Preparation and Characterization of Oligonucleotides Containing *S*-[2-(N^7-Guanyl)ethyl]glutathione†

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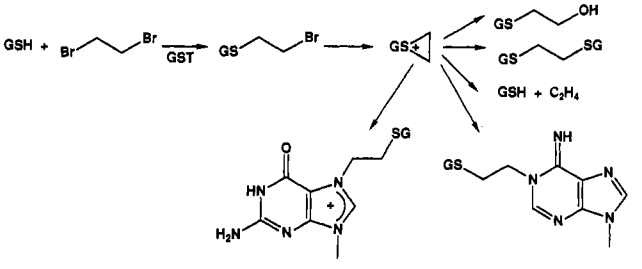
ABSTRACT: *S*-[2-(N^7-Guanyl)ethyl]glutathione is the major adduct derived from modification of DNA with 1,2-dibromoethane in biological systems and is postulated to be a mutagenic lesion [Humphreys, W. G., Kim, D.-H., Cmarik, J. L., Shimada, T., & Guengerich, F. P. (1990) *Biochemistry 29*, 10342–10350]. Oligonucleotides containing this modified base were prepared by treatment of oligonucleotides with *S*-(2-chloroethyl)glutathione and purified by chromatography. The self-complementary oligonucleotide d(ATGCAT), when thus modified at the single guanine, appeared to associate with itself as judged by UV measurements, but CD and NMR measurements indicated a lack of hybridization, with a decrease in the melting temperature of >10 °C. The same lack of self-association was noted when d(ATGCAT) was modified to contain an *N*-acetyl-*S*-[2-(N^7-guanyl)ethyl]cysteine methyl ester moiety. The oligomer d($C_1A_2T_3G_4C_5C_6T_7$) was modified to contain a single *S*-[2-(N^7-guanyl)ethyl]glutathione moiety at the central position, and UV, CD, and ^{1}H NMR studies indicated that this oligomer hybridized to its normal complement d($A_8G_9G_{10}C_{11}A_{12}T_{13}G_{14}$), although the binding was considerably weakened by adduction (imino proton NMR spectroscopy in the presence of H_2O indicated that the hydrogen bond signals seen in the oligomer were all broadened upon modification). All proton resonances were identified using two-dimensional ^{1}H NMR spectroscopy. Adduct formation affected the chemical shifts of the base and 1′, 2′, and 2″ protons of T_3 and C_5, the 2″ proton of C_6, and the 8 and 1′ protons of C_{11}, while little effect was observed on other protons. No cross-peaks were detected between the glutathione and oligomer moieties in two-dimensional nuclear Overhauser enhanced NMR studies. These results suggest that a rather local structural perturbation occurs in the DNA oligomer upon modification and that the glutathione moiety appears to be relatively unperturbed by its placement in the duplex. When the cytosine in the normal d(AGGCATG) complement to d(CATGCCT) was changed to each of the other three potential bases at the central position, no hybridization with the oligomer d(CATGCCT) containing *S*-[2-(N^7-guanyl)ethyl]glutathione was detected. We conclude that these N^7-guanyl derivatives destabilize hybridization and that bases other than cytosine do not appear to show preferential thermodynamic bonding to these adducts, at least in the sequences examined to date. If N^7-guanyl adducts are mutagenic, then either (i) mutations due to the thermodynamically favorable mispairing must occur specifically at sequences prone to mutation or (ii) more complex interactions involving the insertion of the wrong base due to the interaction of the N^7-guanyl adduct with the DNA polymerase need to be invoked.

1,2-Dibromoethane (ethylene dibromide) is a potent carcinogen at a variety of tissue sites (Wong et al., 1982). Activation by conjugation with GSH[1] is necessary for in vitro mutation and DNA alkylation (Rannug, 1980; Rannug et al., 1978; van Bladeren et al., 1981, 1980; Ozawa & Guengerich, 1983; Koga et al., 1986; Cmarik et al., 1990; Kim & Guengerich, 1990), and the reaction is thought to involve formation of *S*-(2-bromoethyl)GSH and the alkylation of DNA via an episulfonium ion intermediate (Peterson et al., 1988) (Scheme I). The major DNA adduct is *S*-[2-(N^7-guanyl)ethyl]GSH, which accounts for >95% of the adducts formed in vitro or in vivo (Ozawa & Guengerich, 1983; Koga et al., 1986; Inskeep et al., 1986; Humphreys et al., 1990).

Scheme I: Formation of DNA Adducts by Conjugation of 1,2-Dibromoethane with GSH



Another DNA adduct is *S*-[2-(N^1-adenyl)ethyl]GSH (~2% total) (Kim et al., 1990). Several lines of evidence suggest that the major *S*-[2-(N^7-guanyl)ethyl]GSH adduct may be mutagenic, at least in a bacterial system, and that structural alterations of the GSH moiety can have dramatic influences on the mutagenicity of such adducts (Kim et al., 1990).

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[1] Abbreviations: GSH, L-glutathione; COSY, two-dimensional (^{1}H homonuclear) correlated NMR spectroscopy; NOESY, two-dimensional nuclear Overhauser enhanced NMR spectroscopy; T_m, melting temperature.

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Further, recent studies indicate that mutant spectra are heavily dominated by transitions at GC base pairs (Cmarik et al., 1991).[2]

N^7-Guanyl derivatives were some of the first DNA adducts described [for review see Hemminki (1983)]. An early hypothesis was that these adducts produced mutations by virtue of the altered pK_a of the N^1 proton imposed by the permanent charge on the imidazole ring and its effect on hydrogen bonding (Lawley & Brookes, 1961; Lawley, 1989; Yamagata et al., 1983). This hypothesis has lost favor, at least in the case of short alkyl derivatives, in light of evidence that alkylating agents that produce only N^7-guanyl adducts (e.g., dimethyl sulfate) are weak mutagens and that O^6-alkylguanines and O^2- and O^4-alkylthymines appear to be more critical lesions in mutagenesis (Lawley, 1984; Guttenplan, 1990). Indeed, many S_N2 alkylating agents such as epoxides preferentially attack the N^7 position of guanine, but other lesions are considered more relevant to mutagenesis and carcinogenicity (Fedtke et al., 1990). However, at least two situations exist where N^7-guanyl adducts may be important. One is the case of 1,2-dibromoethane and related 1,2-dihaloalkanes, considered here—essentially only base pair substitutions are observed (Cmarik et al., 1991; Rannug, 1980). The other involves aflatoxin B_1, which has only yielded N^7-guanyl adducts to date. Both frameshift and base-pair mutations are seen. The basis of base-pair mutation is unknown, and Sambamurti et al. (1988) have suggested that several causes may be operative. The situation is complicated by facile conversion of the N^7-guanyl aflatoxin adduct to the imidazole ring opened product and depurination (Groopman et al., 1981; Baertschi et al., 1989), which may contribute to different phenomena.

The pairing of nucleoside derivatives in organic solvents has led to some insight into modes of interaction in oligomers and DNA [for examples, see Katz and Penman (1966), Newmark and Cantor (1968), and Dyllick-Brenzinger et al. (1980)]. Recently, it has become feasible to synthesize oligomers containing defined adducts and to characterize their three-dimensional structures and interactions with other nucleotides [for examples, see Kalnik et al. (1988, 1989), Norman et al. (1989), Taylor et al. (1990), Gopalakrishnan et al. (1990) and Lepre et al. (1990)]. It may be possible to rationalize observed mutations in terms of preferred thermodynamic pairing in some of these models (Basu & Essigmann, 1988). To date, few physical studies have been carried out with N^7-guanyl adducts in oligomers. Cisplatin forms N^7-guanyl adducts but may not be considered to be a particularly good model for other derivatives because of cross-link formation (Lepre et al., 1990); the aflatoxin B_1 derivative characterized by Gopalikrishnan et al. (1990) is highly intercalated, and such interactions would not be expected with more polar adducts. Part of the difficulty in studying these oligomers containing N^7-guanyl adducts is the result of sensitivity of these modified bases to acid- and base-catalyzed degradation. We have now synthesized several oligomers containing *S*-[2-(N^7-guanyl)-ethyl]thiol derivatives and characterized these using UV, CD, and NMR spectroscopy. The results of these studies are considered in the context of the mutagenicity of 1,2-dibromoethane and the conformational effects of the major adduct on DNA.

