Formation of *S*-[2-(N^7-Guanyl)ethyl] Adducts by the Postulated *S*-(2-Chloroethyl)cysteinyl and *S*-(2-Chloroethyl)glutathionyl Conjugates of 1,2-Dichloroethane†

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ABSTRACT: The formation of *S*-[2-(N^7-guanyl)ethyl]glutathione (GEG) from dihaloethanes is postulated to occur through two intermediates: the *S*-(2-haloethyl)glutathione conjugate and the corresponding episulfonium ion. We report the formation of GEG when deoxyguanosine (dG) was incubated with chemically synthesized *S*-(2-chloroethyl)glutathione (CEG). The depurination of GEG was shown to be first order with a half-life of 7.4 ± 0.4 h at 27 °C. Evidence is also presented for the formation of *S*-[2-(N^7-guanyl)ethyl]-L-cysteine (GEC) in incubation mixtures containing dG and *S*-(2-chloroethyl)-L-cysteine (CEC), the corresponding cysteine conjugate of CEG. This finding demonstrates that this (haloethyl)cysteine conjugate does not require activation by enzymatic action of cysteine conjugate β-lyase but, instead, can directly alkylate DNA. The half-life of the depurination of GEC was 6.5 ± 0.9 h, which is no different from that of GEG. Of the two conjugates, CEC is a somewhat more active alkylating agent toward dG than CEG as N^7-guanylic adduct was detected in reaction mixtures with lower concentrations of CEC than with CEG.

Evidence that nucleic acid binding exhibited by the vicinal dihaloethanes 1,2-dibromethane (EDB) and 1,2-dichloroethane (EDC) is dependent upon their conjugation with glutathione has steadily accumulated (Rannug et al., 1978; Shih & Hill, 1981; Sundheimer et al., 1982; Storer & Conolly, 1985). Recently, a guanine adduct, *S*-[2-(N^7-guanyl)ethyl]glutathione (GEG), was isolated from rats that had been administered EDB (Ozawa & Guengerich, 1983; Koga et al., 1986). The formation of GEG by vicinal dihaloethanes is thought to occur via at least two intermediates. The postulated sequence of events leading to addduct formation includes (a) conjugation of the dihaloethane with GSH via the glutathione *S*-transferases to form the first intermediate, the sulfur half-mustard, (b) cyclization of the half-mustard to form the second intermediate, the electrophilic episulfonium ion (Smit et al., 1979), (c) alkylation by the episulfonium ion at the N^7 position of guanine residues in DNA, and (d) depurination of this alkylation product from the DNA. The intermediate sulfur half-mustard (precursor of the episulfonium ion) would be quite labile at physiological conditions, tending to dehalogenate and react with hydroxyl ions to form the corresponding alcohol. The sulfur half-mustard of EDB, *S*-(2-bromoethyl)glutathione, has never been isolated probably due to its inherent lability and rapid rate of episulfonium ion formation. Inskeep et al. (1986) estimated the half-life of the GSH-dependent alkylating species of EDB to be less than 10 s by observing the decrease in DNA binding in EDB-containing incubates in which added glutathione *S*-transferases had been inhibited with triphenyltin chloride.

The sulfur half-mustard of EDC, *S*-(2-chloroethyl)glutathione (CEG), is more stable than the bromo analogue and has been isolated in relatively pure form in this laboratory (Reed & Foureman, 1986; Schasteen & Reed, 1983). Preliminary studies showed this direct-acting conjugate to be a fairly potent alkylating species toward 4-(nitrobenzyl)pyridine.

The biotransformation of GSH conjugates through the mercapturic acid pathway obligates their existence as cysteine conjugates (Tateishi & Shimizu, 1980). Recently, workers have clearly implicated the cysteine conjugate β-lyase in the nephrotoxicity of several halogenated alkanes and alkenes including chlorotrifluoroethene (Dohn et al., 1985) and hexachlorobutadiene (Nash et al., 1984). The GSH conjugate of EDC could also proceed through the mercapturic acid pathway to the level of a cysteine conjugate, but it is uncertain whether CEC can serve as a substrate for the cysteine conjugate β-lyase (Stevens & Jakoby, 1983). It is thought, however, that the corresponding cysteine conjugate of CEG, *S*-(2-chloroethyl)-L-cysteine (CEC), would be a direct-acting alkylating agent.

The purpose of this study is 2-fold: (a) to ascertain whether the postulated intermediate of EDC metabolism, CEG, can form the N^7-guanylic adduct, GEG, thereby providing confirmation of its status in the metabolic pathway outlined above; (b) to ascertain whether or not the corresponding cysteine conjugate of CEG, CEC, is a direct-acting alkylating agent. To accomplish these ends, we have characterized the reaction products of chemically synthesized CEC and CEG with deoxyguanosine (dG) to determine the main adducts that can be formed from these postulated metabolic products of EDC.

