thus at the reaction temperature (30 °C) and at zero reaction time the polymer was predominantly in the helical conformation. Titration with sodium carbonate during the alkylation reaction will further stabilize this conformation. In a like manner, poly[dA-dT] is double helical in the 0.1 M sodium cacodylate reaction buffer at 37 °C; the midpoint of the helix-coil transition in this buffer occurs at about 63 °C.

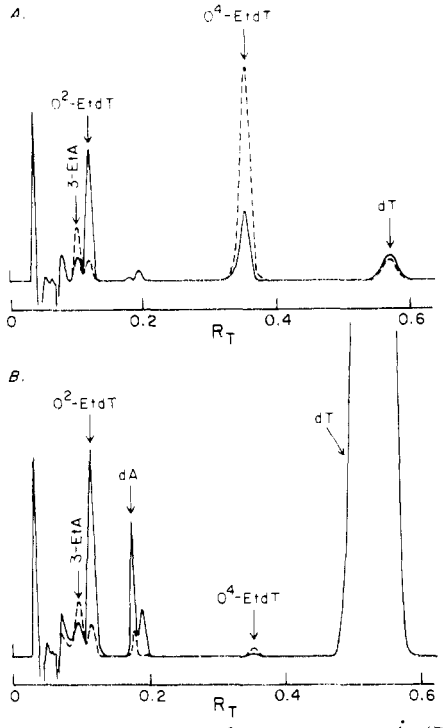

FIGURE 6: (A) Chromatogram of marker compounds. (B) Chromatogram of enzymically digested poly[dA-dT] after reaction with ethylnitrosourea. Same sample as is shown chromatographed in Figure 5B. HPLC elution: 0.07 M NH_4OAc, 1 mM borax, pH 9.5; all other elution conditions as described in Figure 5 legend.

ated and that DNase I displays low activity toward hydrolyzing dTpdA bonds, it is possible that oligomers of the sequence dTpdA(Et)dT are formed in our digestion mixtures which are relatively resistant to further hydrolysis.[3] That we did, nevertheless, observe a substantial yield of the dAp(Et)dT triesters indicates that the low activity of DNase I toward dTpdA linkages was compensated for, to some extent, by the high concentration of DNase I which we employed.

We found that the yields of the two pairs of triester diastereomers achieved greater parity if spleen exonuclease was also included in the enzymic digest (Figure 5B). This enzyme, which operates on nucleotide oligomers in the direction opposite to that taken by venom exonuclease but is also unable to hydrolyze ethylated phosphate linkages, will convert the postulated dTpdAp(Et)dT oligomers to dAp(Et)dT triesters.[4] The increase in the dAp(Et)dT yield is substantial, generally around twofold, when spleen exonuclease is included in the digestion mixture. In the present example (Figure 5) the increase was seen to be about 80%. In contrast, the observed yield of dTp(Et)dA increases very little, if at all; the chromatograms

[3] There are compounds in these preparations which produce very broad, strongly retained peaks in our anion-exchange chromatograms which are not present after the sample is treated with spleen exonuclease. These compounds have not been isolated and characterized but it is possible that they are the postulated, negatively charged oligomers.

[4] Inspection of Figure 5 indicates that the inclusion of spleen exonuclease results in a nearly complete loss of the chromatography peak due to deoxyadenosine. The report that commercial spleen exonuclease has an activity which converts deoxyadenosine to deoxyinosine (Ogilvie & Letsinger, 1968) is supported here with the observation that a new peak appears, concomitant with the loss of the dA peak which has a relative retention time similar to deoxyinosine. This same activity may act on the adenine moiety in the triesters and on the base-modified adenine products; however, we observe no changes in the relative retention times of these compounds or significant changes in yields when spleen exonuclease is included in the digest other than the increase in the dAp(Et)dT triester yield as discussed in the text.