Experimental Procedures

Chemicals. β-Cyanoethyl amidites for DNA oligomer synthesis were purchased from American Bionetics, Inc. (Hayward, CA). *N*-Acetyl-*S*-(2-bromoethyl)cysteine methyl ester was synthesized according to van Bladeren et al. (1981, 1990). *S*-(2-Chloroethyl)GSH was synthesized as described by Humphreys et al. (1990). All other reagents were of the highest quality commercially available.

The 5′-*O*-(*tert*-butyldimethylsilyl)-2′,3′-*O*-isopropylidene derivatives of guanosine, adenosine, uridine, cytidine, and N^7-methylguanosine were synthesized according to published procedures (Ogilvie et al., 1979; Hampton, 1961) and were characterized by their UV and ^1H NMR spectra. The bases were dissolved at 1:1 ratios to a total concentration of 0.2 M in C^2HCl_3 for the NMR studies described in the text.

Chromatography. Ion pair HPLC was used to check the purity of oligonucleotides and to monitor the reaction of oligonucleotides with *S*-(2-chloroethyl)GSH. A mobile phase containing 4 mM $N(n\text{-butyl})_4OH$, 50 mM potassium phosphate (pH 7.0), and 22% CH_3CN (v/v) was used with a Beckman Ultrasphere IP octadecasilyl (C18) column (4.6 mm × 250 mm, Beckman Instruments, San Ramon, CA) at a flow rate of 1.2 mL min^{-1}.

For preparation of DNA oligomers, two types of semipreparative columns were used, both at a flow rate of 3 mL min^{-1}. For strong anion-exchange HPLC, a linear gradient of 500–1400 mM ammonium acetate (in 30% CH_3OH) over 40 min was applied to a Partisil SAX column (10 μm, 9.4 mm × 250 mm, Phenomenex, Torrance, CA). For reverse-phase chromatography, a gradient of 7–11% CH_3CN (v/v, in 0.5 M NH_4HCO_2, pH 6.5) was applied to elute a Beckman Ultrasphere ODS octadecasilyl column (5 μm, 10 mm × 250 mm).

Removal of salt was achieved by gel filtration using an open column packed with Sephadex G-10 (55 × 600 mm, Pharmacia, Piscataway, NJ) and H_2O as the solvent.

DNA Oligomer Synthesis. Oligodeoxyribonucleotides were synthesized using solid-phase phosphoramidite chemistry with a Pharmacia Gene Assembler. The yield of each coupling step was >95%. After deprotection with concentrated NH_4OH, each oligodeoxynucleotide was purified by semipreparative strong anion exchange HPLC, the salt concentration was reduced using Sephadex G-10 chromatography, and then the sample was further purified by reverse-phase HPLC. Purified oligomers were passed over the Sephadex G-10 column and concentrated by lyophilization.

Synthesis and Purification of Derivatized Oligomers. DNA oligomers (20–40 mg) were dissolved in 2 mL of 500 mM potassium phosphate buffer (pH 7.7) and incubated at 37 °C. *S*-(2-Chloroethyl)GSH (600 mg) was added to the solution. The reaction was monitored by ion pair HPLC and judged to be complete after 90 min, giving about 10% yield of the main product. The reaction mixture was cooled to 0 °C, applied on a Sephadex G-10 column to remove salts, and then reduced to dryness by lyophilization.

The residue was dissolved in cold H_2O and loaded on the Phenomenex semipreparative SAX column (vide supra). Retention times of DNA oligomers and adducted DNA oligomers were as follows: t_R 17 min for d(ATGCAT) and t_R 15 min for *S*-{2-[N^7-guanyl-d(ATGCAT)]ethyl}GSH using a 30-min gradient (vide supra), and t_R 32 min for d(CATGCCT) and t_R 27 min for *S*-{2-[N^7-guanyl-d(CATGCCT)]ethyl}GSH using a 35-min gradient [the identities of the oligomers were confirmed by analysis of the hydrolyzed products for *S*-[2-(N^7-guanyl)ethyl]GSH]. After lyophilization, the oligomer adduct fractions were dissolved in H_2O, freed of salts using G-10 chromatography, and further

[2] J. L. Cmarik, W. G. Humphreys, R. S. Lloyd, K. L. Bruner, C. Tibbetts, and F. P. Guengerich, submitted for publication.

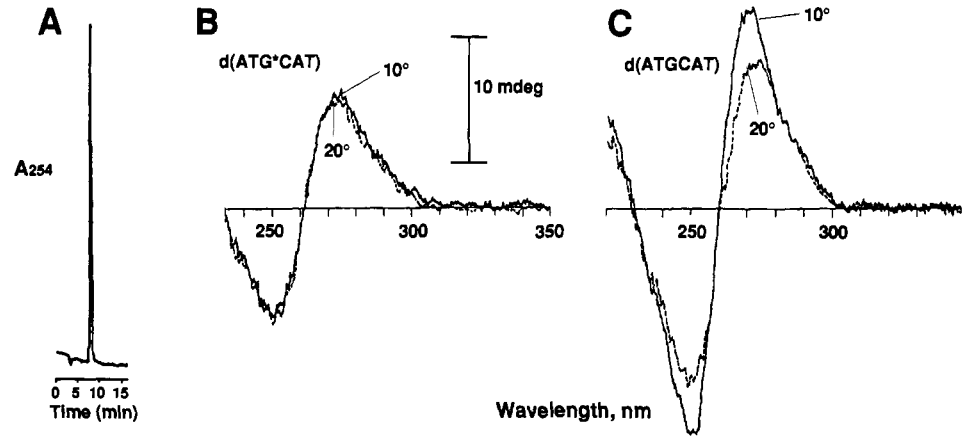

FIGURE 1: CD spectra of d(ATGCAT) and a derivative. (A) Ion pair HPLC of S-{2-[N^7-guanyl-d(ATGCAT)]ethyl}GSH after final reverse-phase purification. Oligomer purity was judged to be >99%. In parts B and C spectra were recorded at both 10 and 20 °C, temperatures that correspond predominantly to the apparent double- and single-stranded forms of each oligomer as judged by the apparent UV melting curves. In all cases the buffer was 50 mM sodium phosphate (pH 7.0) and the oligomer concentration was ~20 μM. (B) d(ATG*CAT), where G* is S-[2-(N^7-guanyl)ethyl]GSH. (C) d(ATGCAT).

purified with the reverse-phase HPLC column. They were then concentrated by lyophilization, dissolved in a minimal amount of H_2O, and passed over the G-10 column. The final yields of adducted DNA oligomers were 5–7%. Purity of adducted DNA oligomers was checked by ion pair HPLC (vide supra).

Spectroscopy. UV melting experiments were done on a Varian Cary 2030 spectrophotometer (Varian Associates, Walnut Creek, CA) equipped with a Neslab water circulating bath (Neslab, Portsmouth, NH) controlled by a Neslab ETP-3 temperature controller. Sample temperatures were raised at 1 °C min^{-1} and monitored with a thermocouple placed in the sample cell. The calculated extinction coefficients (ϵ_{260}, in mM^{-1} cm^{-1}) of the oligonucleotides used in the mixing studies were as follows: d(CATGCCT), 69.6; d(AGGCATG), 86.3; d(AGGAATG), 93.9; d(AGGGATG), 91.9; and d-(AGGTATG), 86.6 (Fasman, 1975). Weight measurements indicated no significant change in the ϵ_{260} of d(CATGCAT) upon modification to include S-[2-(N^7-guanyl)ethyl]GSH.

CD spectra were measured on JASCO J-500 (Tennessee State University) and J-600 instruments (Georgia State University) (Japan Spectroscopic, Tokyo). Both instruments were interfaced to computers for data manipulation.

