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MECHANISM OF ACTION OF THE NITROSOUREAS—IV

REACTIONS OF BIS-CHLOROETHYL NITROSOUREA AND CHLOROETHYL CYCLOHEXYL NITROSOUREA WITH DEOXYRIBONUCLEIC ACID

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Abstract—Calf thymus DNA was reacted with ^{14}C-labeled bis-chloroethyl nitrosourea (BCNU), and chloroethyl cyclohexyl nitrosourea (CCNU), and the nature of the derivatives investigated in an enzymatic digest. In agreement with earlier studies on polyribonucleotides, evidence was obtained for the formation of 7-hydroxyethyldeoxyguanosine, 3-hydroxyethyldeoxycytidine and 3,N^4-ethanodeoxycytidine. In addition, significant amounts of 7-aminoethylguanine were identified in the hydrolysate of DNA treated with BCNU, but not in the hydrolysate of DNA treated with CCNU. Aminoethylguanine was also formed when DNA was reacted with chloroethylamine, suggesting that BCNU produced aminoethylguanine via chloroethylamine as an intermediate. Because both BCNU and CCNU are effective antitumor agents, the formation of aminoethylguanine is probably not an important cytotoxic reaction, but it may have significance as far as mutagenic or carcinogenic activities are concerned.

The chloroethyl nitrosoureas, a group of compounds with a distinctive chemical structure (1-haloethyl-3-alkyl-1-nitrosourea), are useful in the treatment of a variety of neoplasms. Although the mechanism by which they produce their cytotoxic action is not fully understood it was shown early in their development that they have both alkylating [1] and carbamoylating activities [2]. Cheng *et al.* [3] found that the chloroethyl group in the 1 position react with nucleic acids, whereas the constituent at the 3 position is involved in carbamoylation.

Early investigations in our laboratory led us to propose that the chloroethyl group at the 1 position is transferred intact to nucleic acids where it can then react secondarily to produce intra- or interstrand crosslinks [4, 5]. Evidence for the formation of interstrand crosslinks has come from physical studies in Kohn's and Lown's laboratories on DNA exposed to BCNU [6, 7].

Earlier papers in this series have been concerned with the structures of ribonucleoside derivatives obtained from the reaction of nucleosides and polyribonucleotides with haloethyl nitrosoureas [8–10]. These have included: 3-hydroxyethylcytidine, 3-fluoroethylcytidine, 3,N^4-ethanocytidine, 7-hydroxyethylguanosine, 7-fluoroethylguanosine, 1-hydroxyethyladenosine, 1,N^6-ethanoadenosine and 1-fluoroethyladenosine.

In this paper, we examine the products of reaction of BCNU with DNA. In addition to several derivatives that would have been expected from our earlier studies, we found significant amounts of 7-aminoethylguanine, which is evidently formed from the chloroethyl group in the 3 position of BCNU. This derivative was also isolated from DNA reacted with chloroethylamine or chloroethylisocyanate, but not with CCNU. The possible contribution of aminoethylguanine to the cytotoxic or mutagenic effects of BCNU is discussed below.

MATERIALS AND METHODS

Crystalline BCNU, CCNU, 1,3-bis([^{14}C]-2-chloroethyl)-1-nitrosourea and 1-([^{14}C]-2-chloroethyl)-3-cyclohexyl-1-nitrosourea were obtained from Dr. Robert Engle, Drug Research and Development, National Cancer Institute, Division of Cancer Treatment. Deoxycytidine was purchased from P-L Biochemicals (Milwaukee, WI) and deoxyguanosine from ICN Pharmaceuticals (Irvine, CA). Calf thymus DNA, venom phosphodiesterase (EC 3.1.4.1), spleen phosphodiesterase (EC 3.1.4.18), deoxyribonuclease I (EC 3.1.4.5) and bacterial alkaline phosphatase (EC 3.1.3.1) came from the Worthington Biochemical Corp. (Freehold, NJ). Ethylene oxide was purchased from Eastman Organic Chemicals (Rochester, NY), 2-chloroethylisocyanate from the Tridom Chemical Co. (Hauppauge, NY), 2-chloroethylamine–HCl from the Pfaltz & Bauer Chemical Co. (Stamford. CT) and 2-bromoethylamine-HBr from the Aldrich Chemical Co. (Milwaukee, WI).