Materials and Methods

All chemicals used were of the highest grade available from commercial sources. The synthesis of both *S*-(2-chloroethyl)glutathione (CEG) and *S*-(2-chloroethyl)-L-cysteine (CEC) was as reported in Reed and Foureman (1986) and in Schasteen and Reed (1983). The CEG was 90% pure as determined by high-performance liquid chromatography (HPLC), the main contaminant being the hydrolysis product; CEC was >95% pure by the same criteria.

Bulk preparation of the deoxyguanosine adducts for chemical analysis was accomplished by adding, as the dry powder, 227 μmol of either CEC (42 mg) or CEG (85 mg) to 4 mL

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of a solution containing 22.3 μmol (6 mg) of deoxyguanosine. This mixture was buffered to pH 7.4 with 0.1 M sodium cacodylate and incubated overnight at 27 °C. The next morning this mixture was heated to 95 °C for 30 min to complete the depurination, after which 0.1–0.2-mL aliquots were applied to a 5-μm ODS analytical column (Custom LC, Inc., Houston, TX). The chromatography was developed isocratically in 5 mM potassium phosphate, pH 4.5, with 3% acetonitrile at 1.5 mL/min while the eluate was monitored with a variable-wavelength detector set at 283 nm. Fractions containing the adducts were then collected as they eluted, lyophilized, and reapplied to the same column in 3% acetonitrile to desalt the sample; some salt was necessary to resolve the adduct from the parent dG. The fractions containing adduct were again collected, lyophilized, and submitted for various chemical analyses. We estimated the amount of the material recovered from this processing to be about 0.6 mg, which represents about 50% of the adduct originally present in the reacted bulk solution.

For the determination of the depurination rates, incubation mixtures in which the amount of CEC or CEG relative to dG was increased to 33:1 were used. Initiation of the experiment was by addition and dissolution of the dry CEC or CEG; 0.02-mL aliquots were taken at various times and injected directly onto the HPLC; the total volume of these incubates was 0.30 mL. The end point (100% = A_t) for the depurination reaction was obtained from the same incubates which were heated at 95 °C for 30 min after termination of the experiment. Half-lives were then determined from plots of log (A_t − A_{exp}) and time; the variability (r^2) for all plots was >0.97. The first-order rate constants k were then obtained from the relationship $k = 0.693/t_{1/2}$ (Jencks, 1969).

In determining the amount of adduct formed from different molar ratios of either CEC or CEG to dG, incubates similar to those described above were used except that the total volume was 0.06 mL and that the dry CEC or CEG was added in water immediately after the compound was dissolved (approximately 10–15 s). These reaction mixtures were incubated overnight at 27 °C after which they were heated at 95 °C for 30 min; 0.02-mL aliquots were then taken for analysis on HPLC. Standardization of the amount of adduct present was by peak area comparison with a known amount of 7-methylguanine.

Fast atom bombardment (FAB) mass spectroscopy was accomplished on a Varian CH7 mass spectrometer equipped with an Ion Tech FAB gun. Samples were mixed with glycerol on the probe and then dissolved by adding a minimum amount of water.

^{1}H NMR spectra were obtained with a Bruker AM400 NMR spectrometer operating at 400 MHz. All analyses were accomplished with between 0.5 and 2 mg of sample dissolved in 0.5 mL of deuterium oxide. In the case of CEG and CEC, the lyophilized powder was dissolved, and the spectra were obtained within 4 min. Other conditions during data acquisition are listed in Figure 2.

Results

CEC or CEG was incubated with dG in a ratio of 33:1 (alkylating agent:base) for 24 h at 27 °C in 0.1 M cacodylate buffer; the pH of the incubate at the beginning of this period was 7.4 and 7.0 at the end. CEC and CEG each gave a single new UV-absorbing peak that eluted before the parent dG (data not shown); this peak was apparently the only new one produced as a 3%–20% gradient of acetonitrile in 0.025 M potassium phosphate, pH 4.5, ran over 30 min eluted no other UV-absorbing species (283 nm).