NMR spectra were obtained on either a Bruker AM-400 or AMX-500 instrument (Bruker, Billerica, MA) in the Vanderbilt University facility or a Bruker AM-600 at the University of Alabama in Birmingham, AL, operating at 400.13, 500.17, and 600.20 MHz for ^1H, respectively. 3-(Trimethylsilyl)-1-propanesulfonic acid was used as an internal standard for ^1H work, and the C^2HCl_3 peak was used as a reference in ^{13}C work with the monomeric nucleoside derivatives (on the AM-400 instrument). Two-dimensional spectra were measured using COSY and phase-sensitive time-proportional phase incrementation NOESY methods (Marion & Wuthrich, 1983). Spectra were recorded using mixing times ≤0.4 s. NOESY spectra were collected with a 1.8-s repetition delay and a sweep width of 4200 Hz. The carrier frequency was placed on the $^1HO^2H$ signal and irradiated with the decoupler channel. The data sets were collected with 256 t_1 experiments using 2048 complex data points in the t_2 dimension and 64 scans per t_1 increment. The data sets were apodized with a $\pi/32$ shifted sine bell function in the t_1 dimension and a $\pi/16$ shifted sine bell function in the t_2 dimension. COSY spectra were recorded with a 2.4-s repetition delay and a sweep width of 3200 Hz. The spectra were collected with 256 t_1 experiments using 2048 complex data points in the t_2 dimension and 48 scans per t_1 increment. Imino protons were observed by the 1–3–3–1 pulse technique (Hore, 1983). The sample temperature was kept at 5 °C except when stated otherwise.

Results

Synthesis of Oligomers. The preparation of oligomers containing N^7-guanyl adducts presents several potential problems. No procedures exist for the incorporation of N^7-guanyl derivatives in the synthesis of oligomers; the compounds are inherently unstable to both acid and base (and heat). A practical synthesis of S-(2-chloroethyl)GSH and related half-mustards has been achieved (Humphreys et al., 1990), and the strategy used for oligomer preparation involved alkylation of oligomers with these modifiers. The N^7 atom of guanine is the most nucleophilic in DNA (Kikuchi & Hopfinger, 1980), at least in runs of guanines, and the reaction proceeds readily. However, yields are not quantitative and, because of potential difficulties in separations, only oligomers containing a single guanine were used in this work.

The two half-mustards *N*-acetyl-*S*-(2-bromoethyl)cysteine methyl ester and *S*-(2-chloroethyl)GSH were used to alkylate the palindrome d(ATGCAT), a sequence containing alternating purines and pyrimidines, a single guanine, and a length suited to ^1H NMR studies. *N*-Acetyl-*S*-(2-bromoethyl)cysteine methyl ester reacted readily at 0 °C in CH_3OH to give yields of ~90% [based upon starting amount of d-(ATGCAT)]. *S*-(2-Chloroethyl)GSH showed less reactivity: the compound is only sparingly soluble in CH_3OH but did give reaction in an aqueous medium at 37 °C and gave a satisfactory yield (~10%). Apparently, the reaction did not proceed to completion because of hydrolysis and the accumulation of salts and side products which interfered. A number of other unidentified products were obtained—in DNA the enhanced nucleophilicity at runs of guanines (Humphreys et al., 1990) appears to drive the reaction toward the main N^7-guanyl product.

The derivatized oligomers were purified using ion-exchange and reverse-phase HPLC (Figure 1); ion pair HPLC exhibited high resolving power and was used as an analytical tool to monitor purification. However, in attempts to use such a procedure in preparative work we found that quaternary ammonium salts could not be removed from the oligomers. N^7-Alkylguanines are unstable, prone to both imidazole ring opening (base catalyzed) and depurination (acid catalyzed);

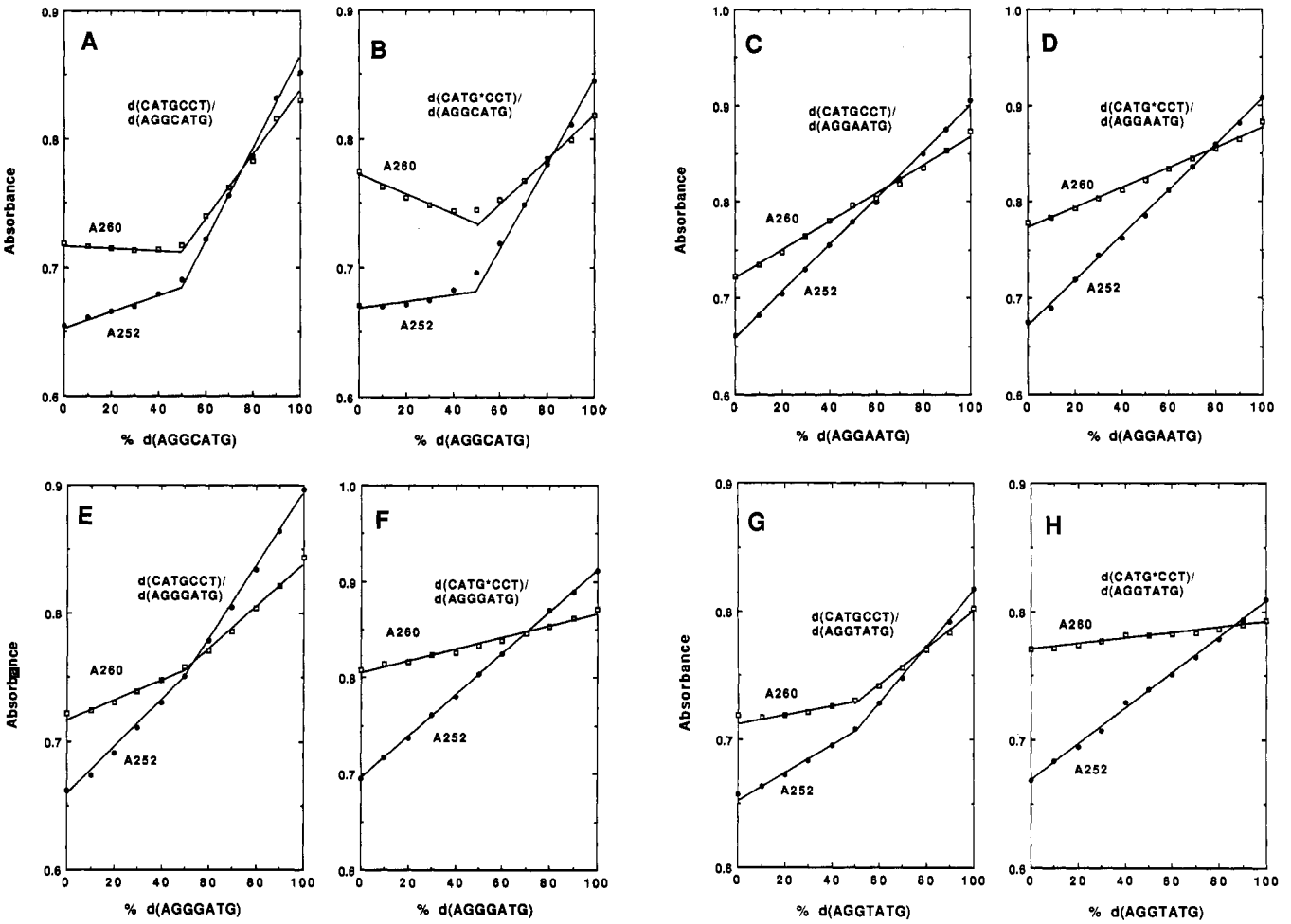

FIGURE 2: UV mixing curves of unmodified and modified d(CATGCCT) with variously substituted complements. d(CATGCCT) or d(CATG*CCT), where G* indicates *S*-[2-(*N*^{7}-guanyl)ethyl]GSH, was mixed with each of the indicated oligomers at the indicated ratios, keeping the total concentration of the two oligomers constant (~20 μM), and UV spectra were recorded (in 50 mM sodium phosphate buffer, pH 7.0). Absorbance at both 252 and 260 nm is plotted as a function of the fraction percent of d(AGGCATG) (parts A and B), d(AGGAATG) (parts C and D), d(AGGGATG) (parts E and F), and d(AGGTATG) (parts G and H).

however, when solutions were carefully handled to avoid heat, acid, and base, the oligomers showed little degradation. Further evidence for the site of DNA modification was provided by the NOESY spectrum of the modified oligomer at pH 4 which showed the cross-peak of *S*-methylene protons of the GSH moiety and the guanyl C^8 proton, thus indicating spatial proximity (data not presented). It should be pointed out that *S*-[2-(*N*^{7}-guanyl)ethyl]GSH is less prone to imidazole ring opening than other derivatives such as those containing aflatoxin products (Baertschi et al., 1989; Kim et al., 1990).

