Reaction of 1,3-Bis(2-chloroethyl)-1-nitrosourea with Synthetic Polynucleotides†

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ABSTRACT: The antitumor agent BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) was incubated with poly(C) and poly(G) in aqueous solution at 37° and pH 7 to produce approximately 0.33 and 0.07% substitution, respectively. Under the same conditions, there was relatively little reaction with poly(A) and poly(U). Polynucleotides reacted with [^{14}C]BCNU were digested by chemical and enzymatic methods, and the derivative nucleotides were isolated by column chromatography. These were identified by a combination of ultraviolet and mass spectroscopy as 3-(β-hydroxyethyl)CMP, 3,N^4-ethano-CMP, and 7-(β-hydroxyethyl)GMP. This would indicate that BCNU generates active two carbon fragments, probably chloroethyl carbonium ions, which are free to react with nucleotides. The production of these substituted bases may be important to the mechanism of action of the therapeutic nitrosoureas since they would probably alter the function of any nucleic acid which contained them.

Many antineoplastic agents, as well as many environmental mutagens and carcinogens, are known to modify cellular DNA and RNA. Such reactions could be responsible for either the therapeutically useful cytotoxic effects or the deleterious side effects of drugs like the nitrosoureas. Accordingly, it is important to elucidate the nature of their interaction with nucleic acids.

Chemical identification of the nucleotide modifications is a preliminary step in determining the significance of these reactions. In the past, this identification has been based largely on comparison with model compounds. Although this approach works well for the identification of simple derivatives like the methylated nucleotides, it is advantageous to use independent methods such as mass spectroscopy to establish the structure of more complicated derivatives.

This approach is illustrated here by the reaction of the antitumor agent, BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea)[1] with synthetic polynucleotides. Although this compound is not a typical alkylating agent in a biological sense, it does possess some alkylating activity (Wheeler and Chumley, 1967). At the same time, BCNU is closely related to the carcinogen, methylnitrosourea, which is known to methylate DNA and RNA (Lawley, 1972). Thus, elucidation of the interaction of BCNU with nucleic acids is of primary importance.

Experimental Procedures

The polynucleotides used in these studies were obtained from Miles Laboratories or Schwarz Bioresearch. Crystalline BCNU (NSC-409962) was supplied by the Division of Cancer Treatment, Drug Research and Development, National Cancer Institute. BCNU, labeled with ^{14}C equally in the two chloroethyl groups (specific activity, 10 Ci/mol), was also supplied by the National Cancer Institute. Venom phosphodiesterase and bacterial alkaline phosphatase came from Worthington; bis(trimethylsilyl)trifluoroacetamide was from Regis Chemical Co.; and other reagents were from standard sources.

Polynucleotides were reacted with ^{14}C-labeled BCNU at 37° in pH 7 cacodylate buffer. At appropriate time intervals, 50-μl aliquots were withdrawn from the 0.5-ml reaction mixture, applied to 2.3-cm discs of Whatman 3 MM paper (Bollum, 1966), washed free of unbound radioactivity, and counted as described previously (Kramer et al., 1974). An alqiuot of the original reaction mixture for each polymer was also applied to a filter paper disc, dried without washing, and counted. Since the amount of polymer in each washed sample and the amount of BCNU in each unwashed sample were both known, the percent substitution could be calculated from the assumption that the same quench correction applied to all samples.

In order to identify the products of reaction of BCNU with polynucleotides, the reacted polymers were digested to the nucleotide level, and derivatives were separated from unreacted nucleotides by column chromatography. Polynucleotides reacted with [^{14}C]BCNU were washed free of radioactive residues of BCNU and its breakdown products by repeated ethanol precipitations from buffered aqueous solution before digestion.

Poly(cytidylic acid) which had been reacted with BCNU was digested by both chemical and enzymatic methods. Acid hydrolysis was performed by heating in HCl at 100° for 30 min. Enzymatic hydrolysis was performed in 0.1 M Tris-HCl buffer (pH 7.5) at 37°. Solutions containing 4 mg/ml of polymer were incubated for 14 hr in buffer containing 9 mM Mg^{2+} and 0.1 mg/ml of venom phosphodiesterase. Bacterial growth was inhibited with $CHCl_3$.