Markers for the expected deoxynucleoside derivatives were synthesized by published methods or by established reactions for the corresponding ribonucleoside derivatives. Thus, 7-β-hydroxyethyldeoxyguanosine was prepared by a slight modification of the procedure of Roe *et al.* [11]:

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10 mg deoxyguanosine was reacted with approximately 100 μl ethylene oxide in 1 ml dimethylsulfoxide (DMSO) overnight at 37°. The reaction mixture was separated on an SP-Sephadex C-25 column; fractions corresponding to the product peak were pooled and concentrated by lyophilization. The major derivative, 7-β-hydroxyethyldeoxyguanosine, was purified by reverse phase high pressure liquid chromatography (h.p.l.c.), and its identity confirmed by ultraviolet spectroscopy. 7-β-Hydroxyethylguanine was prepared by heating the corresponding deoxynucleoside for 15 min in 0.1 N HCl at 100°.

3-β-Hydroxyethyldeoxycytidine was prepared by a similar reaction between deoxycytidine and ethylene oxide. The product was separated and purified in the same way, and was shown to have the same ultraviolet spectrum as 3-β-hydroxyethylcytidine [4].

3,N^4-Ethanodeoxycytidine was prepared by a method analogous to that used by Tong and Ludlum [10] to prepare 1,N^6-ethanoadenosine. Deoxycytidine (100 mg) was reacted with 100 μl of 1,2-dibromoethane in 1 ml DMSO for 24 hr at 37°. Again, the major product was separated from unreacted deoxycytidine on an SP-Sephadex C-25 column, purified further by h.p.l.c., and shown to have the same ultraviolet spectrum as 3,N^4-ethanocytidine [4].

Reactions with DNA were carried out in aqueous solutions. BCNU, labeled with ^{14}C in both chloroethyl groups (9.63×10^{-3} mmole, 0.346 mCi/mmole), was incubated with 2 mg of calf thymus DNA in 1 ml of 25 mM sodium cacodylate buffer, pH 7.0, for 24 hr at 37°. Then, the DNA was precipitated by the addition of 50 μl of 6 M NaCl and 2 vol. of ice-cold ethanol, collected by centrifugation, and redissolved in 1 ml of water. This precipitation procedure was repeated three times, which reduced the radioactivity in the supernatant fraction to background levels.

Finally, DNA was adjusted to a concentration of approximately 1 mg/ml and digested in 50 mM sodium cacodylate buffer, pH 7.0, that contained 1 mM NaCl and 15 mM $MgCl_2$, with 0.4 unit of venom phosphodiesterase, 0.2 unit of spleen phosphodiesterase, 2 units of deoxyribonuclease I and 1 unit of bacterial alkaline phosphatase. After incubation at 37° for 48 hr, the digest was separated on SP-Sephadex C-25.

DNA was reacted with 1-([^{14}C]-2-chloroethyl)-3-cyclohexyl-1-nitrosourea (8.6×10^{-3} mmole, 0.917 mCi/mmole) in the same manner and was then washed, digested and separated on SP-Sephadex C-25 as described above.

Isolation and identification of derivatives depended on reverse phase high pressure liquid chromatography. This was performed on a modular apparatus consisting of a Milton Roy 5000-PSI minipump, a Laboratory Data Control model 709 pulse damper, a Waters Associates μ-Bondapak C_{18} column, a Perkin Elmer LC-55 u.v. detector and a Perkin Elmer Sigma 10 Data System.

Ultraviolet spectra were obtained on all derivatives in 0.1 N HCl, in 0.1 M sodium cacodylate buffer, pH 7.0, and in 0.1 N NaOH, on a Beckman model 35 spectrophotometer.

Mass spectroscopy was performed for us by Mr. Marion Kirk of the Southern Research Institute, Birmingham, AL. A good spectrum was obtained on underivatized 7-β-aminoethylguanine in a Varian MAT 311A instrument using the electron impact technique. The material was introduced directly on the probe and the spectrum was recorded at a source temperature of 110° with 70 eV.

RESULTS

BCNU and CCNU reacted readily with calf thymus DNA under the conditions given above. The extents of alkylation, calculated from the optical density and radioactivity of the digested DNA and the specific activity of the nitrosourea, were 2.1 per cent of the total DNA bases for BCNU and 0.5 per cent for CCNU.