Characterization of Modified d(ATGCAT). When d(ATGCAT) was modified to contain either *S*-[2-(*N*^{7}-guanyl)ethyl]GSH or *N*-acetyl-*S*-[2-(*N*^{7}-guanyl)ethyl]cysteine methyl ester, examination of the apparent melting curves suggested that the T_m had not been altered (results not shown). CD spectroscopy provides an alternative means of evaluating the degree of hybridization of oligomers—when the oligomers under consideration were examined, underivatized d(ATGCAT) showed an increased amplitude in ellipticity at 250 and 270 nm when the temperature was decreased from 20 to 10 °C, but when an *S*-[2-(*N*^{7}-guanyl)ethyl]GSH adduct was present, no temperature-dependent change was seen (Figure 1). In addition, no change was seen with the *N*-acetyl-*S*-[2-(*N*^{7}-guanyl)ethyl]cysteine methyl ester derivative. These studies suggested that the d(ATGCAT) derivatives containing the *N*^{7}-alkyl derivatives were not double-stranded, in contrast to the UV data.

The sharp nature of the peaks in the one-dimensional ^{1}H NMR spectrum (5 °C) also supported the view that the derivatized oligomer was not hybridizing (data not shown), and the lack of temperature dependence of the spectrum confirmed this view. It should be pointed out that the protons in the GSH moiety showed similar shifts and splitting to those of free *S*-[2-(*N*^{7}-guanyl)ethyl]GSH (Koga et al., 1986); the protons in the d(ATGCAT) moiety also showed similar shifts to those of d(ATGCAT) at higher temperature (37 °C), suggesting a lack of interaction of GSH with the oligonucleotide.

Characterization of Modified d(CATGCCT). (*A*) *UV Spectroscopy.* The lack of hybridization of the d(ATGCAT) derivatives indicates that guanine N^7-alkylation is disruptive, and attention was turned to an alternative oligonucleotide, d(CATGCCT)—it has more GC pairs, no self-complementarity, and greater length. Modification of the single guanine dramatically affected hybridization to the complement—the T_m shift appeared to be from about 35 to 20 °C under the conditions used (results not shown). (About 5–10% decomposition of the modified oligomers occurred after three heating/cooling cycles over the range of 2–42 °C).

Mixing curves were used to examine the possibility that bonding of the N^7-substituted guanine to another base was preferred, in light of the decreased pairing to a cytosine (Figure 2). The normal oligomer d(CATGCCT) showed some evidence for weak pairing (as judged by an inflection point near 50%) when a guanine or thymine was placed across from it

in the complement (Figure 2E,G). However, the derivative containing S-[2-(N^7-guanyl)ethyl]GSH *only* showed evidence of pairing when a cytosine was placed across from it in the complement—no inflection was seen in the plots presented in panel D, F, or H of Figure 2. Thus, although S-[2-(N^7-guanyl)ethyl]GSH pairs less tightly to cytosine than does the normal guanine, there is no evidence for thermodynamically preferred bonding to another base.

(*B*) *CD Spectroscopy.* Because of the anomolous UV behavior of the d(ATGCAT) derivative (vide supra), CD spectroscopy was also used to examine the behavior of the heptamers (Figure 3). The normal pair of d(CATGCAT)/d(AGGCATG) showed the expected behavior associated with the temperature change (Figure 3A; see also Figure 2). When the d(CATGCCT) oligomer containing S-[2-(N^7-guanyl)ethyl]GSH was examined, a temperature-dependent change was seen, and hybridization is believed to occur, as judged by the decrease in ellipticity at 250 nm (Figure 3B). However, the spectral amplitude was considerably less than in the spectrum shown in Figure 3A, suggesting weaker or partial hydrogen bonding. When a thymine was placed across from the guanine in d(CATGCCT), some interaction was observed, as noted by the blue shift in the peak upon lowering the temperature (Figure 3C). No real change was seen when the oligomer containing S-[2-(N^7-guanyl)-ethyl]GSH was examined under these conditions (Figure 3D; note the increased sensitivity of the scale in Figure 3C,D).

These results are consonant with those obtained with UV spectroscopy (Figure 2), where the bonding of d(CATGCCT) to its normal complement is considerably weakened by modification of the guanine, and the bonding to a thymine to a guanine is even weaker after its modification. The experiment with the thymine-substituted complement [d(AGGTATG)] was repeated at pH 7.7, and similar results to those found at pH 7.0 were obtained (data not shown).

Characterization of Modified d(CATGCCT). NMR Spectroscopy. In contrast to the situation with the derivative of d(ATGCAT), the one-dimensional NMR spectrum indicated that the d(CATGCCT) derivative containing S-[2-(N^7-guanyl)ethyl]GSH was at least partially paired to d(AGGCATG), as judged by the chemical shift changes upon titration with the complement oligomer at 5 °C (results not shown). The COSY and NOESY spectra were used to assign the protons (Figures 4 and 5 and Table I).

Information relevant to the secondary structure was obtained from the NOESY spectra of the normal d($C_1A_2T_3G_4C_5C_6T_7$)/d($A_8G_9G_{10}C_{11}A_{12}T_{13}G_{14}$) pair and the derivative in which the single guanine (G_4) in the former strand is replaced by S-[2-(N^7-guanyl)ethyl]GSH, recorded at 600 MHz (Figures 4 and 5). Assignment of all protons except for C^2 of A_7 could be done using normal sequential assignment procedures (Table I), as shown for the sugar 1′–base proton connectivities in Figure 5.[3] This study shows that the DNA is probably in the B-form in both cases but that there is some chemical shift perturbation seen on both strands of the modified oligomer. The base–base connectivities can be traced completely through the normal d($C_1A_2T_3G_4C_5C_6T_7$)/d-($A_8G_9G_{10}C_{11}A_{12}T_{13}G_{14}$) pair. In the derivative, although the connectivity of T_3G_4 and G_4C_5 would be expected to be lost due to deuterium replacement of the C^8 proton of G_4 at neutral pH, the connectivities are also lost at C_5 through T_7 (Figure 5C). No cross-peaks involving protons in the GSH moiety and oligomer protons were observed in the NOESY spectrum (with a mixing time of 0.4 s, Figure 5D), although several of the