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[1] Abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; poly(C), poly(cytidylic acid); poly(G), poly(guanylic acid); poly(U), poly(uridylic acid).

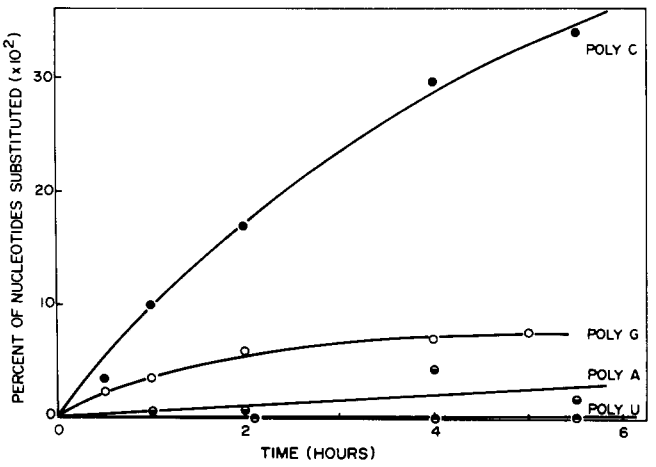

FIGURE 1: Reaction of polynucleotides with BCNU at 37°. [^{14}C]BCNU, 3.5 Ci/mol, was dissolved in 25 μl of ethanol and added to 0.475 ml of polynucleotide solution buffered at pH 7. Final concentrations: polynucleotide, 0.5 mg/ml; BCNU, 0.061 mg/ml; 0.05 *M* sodium cacodylate buffer (pH 7); 0.1 *M* NaCl. Aliquots were withdrawn at the indicated times and analyzed as described in the text.

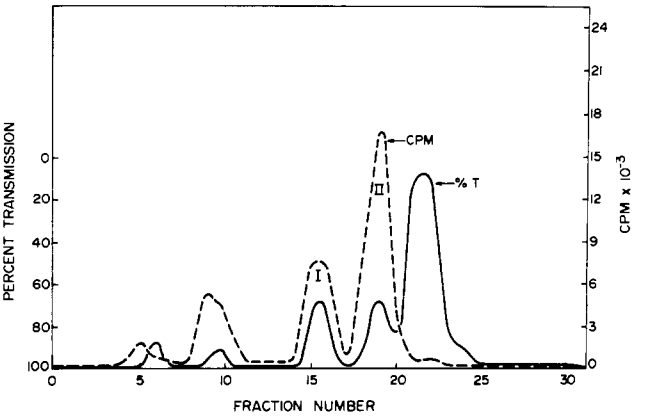

FIGURE 2: DEAE-Sephadex columns on an HCl hydrolysate of poly(C) which had been reacted with [^{14}C]BCNU. Approximately 1 mg of hydrolyzed polymer was applied to a (1.1 × 10 cm) column and eluted with a linear (0.05–0.5 *M*; total volume, 150 ml) gradient of triethylammonium bicarbonate (pH 7.5). Percent transmission was monitored at 260 nm. Ten-minute fractions (2.5 ml) were collected and 25-μl samples were counted in 10 ml of Bray's solution.

Chemical hydrolysis of substituted poly(G) to its constituent nucleotides requires a high pH where 7-substituted guanylic acids are unstable. Therefore we attempted to digest this polymer with a combination of T_1 and venom phosphodiesterase. The resistance of this polymer to complete digestion led us to isolate substituted guanine nucleotides from poly(U,G) which had been reacted with BCNU. Poly(U,G) was digested under the conditions used for poly(C) except that the venom phosphodiesterase concentration was 25 μg/ml and T_1 was added at a concentration of 5 μg/ml. This process was monitored on a small Sephadex G-100 column to demonstrate that complete digestion had been achieved.

Poly(C) hydrolysates were separated into component nucleotide peaks by column chromatography on A-25, DEAE-Sephadex. Samples from polymers which had been reacted with [^{14}C]BCNU and which contained approximately 2 mg of nucleotides were separated on a 1.1 × 10 cm column. Nucleotides were eluted with a linear, 0.05–0.5 *M*, gradient of pH 8.5 triethylammonium bicarbonate. Fractions containing 2.5 ml were collected at 10-min intervals, and 25-μl samples were counted in 10 ml of Bray's solution.