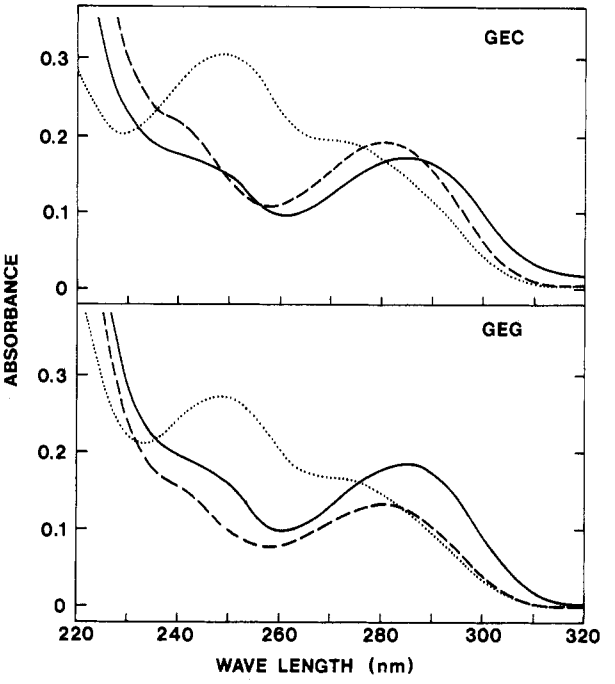

FIGURE 1: UV spectra of the alkylation products formed from dG and CEC (GEC) and from dG and CEG (GEG). Spectra for each sample were taken in water (—), 0.1 N HCl (···), and 0.1 N KOH (– –). Spectra were obtained from a Cary Model 15 spectrophotometer in a 0.05-cm path-length cell with approximately 20 μg of GEC and 35 μg of GEG.

UV spectroscopy of the new material derived from both CEC and CEG yielded spectra with shifts quite similar to one another (Figure 1) and to those of authentic 7-ethylguanine (Singer, 1975). The λ_{max} of the new material from both CEC and CEG was in the region of 282–284 nm, qualitatively different from the λ_{max} of the parent dG at 254 nm. From these data it could be deduced that these new peaks were both N^7-guanine adducts derived from CEC and CEG. The absence of the sugar moiety from the starting nucleoside was confirmed as the spectral characteristics of dG adducts differ markedly from the corresponding guanine adducts (Singer, 1975). Questions involving the presence of the sugar and the nature of the adduct-forming species were also resolved by NMR. These new peaks were collected, processed as outlined under Materials and Methods, and submitted for NMR analysis, the results of which are presented in Figure 2. The 1′ proton on the ribose sugar of dG has a characteristic triplet at 6.4 ppm (Figure 2a). Also, it should be noted that the only signal from the guanine moiety is the C-8 proton, present as a singlet at 8.1 ppm. In the 400-MHz NMR spectra of both the CEG- (Figure 2b) and CEC- (Figure 2c) derived material, the 1′ ribose signal at 6.5 ppm was absent whereas the guanylic C-8 singlet at 8.1 ppm was present. We conclude from these observations that the new products are guanine adducts and that depurination of the nucleoside has taken place.

Signals upfield of the water peak (4.8 ppm) are associated with the species attached to the guanine. In the spectrum of unreacted, intact CEC (Figure 3a) the signal from the cysteinyl methylene was split into a pair of doublets at 3.1–3.3 ppm whereas the α-hydrogen of cysteine was present as a multiplet at 4.1 ppm. The presence of these two signals in the adduct (Figure 3b) at approximately the same chemical shifts and with the same splitting patterns indicated the entire cysteine moiety to be present in the adduct. Returning to consideration of CEC (Figure 3a), the signal from the methylene next to the chlorine was present as a triplet of doublets at approximately 3.8 ppm whereas the signal from the