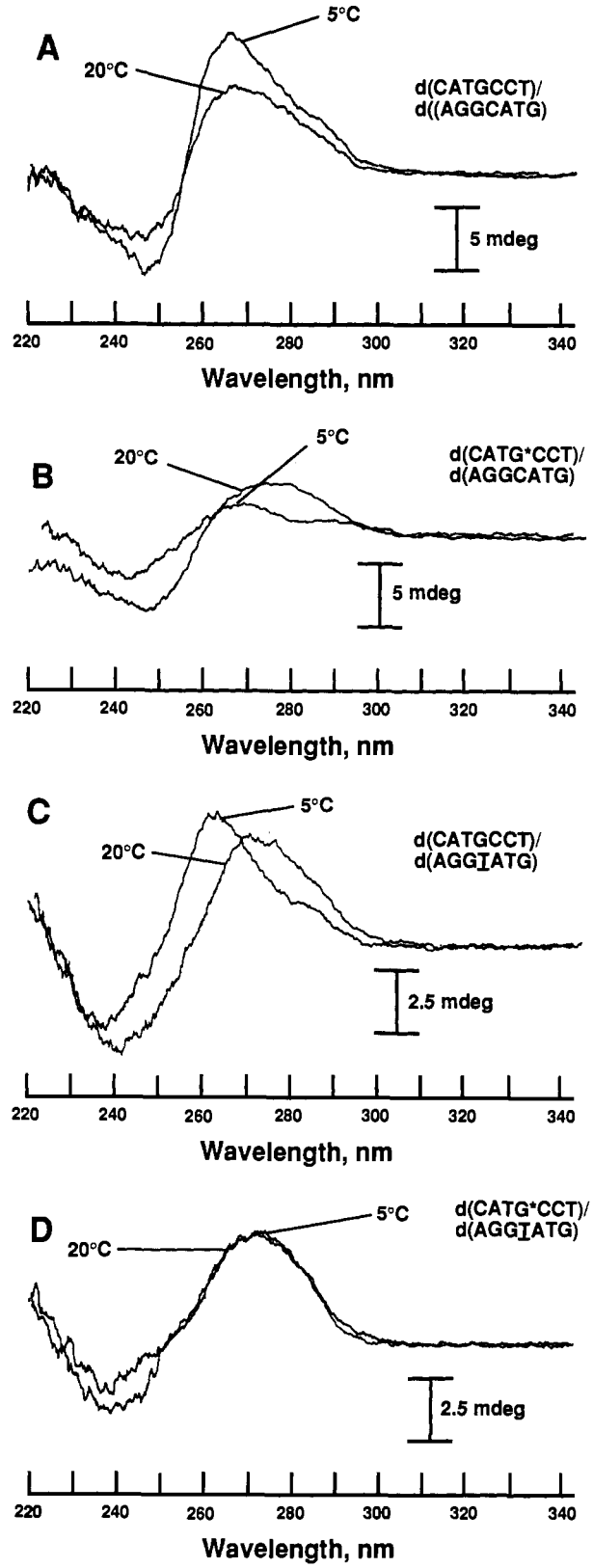

FIGURE 3: CD spectra of d(CATGCCT) and a derivative mixed with potential complements. The spectra of the indicated equimolar mixtures (each oligomer at a concentration of ~10 μM) were recorded in 50 mM sodium phosphate (pH 7.0) at 5 and 20 °C, temperatures that should favor double- and single-stranded oligomers, respectively. G* indicates S-[2-(N^7-guanyl)ethyl]GSH. The different parts show the spectra obtained when a dC (parts A and B) or a dT (parts C and D) was positioned across from the dG (or dG*) in d(CATGCCT).

[3] The C^2 protons of A_2 and A_{12} could be assigned by using one-dimensional NOE experiments in H_2O. The assignments are as follows: A_2 δ 7.87 and A_{12} δ 7.73 in the unmodified oligomer, A_2 δ 7.91 and A_{12} δ 7.83 in the modified oligomer.

Table I: Chemical Shift Assignments for d(CATGCCT)/d(AGGCATG) and d(CATG*CCT)/d(AGGCATG)[a]

	H8/H6			H5/CH_3			H1′			H2′		
	−GS	+GS	Δδ	−GS	+GS	Δδ	−GS	+GS	Δδ	−GS	+GS	Δδ
C1	7.733	7.742	0.009	5.943	5.941	−0.002	5.668	5.668	0.000	2.477	2.478	0.001
A2	8.452	8.451	−0.001				6.335	6.343	0.008	2.993	2.975	−0.018
T3	7.187	7.346	0.159	1.440	1.418	−0.022	5.780	5.974	0.194	2.465	2.295	−0.170
G4	7.895	9.680	1.785				5.871	5.827	−0.044	2.692	2.693	0.001
C5	7.424	7.681	0.257	5.330	5.750	0.420	5.968	6.155	0.187	2.473	2.692	0.219
C6	7.668	7.719	0.051	5.700	5.783	0.083	6.075	6.119	0.044	2.498	2.507	0.009
T7	7.594	7.615	0.021	1.742	1.772	0.030	6.268	6.288	0.020	2.303	2.320	0.017
A8	8.018	8.047	0.029				5.949	5.924	−0.025	2.617	2.555	−0.062
G9	7.847	7.839	−0.008				5.532	5.527	−0.005	2.718	2.737	0.019
G10	7.735	7.760	0.025				5.942	5.915	−0.027	2.745	2.710	−0.035
C11	7.407	7.495	0.088	5.368	5.542	0.174	5.680	5.478	−0.202	2.468	2.400	−0.068
A12	8.373	8.424	0.051				6.283	6.348	0.065	2.953	2.960	0.007
T13	7.172	7.198	0.026	1.510	1.572	0.062	5.812	5.833	0.021	2.333	2.337	0.004
G14	7.897	7.909	0.012				6.140	6.143	0.003	2.602	2.622	0.020

	H2″			H3′			H4′		
	−GS	+GS	Δδ	−GS	+GS	Δδ	−GS	+GS	Δδ
C1	2.093	2.120	0.027	4.738	4.737	−0.001	4.077	4.082	0.005
A2	2.785	2.820	0.035	5.045	5.046	0.001	4.447	4.448	0.001
T3	2.072	1.780	−0.292	4.890	4.902	0.012	4.405	4.340	−0.065
G4	2.661	2.693	0.032	4.983	4.977	−0.006	4.408	4.460	0.052
C5	2.162	2.216	0.05	4.804	4.823	0.019	4.273	4.255	−0.018
C6	2.304	2.140	−0.164	4.867	4.867	0.040	4.193	4.190	−0.003
T7	2.303	2.320	0.017	4.580	4.588	0.008	4.182	4.187	0.005
A8	2.449	2.400	−0.049	4.832	4.812	−0.020	4.198	4.185	−0.013
G9	2.622	2.678	0.056	4.990	4.968	−0.022	4.368	4.355	−0.013
G10	2.611	2.568	−0.043	5.012	4.990	−0.022	4.445	4.433	−0.012
C11	2.133	2.088	−0.045	4.893	4.881	−0.012	4.301	4.220	−0.081
A12	2.703	2.773	0.070	5.035	50.62	0.027	4.447	4.490	0.043
T13	1.893	1.912	0.019	4.861	4.860	−0.001	4.305	4.292	−0.013
G14	2.301	2.349	0.048	4.680	4.689	0.009	4.195	4.203	0.008

	H5′			H5″		
	−GS	+GS	Δδ	−GS	+GS	Δδ
C1	3.747	3.750	0.003	3.747	3.750	0.003
A2	4.175	4.162	−0.013	4.055	4.055	0.000
T3	4.298	4.215	−0.083	4.210	4.165	−0.045
G4	4.158	4.148	−0.010	4.137	4.148	0.011
C5	4.220	4.200	−0.020	4.165	4.078	−0.087
C6	4.090	4.130	0.040	4.085	4.088	0.003
T7	4.090	4.105	0.015	4.060	4.065	0.005
A8	3.696	3.678	−0.018	3.696	3.678	−0.018
G9	4.119	4.080	−0.039	4.091	4.065	−0.026
G10	4.212	4.180	−0.032	4.173	4.150	−0.023
C11	4.217	4.175	−0.042	4.170	4.140	−0.030
A12	4.190	4.170	−0.020	4.117	4.072	−0.045
T13	4.150	4.145	−0.005	4.135	4.130	−0.005
G14	4.130	4.125	−0.005	4.078	4.087	0.009

[a] Values for d(CATGCCT) are listed under −GS, and values for d(CATG*CCT) are listed under +GS; G* = *S*-[2-(N^7-guanyl)ethyl]GSH; Δδ indicates the difference in chemical shift between the two. The first two sets of columns refer to the H8 (purine)/H6 (pyrimidine) and the H^5/CH_3 (pyrimidine) protons—others refer to the deoxyribose protons. All values are reported in ppm downfield of 3-(trimethylsilyl)-1-propanesulfonic acid.

chemical shifts of the GSH were altered by attachment to the oligomer (Table II).

In the DNA oligomer moiety, substantial shifts (>0.1 ppm) were observed for the base and 1′, 2′, and 2″ protons of T_3 and C_5 and the 2″ proton of C_6, while the signals of other protons including the modified G_4 nucleotide moiety did not shift drastically (≤0.05 ppm except for H8 of the modified guanine) (Figure 6). The 8 and 1′ proton chemical shifts of C_{11} in the opposite strand were also altered. These observations suggest that the base modification caused only local disruption of the structure within the duplex.