Two major radioactive peaks appeared ahead of CMP in the acid hydrolysate. Larger quantities of these were isolated on a 1 × 20 cm column of A-25 which was eluted stepwise with 0.05 and 0.1 *M* triethylammonium bicarbonate (pH 8.5).

Although hydrolysates of poly(U,G) which had been reacted with [^{14}C]BCNU could also be separated on DEAE-Sephadex, 7-substituted GMP derivatives are known to be unstable under alkaline conditions. Consequently, this derivative was separated from UMP and GMP on Dowex 50-X8 (–400 mesh). A sample containing approximately 2 mg of nucleotides was dissolved in 0.01 *M* acetic acid and eluted from a 0.05 × 5 cm Dowex-50 column with 0.01 *M* acetic acid as eluent. Unreacted GMP and UMP appeared in a large peak near the front followed immediately by the GMP derivative in a second sharp peak.

Fractions from the A-25 columns which contained CMP derivatives and fractions from the Dowex-50 columns which contained GMP derivatives were pooled and concentrated by lyophilization. CMP derivatives were contaminated with small amounts of UMP products which arose by deamination. These were separated by chromatography on Dowex-50 with H_2O as eluent.

Purified derivatives were characterized by paper chromatography on Whatman No. 1 paper in methanol–concentrated HCl–H_2O (8:1:1, v/v), isobutyric acid–concentrated NH_4OH–H_2O (66:1:33, v/v), and 2-propanol–concentrated NH_4OH–H_2O (7:1:2, v/v). Ascending chromatography was used with the first solvent, and descending chromatography, with the other two. Ultraviolet spectra were obtained on the purified and chromatographically homogeneous derivatives in dilute HCl, dilute NaOH, and 0.05 *M* Na^+ cacodylate buffer (pH 7). Spectra were recorded on a Beckman DB-G spectrophotometer.

Mass spectrometric analysis was performed on nucleosides obtained from the corresponding nucleotides by alkaline phosphatase treatment. Approximately 0.3 mg of each nucleoside was converted to the trimethylsilyl derivative by treatment with 20 μl of bis(trimethylsilyl)trifluoroacetamide for an hour in 20 μl of anhydrous pyridine. Cytidine and analogues were reacted at room temperature. Alkylated guanosine was treated at 100°C. Solvent and excess reagent were removed under vacuum and mass spectra were obtained on the residue with a CEC 21-110 instrument. Samples were introduced directly on the probe, and spectra were obtained with the source temperature at 225°, using 70-eV ionizing electrons.

Results

Reaction with Polynucleotides. Figure 1 shows the extent of reaction of the four homopolyribonucleotides with BCNU at 37° in aqueous pH 7 buffer. Under these conditions there was little or no reaction with poly(A) or poly(U), while there was significant substitution of poly(G) and, especially, poly(C). Thus, cytosine and guanine were substituted more easily than adenine and uracil. Further quantitative comparison is difficult, however, because the extent of reaction is presumably affected by the secondary structure of the polymer.

Reacted polynucleotides were hydrolyzed to the monomeric level for studies on the chemistry of substitution. Methods of hydrolysis that release nucleotides were chosen

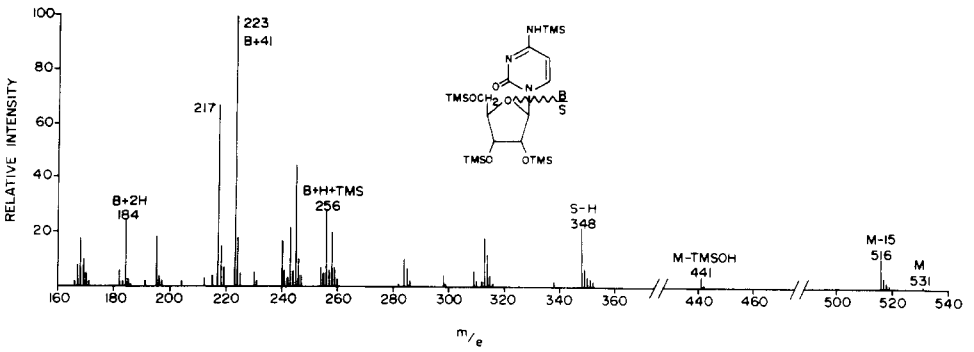

FIGURE 3: Mass spectrum of the trimethylsilyl derivative of cytidine.