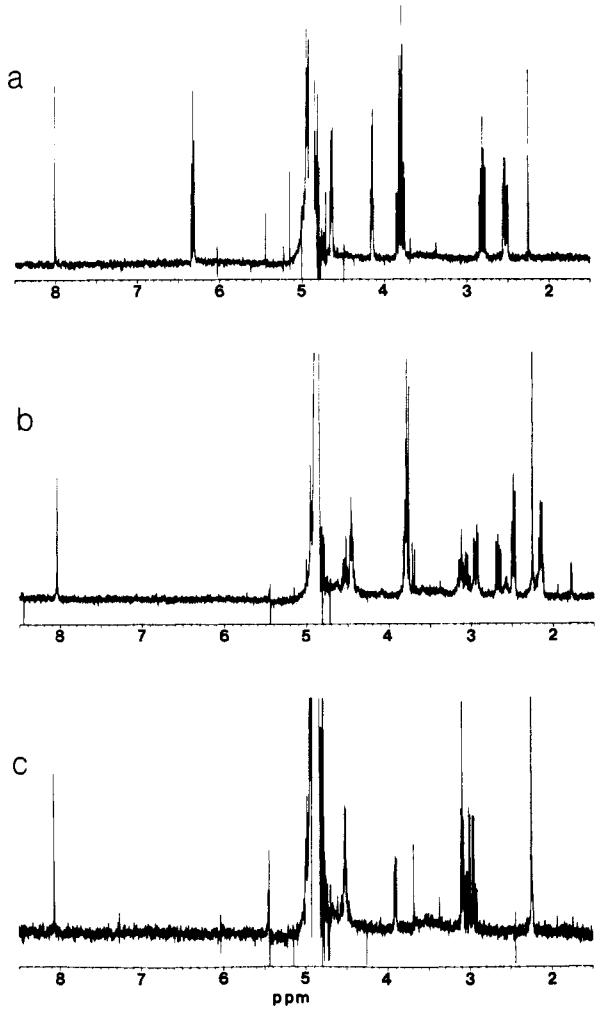

FIGURE 2: The 400-MHz ^{1}H NMR spectra of (a) deoxyguanosine, (b) *S*-[2-(N^7-guanyl)ethyl]glutathione, and (c) *S*-[2-(N^7-guanyl)-ethyl]-L-cysteine. The spectra were obtained under the following conditions: flip angle 74°; sweep width 6000 Hz; filter width 7600 Hz; number of scans 64; number of data points 16 000; acquisition time 1.4 s; relaxation delay 2 s. *tert*-Butyl alcohol was used as the internal standard. The signal at 2.3 ppm in these spectra and in those of Figures 3 and 5 is an unknown contaminant.

methylene next to the sulfur was present as a triplet at 3.0 ppm; the assignment of these two signals was based on their electronic characteristics as chlorine is more deshielding than is sulfur (Gordon & Ford, 1972). In the adduct (Figure 3b), both methylenic signals were present, but at different chemical shifts. The signal from the methylene next to the sulfur, identifiable by its triplet splitting pattern, is shifted downfield from about 3.0 ppm in CEC to 3.1 ppm in the adduct. The characteristic signal (triplet of doublets) from the methylene next to the chlorine in CEC is identifiable in the adduct but was shifted far downfield, from 3.8 to 4.5 ppm. As the guanylic system is deshielding and as the methylene formerly next to the chlorine was more deshielded than was the methylene next to sulfur, it is totally consistent to assign the guanylic linkage through the S-2 methylene. As an ancillary confirmation of its structure, the adduct was submitted to positive ion FAB mass spectroscopy (Figure 4). The predicted molecular ion (CEC + guanine − Cl) was observed at m/z 299 (M + 1) as well as m/z 321 (M + 1 + Na) and m/z 337 (M + 1 + K). Thus, UV, NMR, and MS data confirm the structure of the CEC–guanine adduct as *S*-(2-[N^7-guanyl]-ethyl)-L-cysteine.

The NMR of the CEG adduct may be explained in a manner analogous with the CEC adduct. Figure 5a is the

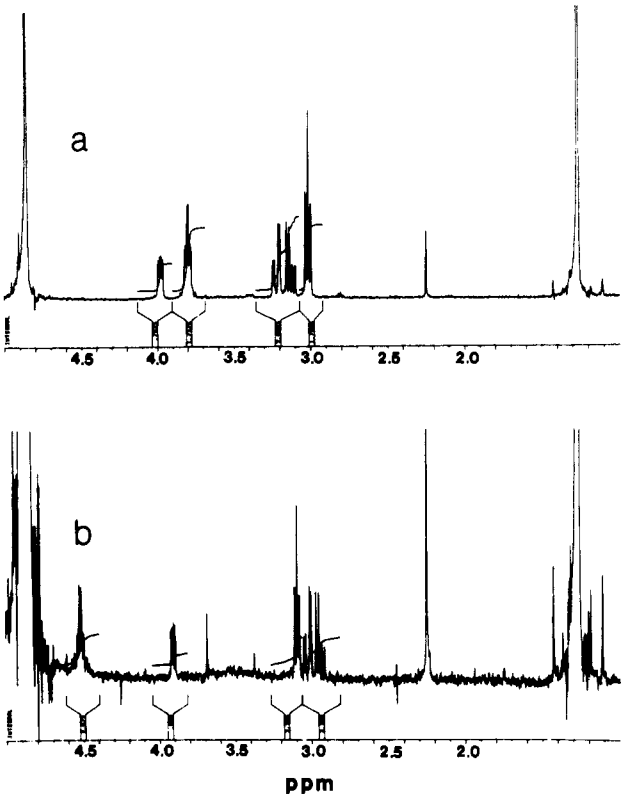

FIGURE 3: The 0–5.0 ppm portion of the 400-MHz spectra of (a) *S*-(2-chloroethyl)-L-cysteine and (b) *S*-[2-(N^7-guanyl)ethyl]-L-cysteine. Data acquisition parameters were as listed in Figure 2.

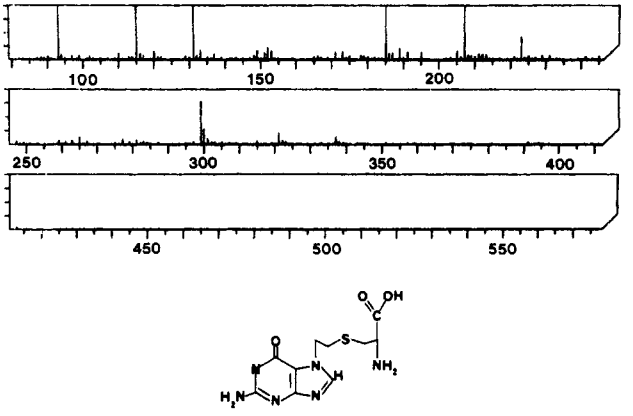

FIGURE 4: Positive ion FAB mass spectrum of *S*-[2-(N^7-guanyl)-ethyl]-L-cysteine. Glycerol was used as the sample matrix.