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

product	% yield[b]	yield rel to total alkylation
3-EtA	0.09	0.04
1-EtdA	0.03	0.01
3-EtdT	0.02	0.01
O^2-EtdT	0.66	0.30
O^4-EtdT	0.07	0.03
TEA_1	0.43	
TEA_2	0.23	
AET_1	0.42	
AET_2	0.22	
total triesters	1.30	0.60
total alkylation	2.17	

[a] This compilation is derived from the chromatograms shown in Figures 5 and 6. [b] As determined from the yields of the compounds listed and unmodified dA and dT.

in Figure 5 indicate a 5% increase in total dTp(Et)dA triester. (The triester yields listed in Table III are based on the chromatogram shown in Figure 5B.) In all of our poly[dA-dT] ethylation experiments using ethylnitrosourea, we found that the dAp(Et)dT and dTp(Et)dA triester yields were approximately equal (using the complete complement of enzyme), the greatest difference observed amounting to about 10%.

It is also of interest to note that, within a triester diastereomeric pair derived from an alkylated poly[dA-dT] digest, the yield of the second diastereomer to elute is much less than that of the first. In the run we are using as an example (Figures 5 and 6 and Table III), the difference is about twofold for both the dAp(Et)dT and dTp(Et)dA triesters. Generally the yield of the second diastereomer is in the range of 50–65% of the first.

Base Modifications in the Poly[dA-dT]-ENU Reaction. Our anion-exchange HPLC technique will resolve the enzymic hydrolysis products due to ethylation at the 1- and 3-position nitrogen on adenine as well as the 3-position nitrogen and the 2- and 4-position oxygens on thymine. It is known that alkylation can occur at the 7-position nitrogen on adenine but we have not, as yet, identified the peak representing this product in our chromatograms. Previous reports indicate, however (for example, the work of Sun & Singer, 1975), that the ethylation of the 7-position nitrogen on adenine is quite low relative to that observed for the N-3 of adenine when using double-helical DNA as the reaction substrate.

The results listed in Table III are quantitatively typical of the many poly[dA-dT]-ENU reactions we have analyzed. Phosphate group ethylation, as represented by the two pairs of diastereomeric triesters, was the predominant reaction. Of the sites on the nucleotide bases, the 2-position oxygen on thymine was the most heavily ethylated. When the reaction was carried out in simple buffer, the O^2-EtdT yield was routinely 40–50% of that observed for triester. Alkylation of the 1-adenine and 3-thymine nitrogen sites was observed to be quite low, 1% or less of the triester yield, in all of our experiments. The yield of 3-EtA was also low, 4–7% of the triester yield.

It is known that alkylation of the 3-position of adenosine destabilizes the glycosyl bond resulting in spontaneous depurination. Sun & Singer (1975) have attempted to quantitate the loss of this ethylated derivative from natural DNA in vitro and have found that 29–45% of the 3-EtA residues are released spontaneously during the alkylation reaction under their

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

	poly[dA-dT] $\times 10^4$ (M)	ENU (M)	% yield[b] 3-EtA	O^2-EtdT	triester[c]
control	8.64	0.10	0.05 (0.05)[d]	0.46 (0.43)	1.07 (1.00)
control	4.29	0.10	0.05 (0.04)	0.50 (0.42)	1.18 (1.00)
0.10 M NaCl	8.64	0.10	0.04 (0.05)	0.31 (0.42)	0.74 (1.00)
0.50 M NaCl	8.64	0.10	0.02 (0.04)	0.18 (0.41)	0.44 (1.00)
0.50 M TMACl	8.64	0.10	0.02 (0.03)	0.14 (0.23)	0.59 (1.00)
0.50 M TMACl	4.29	0.10	0.02 (0.02)	0.14 (0.32)	0.44 (1.00)
0.50 M TMACl	8.64	0.05	0.01 (0.03)	0.09 (0.29)	0.32 (1.00)
0.05 M $MgCl_2$	8.64	0.10	0.02 (0.04)	0.17 (0.33)	0.52 (1.00)
0.05 M $MgCl_2$	4.29	0.10	0.02 (0.04)	0.17 (0.34)	0.50 (1.00)
0.05 M $MgCl_2$	8.64	0.05	0.01 (0.04)	0.12 (0.38)	0.31 (1.00)