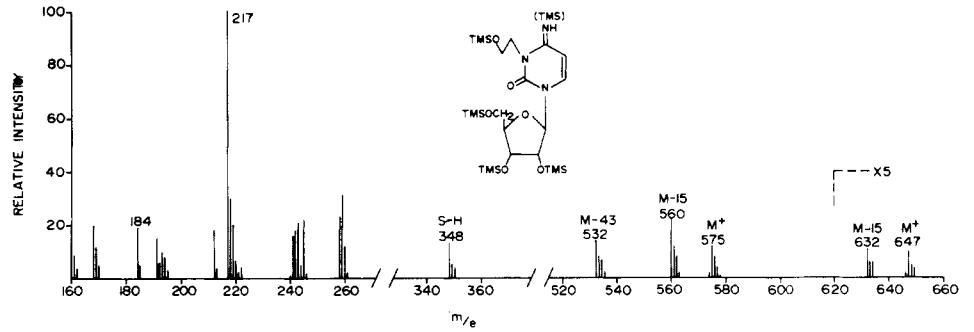

FIGURE 4: Mass spectrum of the trimethylsilyl derivative of 3-β-hydroxyethylcytidine.

over methods that release bases so that any possible attack on the sugar hydroxyls would be detected.

Cytidine Derivatives. Poly(cytidylic acid) which had been reacted with [^{14}C]BCNU was hydrolyzed with 1 *N* HCl at 100° for 30 min. Approximately 1 mg of hydrolyzed polymer was applied to a DEAE-Sephadex column and eluted as shown in Figure 2. The recovery of counts from this column varied between 90 and 110% of the applied radioactivity.

Two major peaks bearing a ^{14}C label were eluted in front of cytidylic acid and were designated derivatives I and II. Similar separations were performed on a larger scale, and fractions containing the two derivatives were pooled and characterized by paper chromatography and ultraviolet spectroscopy as described previously (Kramer et al., 1974).

Chromatography in the three systems described above showed that derivatives I and II were pure compounds with a small amount of a common contaminent. This contaminent had an ultraviolet spectrum which was very similar to the spectrum of 3-methyl-UMP and was assigned the structure 3 hydroxyethyl-UMP as described below.

Derivative I had a spectrum similar to that of 3-methyl-CMP and was assigned the structure 3-hydroxyethyl-CMP with the aid of mass spectral analysis. Derivative II had a spectrum similar to that of 1-methyl-3,N^4-ethanocytosine (Ueda and Fox, 1963) and was eventually assigned the structure, 3,N^4-ethano-CMP.

Mass spectroscopy was initiated by a background study of cytidine. This compound was converted to tetrakis(trimethysilyl)cytidine and a spectrum was obtained as shown in Figure 3. The molecular weight is given by the molecular ion peak at *m/e* 531, and confirmed by the M − 15 peak at *m/e* 516 and the M − 90 peak at *m/e* 441. These are characteristically generated by the elimination of CH_3 (15 mass units) and $HOSi(CH_3)_3$ (90 mass units) from trimethylsilyl derivatives. The peak at *m/e* 184 arises from silylated cytosine which is formed by cleavage of the N–C bond between the base and sugar moieties with transfer of two hydrogen atoms. Scission of this same bond leads to silylated sugar ions (S − 1) of mass 348 and silylated base ions (B + H + Me_3Si) to which a hydrogen atom and an additional trimethylsilyl group have been transferred (*m/e* 256). The most intense peak in the spectrum occurs at *m/e* 223 and corresponds to base plus HC_2O ions (B + 41). Formation of B + 41 ions has been studied by Liehr et al. (1974) who point out that alkylation at N-3 suppresses this fragmentation. Mass spectra fragmentation of trimethylsilyated cytidine and other nucleosides has been reviewed recently by McCloskey (1974).