400-MHz spectrum of unreacted, intact CEG. The glutamyl methylenes were apparent at 2.2 and 2.6 ppm, as were the cysteinyl α-hydrogen at 4.6 ppm and a portion of the characteristic signal pattern of the cysteinyl methylene (pair of doublets) at 3.2 ppm. The glycyl signal at 4.0 ppm and the glutamyl α-hydrogen at 3.9 ppm were nicely resolved. The *S*-ethyl signals were located at about the same chemical shifts as in CEC, at 3.8 ppm (methylene next to Cl; triplet) and 2.9 ppm (methylene next to S; multiplet). This latter signal was obscured by the upfield portion of the cysteinyl methylene signal; the integration (indicating three protons) and 2D NMR COSY experiment confirmed that protons in this multiplet were coupled to the cysteinyl signals at 3.2 ppm and to the other *S*-ethyl methylene at 3.8 ppm (data not shown).

In the NMR spectrum of the CEG-generated adduct (Figure 5b) the glutamyl methylenes at 2.2 and 2.5 ppm were easily discernible as was the cysteinyl methylene, now totally resolved and moved upfield to 2.7–2.9 ppm. The 2D NMR

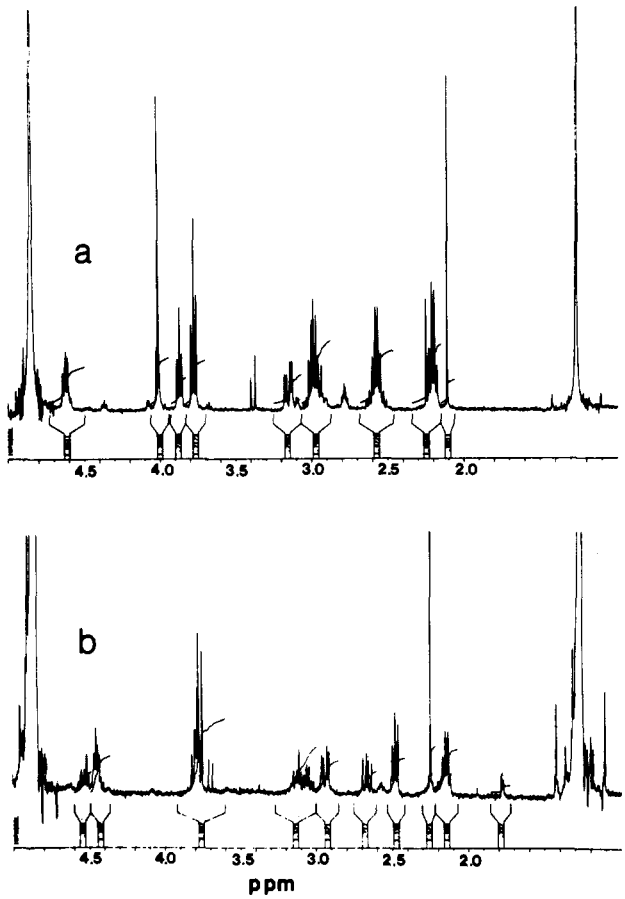

FIGURE 5: The 0–5.0 ppm portion of the 400-MHz spectrum of (a) *S*-(2-chloroethyl)glutathione and (b) *S*-[2-(N^7-guanyl)ethyl]glutathione. The data acquisition parameters were as described in Figure 2.

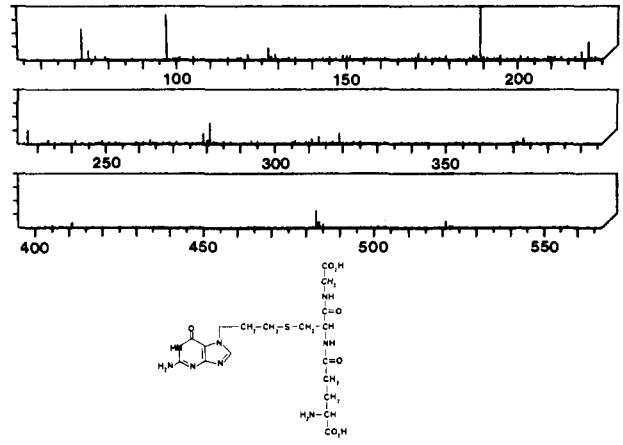

FIGURE 6: Negative ion FAB mass spectrum of *S*-[2-(N^7-guanyl)-ethyl]glutathione. Glycerol was used as the sample matrix.