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

conditions (4 h, room temperature, pH 7.0, 40 mM sodium acetate). In our studies any 3-EtA released from poly[dA-dT] during the ethylation reaction would have been removed during the isolation of the polymer from the reaction mixture and thus lost to the analysis. We kept our reaction time (2 h) and polymer work-up time (<5 min) short and the reaction pH slightly basic (around 7.7) to decrease the extent of this loss. Nevertheless it is possible that the yield of 3-EtA could be up to 50% underestimated. Even if this were the case, ethylation of the 3-position of adenine by ethylnitrosourea would appear to be a minor reaction.

The alkylation of the 4-position oxygen on thymine is also of some interest. As is shown in Figure 5, the enzymic digestion product which represents this alkylation, O^4-EtdT, was poorly resolved from thymine under the standard elution conditions. As indicated in Figure 6, excellent resolution of this compound was attained by slightly modifying the elution buffer.[5] Over a range of reaction conditions (see below) we found the yield of O^4-EtdT to be very low, 7–10% of the O^2-EtdT yield.

It should be mentioned here that the compounds of primary concern, 3-EtA, O^2-EtdT, O^4-EtdT, and triesters, were stable in the standard HPLC buffer at 70 °C for at least up to 6 h. In our analysis these were the most extreme conditions to which the compounds were subjected. In addition, the lowest pH encountered in our work was 7.2. We believe, then, that quantitation errors due to depyrimidination or dealkylation of O^4-EtdT and O^2-EtdT are minimal.

Effect of Solvent Additives on the Poly[dA-dT]-ENU Reaction Product Yields. One of the aims of this investigation is to sort out the structural and functional effects of DNA phosphate alkylation from those due to alkylation at other sites. Thus it would be useful to be able to prepare poly[dA-dT] samples with markedly different base alkylation to phosphate alkylation ratios. Several alkylation experiments were done to determine if the addition of various small DNA binding cations to the reaction mixture resulted in a change in the observed base alkylation, particularly at the O^2 position of thymine, relative to phosphate group alkylation. Trials were carried out using high concentrations of sodium ion, a general DNA counterion, tetramethylammonium ion, a molecule believed to preferentially bind in the grooves of DNA, and magnesium ion, which apparently complexes strongly with the DNA phosphate charges.[6] The results of these experiments are listed in Table IV.

Noting the yield of base-modified products relative to triesters it is seen that increasing concentrations of sodium ion have little effect. Tetramethylammonium ion does decrease the base alkylation to phosphate alkylation ratio to some degree. The relative yield of O^2-thymine alkylation decreases in the range of 24–44% indicating that tetramethylammonium chloride may be of some utility for producing variously modified poly[dA-dT] samples. The inclusion of magnesium chloride also produces a change in relative product yields but in the direction opposite from that anticipated. Magnesium ion appears to decrease the relative yield of base alkylations, particularly at the O^2 position of thymine.

The data in Table IV indicate that addition of any of these salts to the poly[dA-dT]-ENU mixture results in a general decrease in the total alkylation yield. It is not known what fraction of this decrease is due to the formation of complexes between poly[dA-dT] and the added counterions or what fraction is due to the addition of anions which will effectively compete with DNA sites for the ethylating species. In any case the salt effect on product yield appears to influence the reactions at all the poly[dA-dT] alkylation sites considered roughly in parallel. Comparison of the sodium chloride results (0.50 M) with those obtained with tetramethylammonium chloride indicates that these salts are approximately equal in their effectiveness at lowering the total alkylation yield. Magnesium chloride at 0.05 M appears to be somewhat more effective than 0.10 M sodium chloride, suggesting that magnesium binding to poly[dA-dT] may more strongly influence the alkylation

[5] These elution conditions prove to be impractical for routine analysis because the final peak (dI) elutes in 15 h. While column elution with a salt gradient would be convenient in this case, gradient formation with our equipment was not reproducible at the low flow rates we were required to use.