The mass spectrum of cytidine derivative I is shown in Figure 4. The peaks in the high mass range suggest that the sample is a mixture of the tetrakis(trimethylsilyl) and pentakis(trimethylsilyl) compounds with molecular weights of 575 and 647, respectively. Both molecular ion peaks are accompanied by M − 15 peaks, at *m/e* 632 and 560. The addition of five trimethylsilyl groups to the derivative under conditions where four are added to cytidine suggests that cytidine derivative I contains a functional group which can be silylated.

A comparison of the spectra in Figures 3 and 4 shows that tetrakis(trimethylsilyl) derivative I weighs 44 mass units more than tetrakis(trimethylsilyl)cytidine. Since an alkyl substituent added to N-3, as suggested by the ultraviolet spectrum, displaces a hydrogen from the amino group, its weight is actually 45 mass units. The M − 43 ions seen in the derivative spectrum (*m/e* 532) are not present in the spectrum of silylated cytidine and are probably formed by elimination of most of the new alkyl substituent.

Consideration of this spectrum and the ultraviolet data leads to the structure, 3-(β-hydroxyethyl)cytidine for derivative I. The β-hydroxyethyl group has the requisite 45 mass units and its hydroxyl group would be converted to a tri-

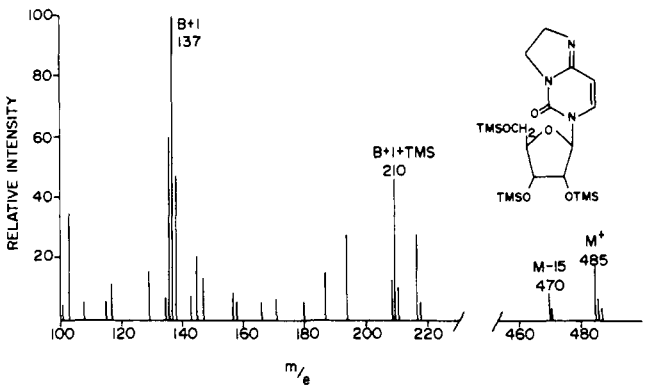

FIGURE 5: Mass spectrum of the trimethylsilyl derivative of 3,N^4-ethanocytidine.

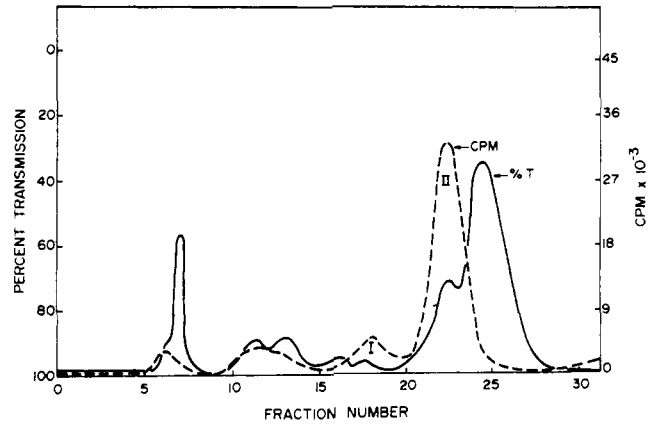

FIGURE 6: DEAE-Sephadex column on enzymatic hydrolysate of poly(C) which had been reacted with [^{14}C]BCNU. The column was eluted under conditions similar to those in Figure 2.

methylsilyl ether as required by the spectrum. The fragment ions which contain the silylated base moiety (B + 2 H, *m/e* 184) do not carry the alkyl substituent. The β-hydroxyethyl group on N-3 has been cleaved as well as the sugar moiety on N-1. This commonly occurs with *N*-alkyl groups longer than methyl (Ohashi et al., 1974).

Although the substituent was placed on N-3 initially on the basis of ultraviolet spectroscopic data, mass spectral fragmentation also supports this assignment. Falch has reported (Falch, 1970) that ethyl and propyl substituents on N-1 of the uracil ring are lost with transfer of one hydrogen atom to the heterocycle whereas the same substituents on N-3 are eliminated with transfer of two hydrogen atoms back to the ring. An analogous elimination is proposed from N-3 of the molecular ion of trimethysilylated cytidine derivative I, with transfer of one hydrogen and the trimethylsilyl group to the ring. This would result in the net loss of 43 mass units observed (*m/e* 532). Similar trimethylsilyl group migrations are well documented (for example, Diekman et al., 1967).