The entire digest was separated on SP-Sephadex C-25 as shown in Fig. 1A for BCNU and Fig. 1B for CCNU. Underivatized deoxynucleosides, which are uncharged at pH 6, appeared in the front. Most of the substituted deoxynucleosides, which are positively charged, were eluted later with the sodium formate gradient. A qualitative difference is apparent in the analysis of the BCNU digest (Fig. 1A) and the CCNU digest (Fig. 1B); there were two retained peaks (II and III) with BCNU and only one with CCNU.

Fractions containing radioactivity were pooled separately for each digest and concentrated by

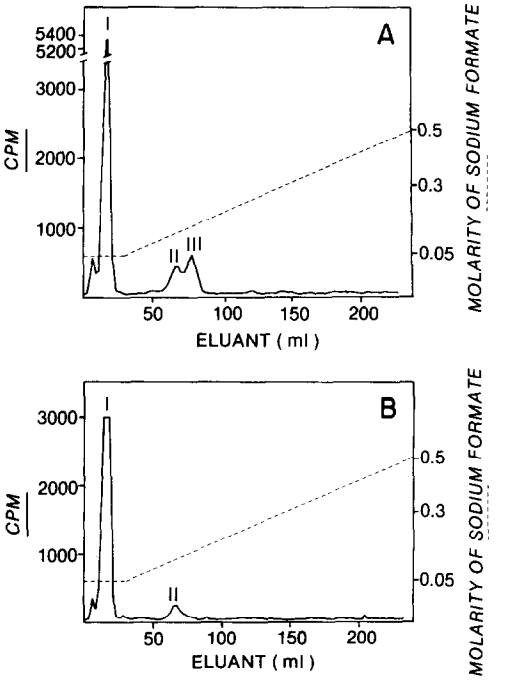

Fig. 1. Panel A: Separation of [^{14}C]BCNU-DNA digest on SP-Sephadex C-25. The digestion mixture was applied to an SP-Sephadex C-25 column (0.9 × 20 cm) and eluted at a flow rate of 1 ml/min with 0.05 M sodium formate, pH 6.0, until all of the uncharged compounds had appeared (peak I). Charged species were then eluted with a 200 ml linear gradient of sodium formate 0.05 M–0.5 M, pH 6.0. Two-minute fractions were collected and a 1-ml aliquot of each fraction was used for counting. Panel B: Separation of [^{14}C]CCNU-DNA digest on SP-Sephadex C-25. Eluted as in Fig. 1A.

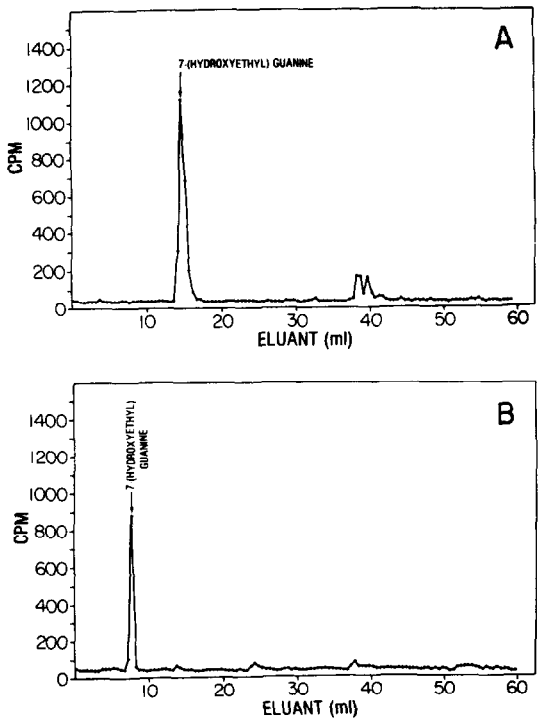

Fig. 2. Panel A: High pressure liquid chromatography analysis of peak I from Fig. 1A (BCNU). The sample was applied to a μ-Bondapak C_{18} column (4 mm × 30 cm) and eluted for 30 min with 1% acetonitrile in 0.02 M KH_2PO_4, pH 2.5, at a flow rate of 1 ml/min, and then with 10% acetonitrile in the same buffer for an additional 30 min. Panel B: High pressure liquid chromatographic analysis of peak I from Fig. 1B (CCNU). Separated as in Fig. 2A except that the column was eluted for 30 min with 1% acetonitrile in 0.05 M KH_2PO_4, pH 7.5, at a flow rate of 1 ml/min, and then with 10% acetonitrile in the same buffer for an additional 30 min.