[6] The cation of tetramethylammonium chloride is believed to bind in the grooves of the double-helical DNA structure and to interact to some extent with the phosphate charges (Shapiro et al., 1969). This salt strongly stabilizes the helical structure relative to the denatured form and has been shown not to modify the predominant helical B conformation to any great extent (Melchior & von Hippel, 1973). Consideration of the increase in the temperature of the DNA helix-coil transition as a function of TMACl concentration and DNA base composition (Melchior & von Hippel, 1973) suggests that, at the tetramethylammonium chloride concentration used in the present study (0.50 M), the poly[dA-dT] helix is at least 75% and perhaps up to 95% saturated with TMA^+. Magnesium ion binds stoichiometrically to the DNA phosphate charges (Felsenfeld & Huang, 1959), the amount bound being a function of binding density and the concentration of competing sodium ions (Archer et al., 1972) but saturating at one magnesium ion per two nucleotide residues. Using the equations and parameters reported by Archer et al. (1972) for the sodium ion concentration in our reaction mixtures (Table IV), we calculate that at the $MgCl_2$ concentration used (0.05 M) the phosphate sites on our poly[dA-dT] samples were essentially saturated with magnesium ion.

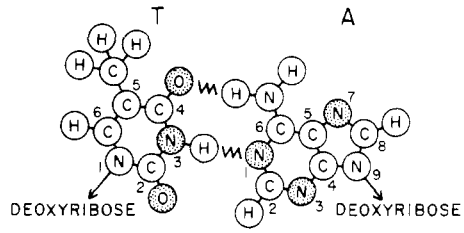

FIGURE 7: Base pairing of dA and dT. Shaded atoms are those having at least one pair of unshared electrons.

yield than the complex between the polymer and sodium ions.[7]

Discussion

The poly[dA-dT] system described in this paper and the next in this series can be used to assess the ability of any spontaneously decomposing ethylating or methylating compound to alkylate DNA phosphate groups. Conceptually the same procedures can be used to determine if any agent will react with the phosphate oxygens in a polynucleotide chain. The only requirement is that the modified phosphate linkage be nuclease resistant. Our work with high pressure anion exchange columns indicates that the triesters produced in an enzymic digest are strongly retained and suggests that the longer the aliphatic carbon chain or the more hydrophobic in character the adduct, the longer will be its retention time. Verification of suspected triester elution peaks can be achieved by the treatment of peak fractions with alkali. Any dA, dT-containing triester will yield, upon base hydrolysis and chromatography, one peak corresponding to the parent dideoxynucleoside monophosphate and peaks representing dA and dT. The other hydrolysis products will be adduct–deoxynucleoside phosphodiesters. In the absence of authentic markers these phosphodiesters can be verified and identified by utilizing venom exonuclease and spleen exonuclease as described here.

Our study of the ethylation of poly[dA-dT] using ethylnitrosourea indicates that the reaction at the phosphate group is base sequence independent, at least at this level of sequence complexity. Some reaction preference for one of the oxygens in the phosphate group relative to the other is observed. When the poly[dA-dT] polymer is in the double-helical conformation, this preference is reflected in a triester diastereomer ratio of about 1:0.6. A similar preference occurs in the de novo synthesis of triesters. At the dinucleoside level the reaction selectivity for one oxygen of the pair is slightly less (1:0.75).

Recently Sun & Singer (1975) have published data on the degree of alkylation at several kinds of sites on HeLa DNA after treatment with ethylnitrosourea in vitro and in cell suspension. We can extrapolate from these data and predict the relative yield of 3-position adenine and phosphate ethylation which might occur in their system if the DNA polymer contained only A-T base pairs. Doing this we find that the expected ratio of 3-position adenine to phosphate oxygen alkylation would be 0.12–0.14. This is a somewhat higher ratio than we observe; over all of our experiments the ratio is in the range of 0.03–0.05. However, considering the differences in the systems and in the assay methods used and the lability of the glycosyl bond in deoxyadenosine alkylated in the 3 position, we think that the correspondence in these results is quite good. Thus the very high level of natural DNA phosphate group ethylation by ethylnitrosourea reported by Sun & Singer (1975) is corroborated in the present study.