Additional confirmation of alkylation on N-3 is found in the absence of B + 41 ions in the spectrum. As discussed above this fragmentation occurs in cytidine and many of its analogues, but is suppressed by alkylation at N-3 (Liehr et al., 1974).

The spectrum of cytidine derivative II is shown in Figure 5. The M^+ and M − 15 peaks give a molecular weight of 485 indicating that the molecule carries one less trimethylsilyl group than tetrakis(trimethylsilyl)cytidine. The molecular weight is, in fact, 26 mass units above that of tris-(trimethylsilyl)cytidine. The occurrence of a B + 1 peak at *m/e* 137 and a B + 1 + Me_3Si peak at *m/e* 210 confirms the fact that 26 extra mass units have been attached to the cytosine ring. Again the absence of B + 41 ions in the spectrum suggests N-3 alkylation. The absence of fragmentation in the side chain suggests a stabilized structure. The cyclized structure proposed in Figure 5 accounts for both the mass spectral and uv characteristics of cytidine derivative II and is related to derivative I. Both of these derivatives can be deaminated to 3-hydroxyethyl-UMP which was noted throughout as a contaminant of these derivatives.

The unexpected structure of derivative II raised the question of whether this compound arose as an artifact of high temperature acid hydrolysis. Accordingly, a separate sample of poly(C) which had been reacted with [^{14}C]BCNU was digested enzymatically and separated on DEAE-Sephadex as shown in Figure 6. The same two derivatives were present in this hydrolysate, but the cyclized structure actually predominated. We can conclude, therefore, that this derivative is present as such in the substituted polynucleotide.

Guanosine Derivatives. As described below, 7-hydroxyethyl-GMP was identified in hydrolysates of BCNU-treated poly(U,G). Since this compound is unstable under the mildly alkaline conditions required for enzymatic hydrolysis and DEAE-Sephadex chromatography, its identity was first established in studies at the monomeric level.

BCNU, 16 mg/ml, was suspended on 0.05 *M* cacodylate buffer containing 15 mg/ml of GMP at pH 7. The mixture was incubated overnight at 37°, diluted with 20 ml of water, and applied to a 1.3 × 4.5 cm column of Dowex 50-X8 (~400). This was eluted with 0.01 *N* acetic acid which separated GMP as an early peak from a second derivative peak.

Fractions containing the derivative were pooled and evaporated to dryness. The product was then characterized as a pure compound by paper chromatography in the three solvent systems listed above. Ultraviolet spectroscopy indicated that the product was probably a 7-substituted guanylic acid (pH 2, λ_{max} 259, λ_{min} 232 nm; pH 7, λ_{max} 258, λ_{min} 237 nm; pH 12, λ_{max} 266, λ_{min} 246 nm). Spectral changes characteristic of cleavage of the imidazole ring in such compounds (Lawley and Brookes, 1963) were noted on standing in alkaline solution.

This derivative was then converted to the nucleoside with alkaline phosphatase for study by mass spectroscopy. Such a product is a quaternary ammonium salt and thus unsuitable for analysis by electron impact mass spectrometry. A procedure has been developed by McCloskey and his coworkers (Von Minden et al., 1972) for converting 7-alkylated guanosines to trimethylsilyl derivatives of 7-alkyl-8-oxoguanosines. This oxidation, which occurs only when both N-7 and N-9 are substituted, converts the ammonium cation to an un-ionized amine which can be analyzed by mass spectrometry.

In the spectrum (Figure 7) of the unknown guanosine derivative, the peak at *m/e* 775 is assigned as a molecular ion peak; it is accompanied by a M − 15 peak as is always the case with trimethylsilyl derivatives. Other nucleoside cleavage ions are observed at masses consistent with that assignment. Ions formed by loss of the ribose moiety are observed at *m/e* 427 and 428, which confirm that structural alteration has taken place on guanine. Fragmentation of the ribose moiety contributes some of the most abundant ions to

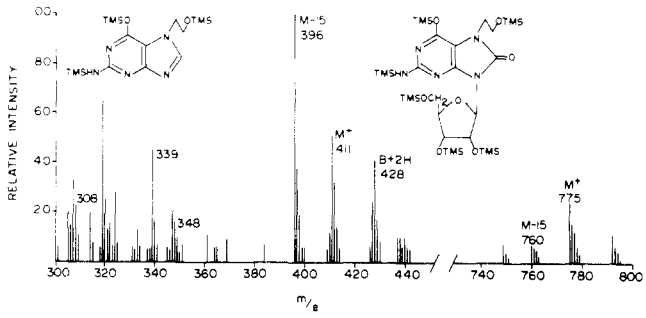

FIGURE 7: Mass spectrum of the trimethylsilyl derivative of 7-β-hydroxyethyl-8-oxoguanosine.

the spectrum, including those of masses 103, 147, and 217.