COSY spectrum showed the multiplet at 3.8 ppm to contain at least one proton that was coupled to the glutamyl methylene signal at 2.2 ppm and other protons that were not coupled to any other signals in the spectrum. As this multiplet integrated at 3, it was concluded that it contained the signals both from the glycyl methylene and from the glutamyl α-hydrogen. The presence of these signals indicates the entire glutathionyl moiety to be present in this adduct.

Major qualitative differences existed between the spectra in Figure 5 at around 4.4 and 3.1 ppm. The distinctive coupled cysteinyl methylene signal was shifted upfield from about 3.15 to 2.95 ppm. The multiplet that obscured one part of the cysteinyl methylene signal (3.0 ppm, Figure 5a) was resolved

Table I: Half-Life and First-Order Rate Constants (k) for the Depurination of the Adducts Formed by Reaction of Deoxyguanosine with *S*-(2-Chloroethyl)glutathione (CEG) and with *S*-(2-Chloroethyl)-L-cysteine (CEC)[a]

compound	$t_{1/2}$ (h)	k ($\times 10^2$ h^{-1})
CEG	7.4 ± 0.3 (n = 4)	9.4 ± 0.4 (n = 4)
CEC	6.5 ± 0.9 (n = 5)	10.7 ± 1.4 (n = 5)

[a] See text for details of the analyses.

Table II: Percent of dG Converted to *S*-[2-(N^7-Guanyl)ethyl] Adducts in Incubation Mixtures with Differing Molar Ratios of *S*-(2-Chloroethyl)-L-cysteine (CEC) or *S*-(2-Chloroethyl)glutathione (CEG)

molar ratio of CEC(G):dG[a]	percent dG converted by	
	CEC	CEG
1:1	0.4 ± 0.1[b]	<0.1
5:1	2.7 ± 0.2	3.0 ± 0.8
10:1	6.7 ± 0.4	6.9 ± 1.0
33:1	20.2 ± 0.2	23.2 ± 1.0

[a] 100% dG = 112 nmol. Incubation mixtures with a volume of 0.3 mL were maintained at pH 7.4 and 27 °C for 12 h after which they were heated in sealed containers for 30 min at 95 °C. Aliquots were then applied directly to the HPLC. Quantitation was by comparison with known amounts of 7-methylguanine. See text for more details. [b] Percent ± SD; n = 3–4.

and present downfield at 3.0–3.2 ppm as a broad multiplet with an integration proportion of 2. Absent from Figure 5b was the triplet signal from the methylene next to chlorine, formerly at 3.8 ppm in Figure 5a; instead, there was a broad multiplet far downfield at 4.4–4.6 ppm that integrated at 3. The 2D NMR COSY spectra of Figure 5b showed this broad multiplet to contain protons that were coupled to the broad multiplet at 3.0–3.2 ppm as well as to the upfield complex of the cysteinyl methylene signal at 2.7 ppm. This new multiplet at 4.4–4.6 ppm contained the signals both from the cysteinyl α-hydrogen and from one of the *S*-ethyl methylenes. In a manner totally analogous to the shift observed with the methylene signals in Figure 3 and in concordance with the expected chemical shift of protons directly next to the deshielding guanine system, the methylene signal present in the complex at 4.4–4.6 ppm was designated as the S-2 methylene, the one formerly next to chlorine.

This GSH-derived adduct was also submitted to negative ion FAB mass spectroscopy (Figure 6) and gave the predicted molecular ion (CEG + guanine − Cl) at m/z 483 (M − 1); the sodium addition product of the molecular ion was also observed at m/z 503 as was the potassium addition product at m/z 521. Thus, UV, NMR, and mass spectral data confirm the structure of the CEG-derived adduct as *S*-[2-(N^7-guanyl)ethyl]glutathione.

The rate of appearance of the N^7-guanyl adducts from incubates containing either CEC or CEG is documented in Figure 7. A peak that appeared in the chromatograms of these incubates may have been the intact nucleoside adduct as it appeared to be maximal at 2 h of incubation and subsequently decreased with time; it was insufficiently resolved, however, from the parent dG to be quantitated. As the half-life of both CEC and CEG under these incubation conditions is on the order of 10–15 min (Reed & Foureman, 1986), the compounds would be essentially nonexistent after 2 h, the time of the first point in this chart. As can be seen, there were no appreciable differences in the rates of depurination between these two adducts. The first-order rate constants were derived from plots such as these and are presented in Table I.