The hydrogen bonds in d(CATGCCT)/d(AGGCATG) and its derivative were examined by utilizing a 1–3–3–1 pulse sequence in the presence of 85% H_2O (15% ^2H_2O) to show the imino protons (Figure 7). Six of the seven hydrogen bonds were seen in the underivatized oligomer, and these were assigned by NOE difference spectra. In the derivative, the imino proton signals could be resolved only at lower temperature (−5 °C) due to chemical exchange of molecular species of the derivatized oligomer, that is, the double-stranded and single-stranded equilibrium expected from UV melting curves (vide supra). The resonances are very broad with respect to the underivatized oligomers due to the aforementioned chem-

Table II: Chemical Shift Assignments for GSH Contained in G* and d(CATG*CCT)/d(AGGCATG)[a]

	δ (ppm)	
	G* adduct[b]	in oligomer
Glu β	1.96	2.21, 2.27
Glu γ	2.31	2.59
SCH_2	2.91, 2.95	3.00, 3.11
Cys β	2.51, 2.77	2.84, 2.98
Gly α	3.62, 3.67	3.60
Glu α	3.61	3.86
NCH_2	4.28, 4.37	4.58
Cys α	4.28	4.21

[a] G* = *S*-[2-(N^7-guanyl)ethyl]GSH. All spectra were recorded in ^2H_2O. [b] From Koga et al. (1986).

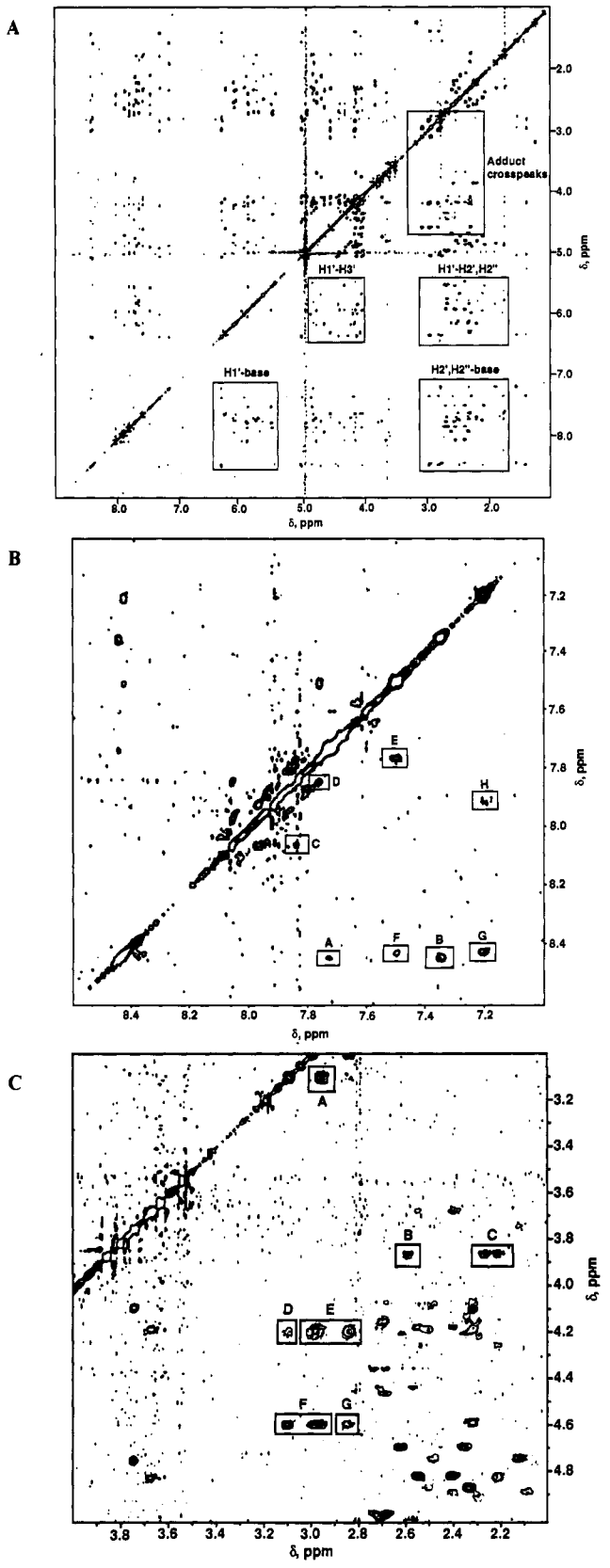

FIGURE 4: ^{1}H homonuclear NOESY NMR spectrum (600 MHz) of a 1:1 mixture of d(CATG*CCT) and d(AGGCATG) in 67 mM sodium phosphate (pH 7.0) and 100 mM NaCl recorded at 5 °C (total oligomer concentration ~4 mM). G* indicates *S*-[2-(N^7-guanyl)-ethyl]GSH. The mixing time was 0.4 s. (A) Contour plot of the whole region of nonexchangeable protons (1–9 ppm). (B) Expanded contour plot of NOESY cross-peaks of base–base protons: (A) $1C_6$–$2A_8$; (B) $2A_8$–$3T_6$; (C) $8A_8$–$9G_8$; (D) $9G_8$–$10G_8$; (E) $10G_8$–$11C_6$; (F) $11C_6$–$12A_8$; (G) $12A_8$–$13T_6$; (H) $13T_6$–$14G_8$. (C) Expanded contour plot of NOESY cross-peaks of the GSH moiety: (A) SCH_2H_A–SCH_2H_B; (B) Glu α–Glu γ; (C) Glu α–Glu β; (D) Cys α–SCH_2; (E) Cys α–Cys β; (F) SCH_2–NCH_2; (G) NCH_2–Cys β.

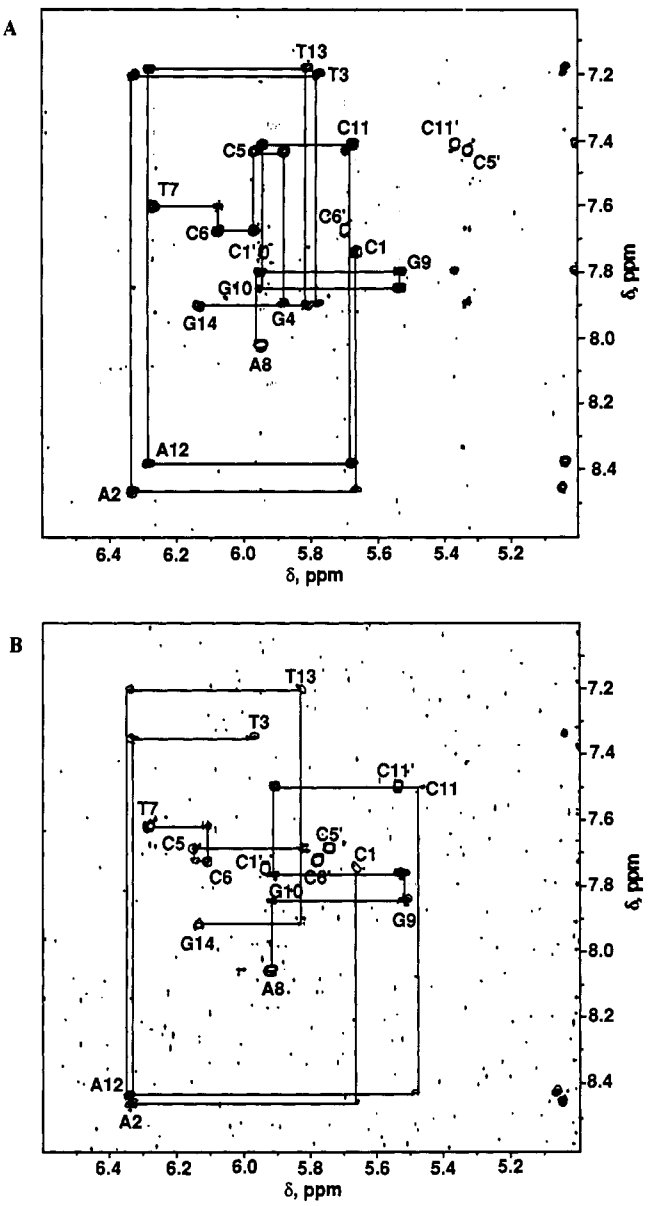

FIGURE 5: Expanded contour plots of the phase-sensitive NOESY spectrum of (A) d(CATGCCT)/d(AGGCATG) and (B) d-(CATG*CCT)/d(AGGCATG), where G* = *S*-[2-(N^7-guanyl)-ethyl]GSH. Connectivities between base protons (7.0–8.6 ppm) and sugar H1′ protons (5.0–6.6 ppm) are plotted. The lines follow the connectivities between adjacent base protons and their intervening sugar H1′ protons. The cytosine H6–H5 cross-peaks are denoted by primed numbers.

ical exchange. Although broadened, the resonances for A_2·T_{13}, T_3·A_{12}, C_5·G_{10}, C_6·G_9, and C_1·G_{14} are all seen at approximately the same chemical shifts as in the underivatized oligomer. There is a large, downfield shift seen for the G_4·C_{11} pair (~ 0.75 ppm), which is most likely due to the increased acidity of the N^1 proton after guanine N^7-alkylation. This effect could possibly even lead to full proton transfer to cytosine, although protonated cytosine hydrogen bonds are usually seen further downfield (Rajagopal & Feigon, 1989). The small shoulder on the upfield side of the C_1·G_{14} resonance is of unknown origin and might reflect a small population of a different duplex conformation. Because of rapid exchange at the C^8 position in N^7-guanyl adducts that proton cannot be observed in ^2H_2O, but the H_2O spectra allow observation and the C^8 proton could be seen at δ 9.68.