The spectrum reveals the presence of several other components in this sample. Peaks at *m/e* 314 and 299 correspond to the M^+ and $(M - 15)^+$ ions from tris(trimethylsilyl)phosphoric acid. Most of this more volatile substance vaporized before the nucleoside derivative did. The small peak at *m/e* 792 reflects contamination by a high mass compound, but does not fit into the fragmentation pattern of a silylated nucleoside.

Peaks at *m/e* 411 and 396 correspond to molecular ions and $(M - 15)^+$ ions formed from the tris(trimethylsilyl) derivative of the altered guanine base. A small amount of the bis(trimethylsilyl) analogue is detected 72 mass units lower, at *m/e* 339 and 324. The molecular weight of the novel tris(trimethylsilyl) base is 44 mass units higher than that of tris(trimethylsilyl)guanine itself (McCloskey, 1974). Thus alkylation by the active agent derived from BCNU has added 44 mass units. The alkyl group occupies a site where one of the three trimethylsilyl groups would be added to guanine, yet three silyl groups are added to the derivative. Thus the drug-derived alkyl substituent must contain a silylatable functional group.

These arguments are analogous to those developed for the structure of cytidine derivative I, and it seemed likely that the same β-hydroxyethyl group had been added to guanine. The loss of 43 mass units, important in the spectrum in Figure 4, is not observed here. Rather, cleavage occurs in the α bond of the N-alkyl group analogous to that reported for N-alkylated lactams (Budzikiewicz et al., 1967). Thus the peak at *m/e* 308 corresponds to $(M - CH_2OMe_3Si)^+$ ions.

The molecular weight of the silylated nucleoside (*m/e* 775 in Figure 7) is 132 mass units higher than that of penta(trimethylsilyl)guanosine. The simplest structural assignment includes the β-(trimethylsilyloxy)ethyl substituent already identified on guanine, and an 8-oxo group introduced by silylation under McCloskey's oxidizing conditions. When this derivative is silylated, it has the required molecular weight. Introduction of the 8-oxo group during high temperature silylation confirms that positions 7 and 9 are substituted. Position 9, of course, carries the ribose group. Position 7, therefore, must carry the β-hydroxyethyl group introduced by reaction with the active species derived from BCNU. Thus we are led to the structure shown in Figure 7 for the derivative isolated from the reaction of BCNU with GMP.

Poly(U,G) which had been reacted with [^{14}C]BCNU was digested enzymatically and the products were separated on Dowex-50. A labeled nucleotide was identified in the derivative peak as 7-hydroxyethyl-GMP by cochromatography in methanol–HCl–H_2O and isobutyric acid–NH_3–H_2O with 7-hydroxyethyl-GMP obtained from the reaction of BCNU with GMP. Several other labeled peaks were also noted which were generated by alkaline treatment of 7-hydroxyethyl-GMP. We concluded, therefore, that this substituted nucleotide was present in BCNU-treated poly(U,G).

Discussion

In agreement with earlier reports on the chemistry of a related agent, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (Cheng et al., 1972), BCNU clearly reacts with polynucleotides. Although the extent of reaction is low compared to that observed for classical, water-soluble alkylating agents (Ludlum, 1966), the nature of the nucleic acid modification is, of course, frequently more important than its extent. Thus, we have found that 3-methylcytosine conveys misinformation when it is incorporated into a polyribonucleotide template and copied by RNA polymerase (Ludlum and Wilhelm, 1968). This finding has been confirmed and extended (Ludlum, 1970a, 1971; Singer and Fraenkel-Conrat, 1970) while O^6-methylguanine has also been shown to convey misinformation (Gerchman and Ludlum, 1973). On the other hand, 7-methylguanine, which is a much more common product when nucleic acids are treated with methylating agents, pairs normally with cytosine (Ludlum, 1970b).