The identity of the ultimate alkylating species derived from ethylnitrosourea and other alkylnitrosamides remains obscure. However, the evidence that the methyl group in methylnitrosourea is transferred intact to the nucleophilic site (Lawley & Shah, 1973) combined with the unlikelihood that methyl or ethyl carbonium ions can exist in aqueous solution (Ingold, 1953) points to the possibility that alkyldiazonium ion or perhaps alkyldiazohydroxide is generated during the chemical decomposition of alkylnitrosamide (reviewed by Magee, 1977b; Lawley, 1976). Either of these molecules could be the alkylating species and the alkylation reaction then proceeds via the S_N2 mechanism (Ingold, 1953). If this is the case, molecular nitrogen and perhaps hydroxide ion would be generated during the transfer of the alkyl group to the nucleophilic site. Since these are excellent leaving groups (March, 1968), the reaction might require a very low activation energy and thus show little nucleophile selectivity. Our observed product distribution upon ethylating poly[dA-dT] with ethylnitrosourea is satisfactorily described within the framework of this model.

With low sensitivity to the relative nucleophilic strength of the several kinds of reaction sites on the DNA helical structure, we would expect the product distribution of the ethylation reaction to reflect clearly the relative concentrations of the reaction sites and their steric accessibility. At two per DNA residue the available phosphate oxygens compose the class of nucleophilic sites of highest concentration and their position in the helical structure makes them quite accessible to the solvent. The high degree of phosphate alkylation by ENU (60–70% of total) may be due to these characteristics. Essentially all of the remaining ethylation of poly[dA-dT] occurs at the 2-position oxygen on thymine residues (about 30% of total). This atom is in the narrow groove of the helical structure, not far in location from the 3-position nitrogen of adenine (see Figure 7), considered to be the most nucleophilic site in the A-T base pair. Inspection of a space-filling model of DNA in the helical B-conformation indicates that the 2-position oxygen of thymine is somewhat more accessible than is the 3-position nitrogen of adenine. The yield of 3-position adenine ethylation in poly[dA-dT] was 3–5% of the products assayed. With regard to concentration, the ratio of O^2-thymine sites to available phosphate oxygens in poly[dA-dT] is 1:4; yet, the observed ratio of product yields with respect to these sites approaches 1:2. Since the phosphate oxygens seem to be somewhat more sterically accessible and are probably more nucleophilic than the 2-position thymine oxygen atoms (Jensen, 1978), these results suggest that there are other influences operative in the reaction. One possibility is that solvent interactions with the phosphate charges reduce the level of reaction at these sites.

The degree of reaction with the 4-position oxygen of thymine in poly[dA-dT] was observed to be quite low. In helical DNA this oxygen atom is exposed in the wide groove, closely corresponding to the position of the very reactive 7-position nitrogen of guanine in the G-C base pair. Other factors, then, besides steric and concentration effects influence the reactivity at this site. One possibility is that hydrogen bonding with adenine in the A-T base pair modifies the availability of the unshared electrons for the nucleophilic reaction. It is relevant to note that

[7] The product yields listed in Table III are from an experiment in which the ENU concentration was about sixfold greater (0.57 M) than the concentration used in the experiments considered in Table IV (0.10 M). Nevertheless the yield of alkylated products was about the same (about 2.1%, Table III, compared with about 1.6%, first control, Table IV). The titration with 2 M Na_2CO_3 used to maintain the pH in the former experiment (Table III) results in a final sodium carbonate concentration in the reaction mixture of about 0.4 M. Since the experimental results listed in Table IV indicate that inclusion of salt causes a general decrease in the alkylation product yield, the relatively low yield in the titration experiment is not unexpected.

Singer (1976) has observed that the yields of 2-position and 4-position thymine oxygen ethylations are equivalent when single-stranded DNA is modified by the ethylnitrosourea reaction.

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