Characterization of Monomeric Ribonucleosides. It is possible to prepare derivatives of monomeric ribonucleosides

Table III: Changes in ^{13}C NMR Chemical Shifts of Base Carbons of Silylated Mononucleoside Derivatives upon Mixing at a 1:1 Ratio in C^2HCl_3[a]

	ΔG	ΔU	ΔG	ΔC	ΔG	ΔA
C2	0.04	0.04	−0.32	−0.88	−0.04	<0.01
C4	0.07	0.07	−0.13	−0.13	0.10	0.04
C5	0.05	0.03	−0.14	−0.97	0.08	0.02
C6	0.03	0.07	−0.67	0.43	0.04	0.04
C8	0.07		0.50		0.07	−0.04

	Δ(N^7-MeG)	ΔU	Δ(N^7-MeG)	ΔC	Δ(N^7-MeG)	ΔA	Δ(N^7-MeG)	ΔG
C2		0.03		−0.22		<0.01		−0.57
C4	0.05	0.18	0.16	0.11	0.03	0.02	0.28	−0.03
C5	−0.04	−0.01	<0.01	0.07	0.03	0.01	0.08	0.10
C6	−0.06	0.15	0.24	0.03	0.33	0.08	−0.01	0.31
C8	0.08		−0.28		0.38	0.01	−0.21	0.15

[a] The 5′-*O*-(*tert*-butyldimethylsilyl)-2′,3′-*O*-isopropylidene derivatives of the bases were mixed at a 1:1 ratio in C^2HCl_3 to a total concentration of 0.2 M. Chemical shifts recorded under these conditions were subtracted from those found for the base alone, and the difference is reported.

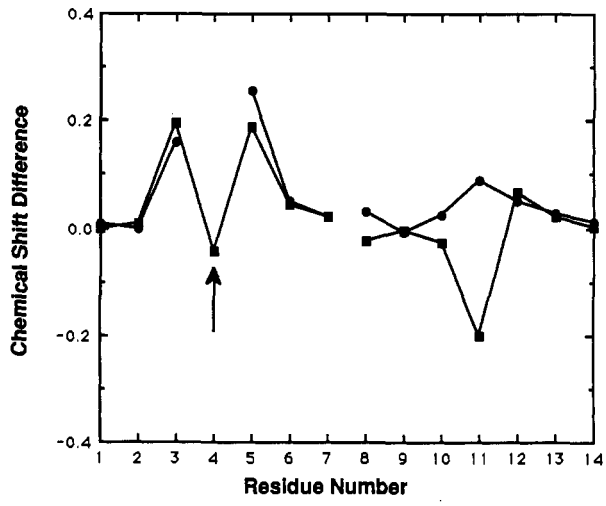

FIGURE 6: Changes in NMR chemical shifts of H1′ and H8/6 base protons in d($C_1A_2T_3G_4C_5C_6T$)/d($A_8G_9G_{10}C_{11}A_{12}T_{13}G_{14}$) resulting from replacement of G_4 with *S*-[2-(N^7-guanyl)ethyl]GSH. H1′ (■), H8/6 (●). The arrow points to the site of modification, where the shift of the H8 proton is not shown because of the large shift due to chemical nature.

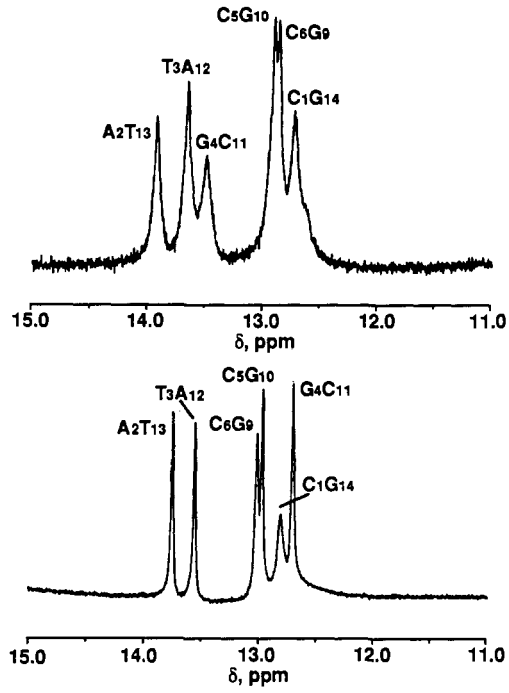

FIGURE 7: Imino proton spectrum of the 1:1 mixtures of (upper trace) d(CATG*CCT)/d(AGGCATG) and (lower trace) d-(CATGCCT)/d(AGGCATG) in H_2O containing 67 mM sodium phosphate (pH 7.0) and 100 mM NaCl at 5 and −5 °C, respectively, and recorded at 400 MHz. G* indicates *S*-[2-(N^7-guanyl)ethyl]GSH. The H_2O signal was suppressed by the 1−3−3−1 pulse method.

and study their pairing in organic solvents such as $CHCl_3$ or $(CH_3)_2SO$ (Katz & Penman, 1966; Newmark & Cantor, 1968). The silylated derivatives of the four natural bases were prepared and examined, along with an N^7-methylguanine derivative, in C^2HCl_3. The changes in the ^1H NMR shifts upon mixing are shown in Table III. The N^7 derivatives of guanine showed attenuated interaction with the cytosine derivative, but no preferred bonding to any of the other base derivatives was seen, a result not inconsistent with that seen in the oligomers (Figure 2).

In another study, the pK_a values of the two guanosyl derivatives containing N^7-methylguanine and *S*-[2-(N^7-guanyl)ethyl]cysteine methyl ester were measured by UV spectroscopy. In H_2O, the pK_a of guanine was ~9 and those of these two N^7-guanyl derivatives were both ~7 (data not presented). When the two N^7-alkylguanines were examined in 80% $(CH_3)_2SO$, the apparent pK_a values were also nearly identical to each other (~8.5 as measured directly with the electrode in this solvent). Thus, the size of the alkyl group present at the N^7-guanyl position does not appear to have a significant influence on the pK_a of the guanine, although the effect of a GSH moiety was not examined in this study. Thus, differences in the tendencies of these different N^7-guanyl substituents to perturb the pK_a of the N^1 proton can probably not be offered as an argument for different biological effects (Humphreys et al., 1990).

Discussion

The oligomer d(CATGCCT) was studied in detail after adduction of the single G residue with *S*-(2-chloroethyl)GSH to form an *S*-[2-(N^7-guanyl)ethyl]GSH residue and subsequent pairing with the normal complementary strand d-(AGGCATG). The NOESY experiments showed several points about the modified duplex structure: (i) the duplex appears to be in a normal B-form; (ii) there are large chemical shift changes seen in the base as well as the sugar protons of the residues flanking the adduct as well as the base directly across the duplex; and (iii) base–base interactions are disrupted on the 3′ side of the adduct on the modified strand. These observations suggest a picture of the structure in which only a localized perturbation has occurred in the sugar–phosphate backbone and possibly characterized by an increased mobility for the bases, especially on the 3′ side of the adduct.