We were, therefore, interested in determining the structural modifications induced by BCNU. The application of mass spectroscopy to this problem is of particular importance since, in combination with ultraviolet spectroscopy, it allowed us to determine structures on a very limited amount of material.

The nucleotide derivatives which were isolated, namely, 3-(β-hydroxyethyl)CMP, 3,N^4-ethano-CMP, and 7-(β-hydroxyethyl)GMP, all involve the transfer of a two carbon moiety from BCNU. Separate studies carried out on the decomposition of this agent (Colvin et al., 1974) lead us to believe that this is a result of attack by a chloroethyl carbonium ion.

If the chloroethyl group were attached, even transiently, to CMP or GMP within a nucleic acid strand, this could confer alkylating activity to the entire molecule. Intramolecular reaction in the case of CMP would, of course, generate the cyclic compound we observed. Interstrand cross-linking might be an additional possibility. We have been unable to detect chloroethyl derivatives of nucleotides, however, and their lifetime may be very short.

The cyclic derivative of CMP which is generated has evidently not been described, although the related base has been synthesized by Ueda and Fox (1963). Current studies to determine the significance of these nucleotide modifications are in progress.

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Physicochemical Properties of DNA Binding Proteins: Gene 32 Protein of T4 and *Escherichia coli* Unwinding Protein†

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ABSTRACT: The single-stranded DNA binding protein coded for by gene 32 of bacteriophage T4 and a similar protein isolated from uninfected *Escherichia coli* both induce characteristic changes in the circular dichroism (CD) of single-stranded nucleic acids. These CD changes have been adapted as an assay of protein–DNA complex formation. Far-ultraviolet CD spectra show the secondary structure of the two proteins to be similar with ~20% α helix, ~20% β structure, and 60% random coil. Both proteins show prominent Cotton effects arising from their aromatic chromophores. Nitration of five of the nine tyrosyl residues of gene 32 protein prevents DNA binding, while prior formation of the DNA complex protects all tyrosyl residues from nitration. The tyrosyl residues may participate in gene 32 protein–DNA binding by intercalation between bases of the single strand. In contrast, no tyrosyl residues can be nitrated in the *E. coli* protein suggesting that surface tyrosyls do not play a part in binding of the *E. coli* protein to DNA. Approximately 50 amino acids can be cleaved from the gene 32 protein with trypsin. This cleavage also occurs spontaneously in infected cell extracts. The remaining protein of mol wt 30000 has the same CD spectra and DNA binding properties as the native protein. The physicochemical properties can be correlated with previous work on the structures and functions of the group of DNA "unwinding proteins".

Since the introduction of DNA-cellulose affinity chromatography by Alberts et al. (1968), a number of proteins exhibiting specific affinity for single- or double-stranded nucleic acids have been isolated from viral, bacterial, and eukaryotic sources. One class of DNA-binding proteins has become known as "unwinding" proteins because of their tight, cooperative, and preferential binding to single-stranded nucleic acids without regard to base composition. This results in a decrease in the melting temperature of double-stranded nucleotide polymers mixed with the purified proteins. A number of proteins in this class, including the gene 5 protein coded for by bacteriophage fd (Alberts et al., 1972), the gene 32 protein coded for by bacteriophage T4 (Alberts and Frey, 1970), and the "unwinding" protein found in uninfected *Escherichia coli* (Molineaux et al., 1974; Weiner et al., 1975) can be isolated in a homogeneous form. The presence of many copies (800–100000) of these proteins per cell as well as the in vitro formation of complexes between these proteins and single-stranded DNA suggests a stoichiometric as opposed to catalytic role for these proteins in the cellular processing of nucleic acids (Mazur and Model, 1973; Sinha and Snustad, 1971; Geider and Kornberg, 1974).

The gene 32 protein has been shown to be necessary for both the replication and recombination of the T4 genome in vivo (Tomizawa et al., 1966). In vitro it facilitates renaturation of native DNA (Alberts and Frey, 1970) and specifically enhances the catalytic rate of purified T4 DNA polymerase (Huberman et al., 1971). The *E. coli* unwinding protein has been found to be a necessary component of in

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