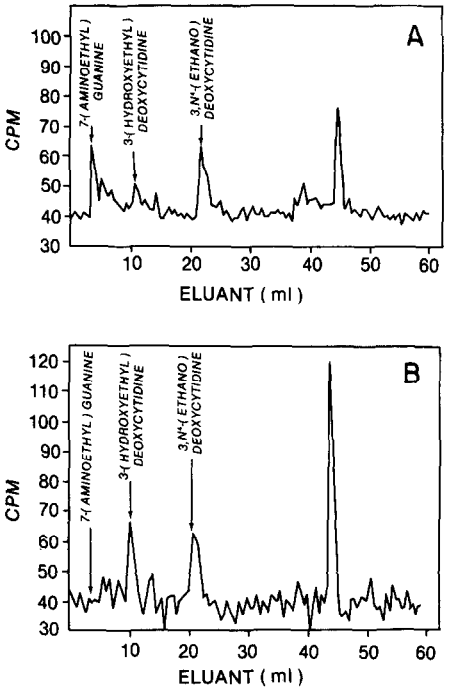

Fig. 3. Panel A: High pressure liquid chromatographic analysis of peak II from Fig. 1A (BCNU). Eluted as in Fig. 2B. Panel B: High pressure liquid chromatographic analysis of peak II from Fig. 1B (CCNU). Eluted as in Fig. 2B.

lyophilization. They were then analyzed further by high pressure liquid chromatography in comparison with optical density markers of the deoxynucleoside derivatives whose synthesis is described above.

Radiolabeled material from the uncharged region (peak I of Fig. 1A and 1B) appeared as a single peak on high pressure liquid chromatography for both BCNU-treated DNA (Fig. 2A) and CCNU-treated DNA (Fig. 2B). Although elution conditions were changed slightly between panel A and panel B of Fig. 2, all of the radioactivity corresponded to a marker of 7-hydroxyethylguanine in each case. Thus, we conclude that 7-hydroxyethyldeoxyguanosine was formed and converted to 7-hydroxyethylguanine during the digestion procedure.

Two of the expected derivatives of deoxycytidine appeared in the h.p.l.c. analysis of peaks II from Fig. 1. Figure 3A shows the analysis of peak II from the BCNU reaction, and Fig. 3B shows the analysis of peak II from the CCNU reaction. Appearance times for 7-aminoethylguanine, 3-hydroxyethyldeoxycytidine, and 3,N^4-ethanodeoxycytidine are indicated on these figures. 7-Aminoethylguanine, which is a product of the BCNU reaction as shown below, is clearly absent from the CCNU analysis in Fig. 3B. 3,N^4-Ethanodeoxycytidine is present in both Fig. 3A and 3B, while 3-hydroxyethyldeoxycytidine is definitely present in Fig. 3B and may be present in Fig. 3A. There is a late peak at 45 ml that still remains to be identified.

A major difference between BCNU and CCNU became apparent when peak III of Fig. 1A was analyzed by high pressure liquid chromatography. This analysis is shown in Fig. 4; most of the radioactivity was in a single peak whose appearance volume was approximately 9 ml.

Larger amounts of this derivative were prepared by repeating the reaction of BCNU with DNA on a 1 g scale. The DNA was digested and separated as before on an SP-Sephadex C-25 column whose elution was monitored by ultraviolet absorption at 254 nm. The O.D. peak corresponding to peak III

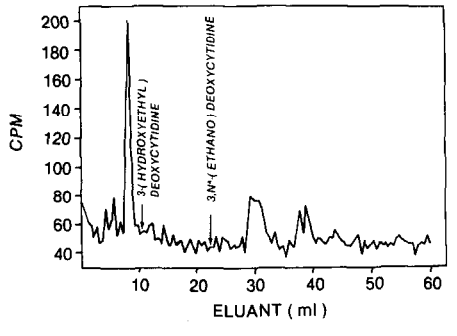

Fig. 4. High pressure liquid chromatographic analysis of peak III from Fig. 1A. The sample was eluted as described in Fig. 2B.