To judge the relative alkylation efficacy of these compounds, dG was incubated with differing molar ratios of either CEC

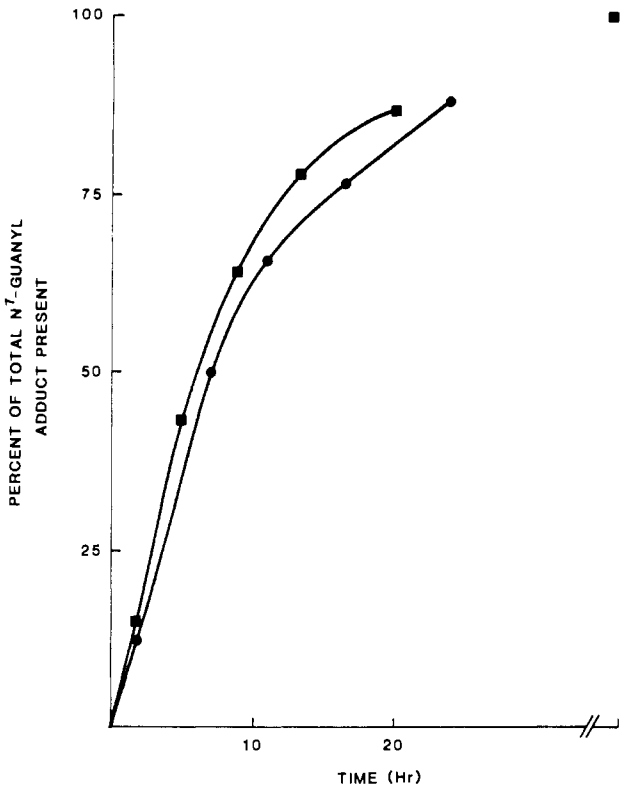

FIGURE 7: Rate of appearance of *S*-[2-(N^7-guanyl)ethyl]glutathione (●) and of *S*-[2-(N^7-guanyl)ethyl]-L-cysteine (■) in incubates containing deoxyguanosine (dG) and either CEC or CEG in a 33-fold molar excess to dG. The pH of the incubates was maintained at 7.4 by 0.1 M cacodylate buffer with the temperature at 27 °C. At the appropriate times, 0.02-mL aliquots were withdrawn from the individual incubates and applied directly to a 5-μm ODS RP column. See text for further details.

or CEG, the N^7-guanylic adducts were quantitated, and the results were tabulated in Table II. It is important to note that adduct formation could be detected with CEC and not with CEG at molar equivalency with dG. This can be taken to mean that, at low concentration, CEC is a somewhat more avid alkylating agent than is CEG. This may possibly be due to differences in the reactivity of the other postulated intermediate, the episulfonium ion, which may arise from either CEC or CEG. This difference seemed to be overcome at higher concentrations.

Discussion

The data presented here demonstrate that the corresponding cysteine conjugate of CEG, CEC, is capable of alkylation. The fact that we were able to detect the CEC–N^7-guanyl adduct at lower concentrations of alkylating agent then we could the corresponding CEG adduct indicates CEC to be a somewhat more potent alkylating agent than CEG, at least toward dG as a substrate. Although a recent report states that CEC was not a substrate for the cysteine conjugate β-lyase (Stevens & Jakoby, 1983), our work demonstrates that activation through this enzyme is not necessary for nucleoside alkylation. Also, cleavage of CEG by those enzymes that catabolize glutathione would apparently produce little change, if any, in the potential of this compound to alkylate DNA.

The half-lives of the depurination or release of the N^7-guanyl adducts from the deoxyguanosine moiety reported here (Table II) are consistent with those reported for depurination of 7-methylated dG (Lawley & Brookes, 1963) and for dG reacted with nitrogen- and phosphoramide-mustards (Kallama & Hemminki, 1986). As it is known that the rates of hydrolysis of 7-alkylguanines at neutral pH are 7-alkyldeoxyguanosine > 7-alkyldeoxyguanosine monophosphate > alkylated DNA (Lawley & Brooks, 1963); the rates we report may reflect those reported by Inskeep et al. (1986) but apparently differ from those reported by Nachtomi and Sarma (1977); this latter report was an in vivo study and probably represents the repair of the N^7-guanyl lesions.

The N^7-guanyl adduct was apparently the only product formed by CEC and CEG in these studies even when alkylating agent to base ratios were as high as 33:1 as no other UV-absorbing material was detected when the HPLC column was flushed with 20% acetonitrile. This fact demonstrates the specificity of this reaction and is consistent with the findings of Koga et al. (1986), who found GEG to be the major stable nucleic acid adduct formed in their in vivo experiments with EDB. It should be noted that the specificity for the N^7 position on guanine was maintained with the free nucleoside where access to the other sites on guanine susceptible to alkylation (O^6 or N^2) were not blocked by hydrogen binding as they would be in the intact DNA (Singer, 1975).

In another study (Reed & Foureman, 1986) we found evidence of extended alkylating capability by CEC and postulated the existence of a reactive species other than the episulfonium ion as being the causal factor. We anticipated but did not find any evidence for a reactive species other than the episulfonium ion in this study; this may be due to the low reactivity of this unknown species toward dG but may also indicate that this reactive species could be involved in other processes, e.g., phosphate ester formation.