The pairing of a guanine to cytosine appears to be considerably weakened by its N^7-alkylation. This effect is clearly noted in the studies with the monomeric nucleoside derivatives

and the oligomers examined here. d(ATGCAT) was found not to self-associate at all after modification, and in the study with the modified d(CATGCCT)/d(AGGCATG) pair, weakened pairing was observed in the UV, CD, and NMR spectra (Figures 3 and 7). This loss of hydrogen bonding may be due at least in part to the altered pK_a of the N^1 proton and subsequent development of negative charge at the N^1 and O^6 positions, although the pK_a (~7) is still in the physiological range and close to that used in most buffered experiments. In space-filling models of B-form DNA the GSH moiety of S-[2-(N^7-guanyl)ethyl]GSH can very easily be accommodated in the major groove, and even two moieties at adjacent sites or on the opposite sides of adjacent base pairs [G*C/CG*, where G* = S-[2-(N^7-guanyl)ethyl]GSH] do not crowd. Further, no cross-peaks involving the GSH or ethylene moieties were seen in the NOESY spectrum. Further details for the loss of pairing seen upon N^7 substitution in this oligomer remain unclear. In the case of the modified d(ATCGAT), two GSH moieties can be accommodated in the large space of the major groove, as revealed by a space-filling model. However, the chemical shifts of the NMR signals of the modified oligomer were not temperature dependent, suggesting that this sequence does not hybridize when the modified guanine is present. Whether very simple N^7-guanyl alkylations (i.e., methylation) will produce similar effects in the oligomers as the alkylations with the cysteine and GSH derivatives seen here is not known.

Although N^7-guanyl derivatives were some of the first DNA adducts identified, there is little background on the physical consequences of these adducts on DNA structure or the biological relevance as manifested in mutations. Mutations are seen with 1,2-dibromoethane or S-(2-chloroethyl)GSH at guanyl sites in both *Salmonella typhimurium* TA100 (Rannug, 1980; Rannug et al., 1978; Humphreys et al., 1990) and bacteriophage M13mp18 (Cmarik et al., 1991). Work with the latter system indicates that base-pair mutations at GC pairs are very dominant, sometimes clustered at runs of guanines, and that the major change is a GC to AT transition (Cmarik et al., 1991).[2] Thus, if a guanine derivative is a miscoding lesion, then it would appear to act by pairing with a thymine. We considered the possibility that the pairing of an N^7-guanyl derivative with a base other than cytosine due to aberrant hydrogen bonding might be responsible for the mutation. We found that N^7-alkylation of the 2',3'-acetonide derivative of guanosine altered the pK_a (at the N^1/O^6 site) downward, consonant with the literature (Lawley, 1984). However, there was no difference between an N^7-methyl derivative and a more complex adduct in which an N-acetylcysteinyl methyl ester group was present. Thus the differing mutagenicity of the different N^7-alkyl derivatives (Humphreys et al., 1990) could not easily be explained by alterations in pK_a values. We also examined the possibility that the mispairing could be understood in terms of the mispairing of silylated nucleoside derivatives in C^2HCl_3. Indeed, evidence for G·C pairs was observed and the N^7-methylation of the guanyl derivative hindered such pairing (Table III). However, no evidence for a more favorable interaction of the modified guanine with another base was seen, particularly not the uridine derivative. However, these studies in aprotic solvents are limited as models for transition states and intermediates in polymerase reactions, where H_2O molecules may be present and engage in hydrogen bonds with nucleotides. As pointed out in the text above, when synthetic oligonucleotides containing a single guanyl residue were modified to include cysteinyl-based N^7-guanyl adducts, hybridization was attenuated and there was no clear evidence for preferential bonding to a base other than the normal cytosine under conditions where it might have been detected.

Duplex formation was seen when N^7-guanyl-adducted d(CATGCCT) was paired with its normal complement, although the base pairing was weakened as noted above. Experiments with complementary strands containing G, A, or T across from the adduct showed no evidence for duplex formation. On the basis of these observations, the stability of several possible base-pairing schemes can be ruled out. If the N^7-adducted guanine were present in the enol tautomer, then a base pair to T should be stable. Further, if wobble base pairs were favored with the enol or enolate tautomer, then A and C should form stable base pairs. On the basis of these arguments the most likely structures in the base pairing of d(CATGCCT) are either an N^7-G·C or an N^7-G·C^+ (where C^+ is a protonated cytosine).

What can be concluded about the basis of mutations regarding S-[2-(N^7-guanyl)ethyl]GSH adducts derived from 1,2-dibromoethane? First of all, the possibility cannot be unambiguously ruled out that an adduct other than S-[2-(N^7-guanyl)ethyl]GSH derived from S-(2-chloroethyl)GSH (Humphreys et al., 1990; Cmarik et al., 1991) is more mutagenic. However, several other adducts that might be considered, including the imidazole ring opened adduct (Kim et al., 1990), S-[2-(O^6-guanyl)ethyl]GSH, S-[2-(N^2-guanyl)-ethyl]GSH, and S-[2-(N^3-cytosinyl)ethyl]GSH, appear not to be responsible.[2] Also, a role for depurination is not consistent with the observed mutation spectrum (Cmarik et al., 1991) and the lack of SOS response (Humphreys et al., 1990). Since >98% of the adducts are accounted for (Kim et al., 1990), any other minor adduct would have to be extremely mutagenic (Humphreys et al., 1990). Another possibility which must still be considered is that S-[2-(N^7-guanyl)-ethyl]GSH is mutagenic because of its ability to direct thermodynamically favorable hydrogen bonds with thymine but does so only within the particular sequences most prone to mutation. These possibilities are testable, and appropriate experiments are being designed.

Currently the most attractive working paradigm is that the major DNA adduct S-[2-(N^7-guanyl)ethyl]GSH is mutagenic but that the mutations are influenced primarily by kinetics as opposed to thermodynamics in the course of the polymerase reaction. That is, the oligomer d(CATG*CCT)/d(AGGCATG) is seen as the reaction product and is not an appropriate model for the complex of d(···CATG*CCT)/d(···AGG), incoming nucleoside triphosphate, and polymerase which is involved in formation of the transition state, the free energy of which determines the reaction. The fact that the N^7-guanyl adduct studied herein does not form a preferential base pair with T does not mean the idea of thermodynamically favored product stability can be completely dismissed. A mutational mismatch need not be as stable as the normal base pair because very low mutational frequencies can still be very important. However, one would expect to see at least some sign of stable product formation with the known in vivo mismatch. In this case, there is even evidence that the normal oligonucleotide forms some duplex that contains a G·T or G·G mismatch, under the same conditions that show no mismatched base pairs with the modified oligomer (Figures 2 and 3). The degree to which certain substitutions "fit" with modified oligonucleotides may not explain the kinetic events which give rise to them. This view is certainly not without precedent. For instance, the thermodynamics of formation of "right" and "wrong" base pairs are not really sufficient to explain the low error rates seen in replication. Polymerases of low fidelity can

readily produce unusual products, in terms of base pairs, and the thermodynamic instability is compensated for by overall duplex formation (Abbotts et al., 1991). Swann (1990) has recently reviewed the literature associated with O^6-alkylguanine and O^4-alkylthymine miscoding. Of interest is the observation that the major mutation directed by an O^6-methylguanine is a GC → AT transition, as in the case of the system under consideration here—also, synthetic oligomers containing an O^6-methylguanine·cytidine pair have higher T_m values than those containing an O^6-methylguanine·thymine pair (Gaffney et al., 1984; Gaffney & Jones, 1989). Possible explanations advanced are that (i) the DNA polymerase might mistake an O^6-alkylguanine for an adenine on the basis of physical similarities or (ii) the most important factor in miscoding is that the O^6-alkylguanine·thymine mispairs retain a Watson–Crick alignment as opposed to a wobble conformation in an O^6-alkylguanine·cytosine pair, either of which suggests that the stability of normal base pairs has been overemphasized in DNA replication (Swann, 1990). The appropriateness of these particular hypotheses in the case of *S*-[2-(N^7-guanyl)ethyl]-GSH is not yet clear, but it is apparent that mechanisms of mutation will need to be understood in the context of the enzymes involved.

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