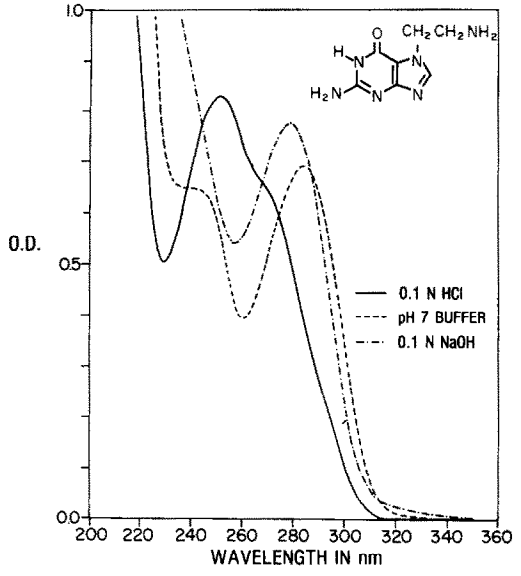

Fig. 5. Ultraviolet spectra of 7-(β-aminoethyl)guanine.

in Fig. 1A was collected and purified by high pressure liquid chromatography under conditions similar to those shown in Fig. 4. The derivative peak was collected, lyophilized to dryness, and desalted by application to an SP-Sephadex C-25 column (0.9 × 20 cm) that had been equilibrated with double-distilled water. The column was eluted with water for 2 hr at a flow rate of 0.9 ml/min; the retained peak of 7-β-aminoethylguanine was then eluted with 10 mM NH_4OH. This purified and desalted material was lyophilized to dryness and used for structural analysis.

Its ultraviolet spectra in acid, base and neutral solution are shown in Fig. 5 and are essentially identical to those of 7-methylguanine [12]. This suggested to us that we had isolated a new 7-alkylguanine that differed from the expected 7-hydroxyethylguanine in being retained on a cation exchange column.

Evidence that this derivative was 7-β-aminoethylguanine came from its mass spectrum as shown in Fig. 6. A molecular ion peak appears at the correct *m/e* ratio of 194. In addition, the fragmentation pattern shows a peak at 164 that corresponds to the loss of $—CH_2NH_2$ and a cluster around 150 that corresponds to the loss of $—CH_2CH_2NH_2$.

Conclusive evidence that our unknown was indeed 7-β-aminoethylguanine was obtained by synthesizing this derivative directly from guanosine and 2-bromoethyl ammonium bromide. These two ingredients were incubated together in DMSO for 2 days at 37°. Separation of this reaction mixture on SP-Sephadex C-25 yielded a major product with the spectrum of a 7-substituted guanosine. Acid hydrolysis gave a compound with the same spectral and chromatographic properties as 7-β-aminoethylguanine isolated from DNA treated with BCNU.

The absence of aminoethylguanine as a derivative when DNA is treated with CCNU indicates that the aminoethyl moiety arises from the 3-chloroethyl group of BCNU. Wheeler and Chumley [1] pointed out that chloroethylisocyanate and chloroethylamine are formed from the decomposition of BCNU, and that both compounds possess alkylating activity. To determine whether 7-aminoethylguanine could have arisen from these decomposition products, parallel reactions were performed in which DNA at a concentration of 2 mg/ml in 0.1 M sodium cacodylate buffer, pH 7, was reacted separately with 10 mM BCNU, 2-chloroethylisocyanate and 2-chloroethylamine·HCl. After reaction at 37° for 24 hr, the DNA from each reaction mixture was precipitated repeatedly with ethanol and then redissolved in cacodylate buffer at pH 7 and depurinated for 10 min at 100°. The reaction tubes were cooled in an ice bath and the DNA was precipitated with 1 N HCl. The supernatant fractions from the three separate reaction mixtures were then concentrated by lyophilization and analyzed as shown in Fig. 7. In addition to the normal purines, guanine (peak 2) and adenine (peak 4), a prominent 7-β-aminoethylguanine peak was present in each supernatant fraction. 7-Hydroxyethylguanine (peak 3) was evident only in the BCNU reaction.