We do not mean to imply that N^7-guanylic adducts are the sole adducts formed by CEC or CEG with DNA. Inskeep et al. (1986) provide chromatographic evidence for GEG and for at least two other unknown products in thermal hydrolysates of DNA isolated from rats exposed to radiolabeled 1,2-dichloroethane. These unidentified products indicate that the metabolites of EDC generate other products in addition to those characterized in this work.

Our study establishes that the postulated intermediates of EDC metabolism, CEC and CEG, are direct-acting alkylating agents, capable of forming *S*-[2-(N^7-guanyl)ethyl] adducts. To our knowledge, this is the first demonstration of a glutathione conjugate acting as an alkylating agent. Further studies are planned in this laboratory to elucidate more conclusive evidence of the other reactive intermediate postulated to occur during alkylation by dihaloethanes, the episulfonium ion.

Acknowledgments

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Registry No. dG, 961-07-9; GEG, 100840-34-4; CEG, 75607-61-3; GEC, 106947-24-4; CEC, 28361-96-8.

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Biochemistry of Terminal Deoxynucleotidyltransferase (TdT): Characterization and Mechanism of Inhibition of TdT by P^1,P^5-Bis(5′-adenosyl) Pentaphosphate†

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ABSTRACT: The catalysis of DNA synthesis by calf thymus terminal deoxynucleotidyltransferase (TdT) is strongly inhibited in the presence of Ap_5A, while replicative DNA polymerases from mammalian, bacterial, and oncornaviral sources are totally insensitive to Ap_5A addition. The Ap_5A-mediated inhibition of TdT seems to occur via its interaction at both the substrate binding and primer binding domains as judged by (a) classical competitive inhibition plots with respect to both substrate deoxynucleoside triphosphate (dNTP) and DNA primer and (b) inhibition of ultraviolet light mediated cross-linking of substrate dNTP and oligomeric DNA primer to their respective binding sites. Further kinetic analyses of Ap_5A inhibition revealed that the dissociation constant of the Ap_5A–enzyme complex, with either substrate binding or primer binding domain participating in the complex formation, is approximately 6 times higher (K_i = 1.5 μM) compared to the dissociation constant (K_i = 0.25 μM) of the Ap_5A–TdT complex when both domains are available for binding. In order to study the binding stoichiometry of Ap_5A to TdT, an oxidized derivative of Ap_5A, which exhibited identical inhibitory properties as its parent compound, was employed. The oxidation product of Ap_5A, presumably a tetraaldehyde derivative, binds irreversibly to TdT when the inhibitor–enzyme complex is subjected to borohydride reduction. The presence of aldehyde groups in the oxidized Ap_5A appeared essential for inhibitory activity since its reduction to alcohol via borohydride reduction or its linkage to free amino acids prior to use as an inhibitor rendered it completely ineffective. With use of a tritiated oxidation product of Ap_5A, a binding stoichiometry of 1 mol of Ap_5A to 1 mol of TdT was observed. Thus, a single Ap_5A molecule seems to span across both the substrate and primer binding site domains in TdT.

Enzymatic synthesis of DNA is a complex reaction that involves multiple components. Of the various DNA polymerases that catalyze this reaction, terminal deoxynucleotidyltransferase (TdT)[1] is a relatively simple polymerase since it does not require template direction, and consequently, all four deoxyribonucleoside triphosphates (dNTPs), with the exception of Mn-dATP, compete for binding to TdT (Bollum, 1974; Modak, 1979). The ability of ribonucleoside triphosphates (rNTPs) to compete with dNTPs for binding to TdT with subsequent enzyme inhibition is a rather unique feature of TdT (Kato et al., 1967; Bhalla et al., 1977; Modak, 1978, 1979). Thus the substrate binding site in this enzyme appeared to be able to bind both dNTPs and rNTPs. Using ultraviolet (UV) light mediated cross-linking of substrate dNTPs and photoaffinity labeling with azido-ATP to TdT, we demonstrated that the 26-kDa subunit of TdT was responsible for binding of both dNTPs and ATP and that the cross-linking of these triphosphates exhibited all the characteristics and requirements that were known to form enzyme–substrate complex (Modak & Gillerman-Cox, 1982; Abraham et al., 1983). While affinity labeling in this manner provides a

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[1] Abbreviations: Ap_5A, P^1,P^5-bis(5′-adenosyl) pentaphosphate; TdT, terminal deoxynucleotidyltransferase; dNTP, deoxynucleoside triphosphate; K_i, dissociation constant; rNTP, ribonucleoside triphosphate; UV, ultraviolet; o-Ap_5A, Ap_5A oxidized at the 2′- and 3′-positions of ribose moieties; Gp_3A, guanosine(5′)triphospho(5′)adenosine; Gp_3G, P^1,P^3-bis(5′-guanosyl) triphosphate; mGp_3G, 7-methylguanosine(5′)triphospho(5′)guanosine; Ap_4A, P^1,P^4-bis(5′-adenosyl) tetraphosphate; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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