DISCUSSION

Although the mechanism of action of the nitrosoureas is not completely established, their reactions

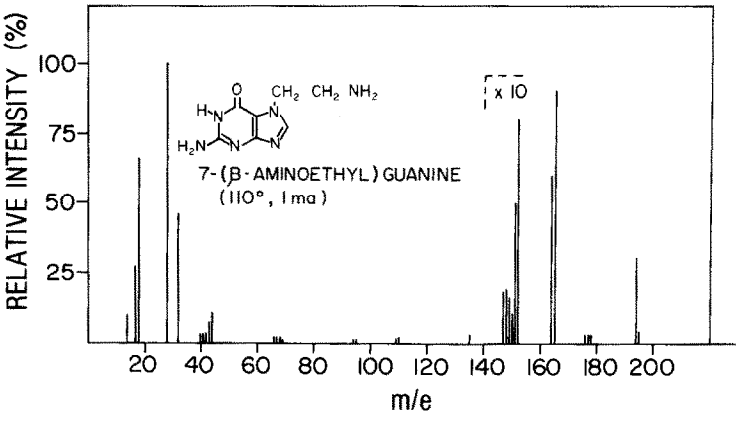

Fig. 6. Mass spectrum of 7-(β-aminoethyl)guanine.

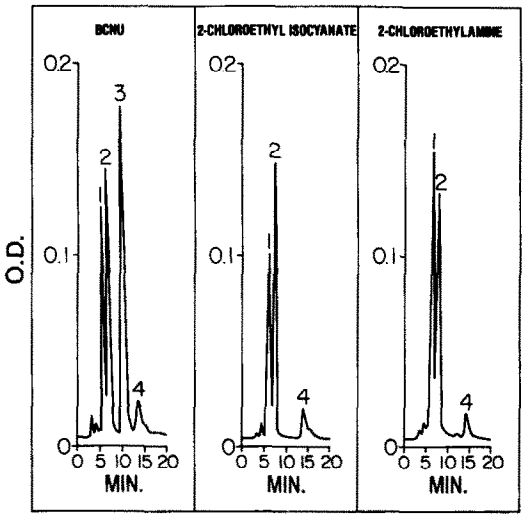

Fig. 7. High pressure liquid chromatographic analysis of purines from DNA which had been reacted with BCNU, 2-chloroethylisocyanate or 2-chloroethylamine. A 200-μl sample containing purines released from DNA (see text) was applied to a μ-Bondapak C_{18} column (4 mm × 30 cm) and eluted isocratically with 1% acetonitrile in 0.05 M KH_2PO_4, pH 6.0. Peak 1 is 7-(β-aminoethyl) guanine, peak 2 is guanine, peak 3 is 7-(β-hydroxyethyl) guanine, and peak 4 is adenine.

with nucleic acids are certainly important. Previous investigations in this series have shown that the haloethylnitrosoureas react with polynucleotides to form haloethyl and hydroxyethyl derivatives by mechanisms which involve the chloroethyl group in the 1 position. The investigations described above indicate that BCNU reacts uniquely to form aminoethylguanine, since it is the only one of the common nitrosoureas to contain a chloroethyl group in the 3 position.

Thus, there are evidently three classes of nucleoside derivatives formed by BCNU: haloethyl nucleosides, hydroxyethyl nucleosides and aminoethyl nucleosides. These probably arise by different mechanisms and may have a different significance at a molecular level.

The haloethyl nucleosides are formed by the direct transfer of a haloethyl group from the 1 position of the nitrosourea to a nucleophilic site on a nucleoside. Such derivatives are apt to be particularly significant as far as the cytotoxic action of the nitrosourea is concerned because they can lead directly to crosslinking.

The hydroxyethyl derivatives evidently arise independently by a different mechanism for we have shown that hydrolysis of the haloethyl nucleosides is too slow to account for the hydroxyethyl derivatives present [9, 10]. We have proposed that they arise instead by a direct attack of the nucleoside on an oxadiazoline intermediate [10].

The formation of 7-aminoethylguanine reported here suggests still a third mechanism by which nucleic acids can be modified. Clearly, the 3-chloroethyl group of BCNU is involved, but we cannot tell from our data whether the reactive species is BCNU itself, chloroethylisocyanate, chloroethylamine, or some other intermediate. In any case, DNA is uniquely substituted by this particular nitrosourea.

Wheeler *et al.* [13] have shown that BCNU, chloroethylamine, and compounds capable of generating chloroethylamine are more cytotoxic to tissue culture cells than are other nitrosoureas. Since other haloethyl nitrosoureas are effective antitumor agents, however, it is somewhat unlikely that the formation of 7-aminoethylguanine is a significant cytotoxic reaction *in vivo*. It is possible that this lesion may have some significance as a mutagenic or carcinogenic event, especially if its formation is followed by depurination or incorrect repair. Its importance can be evaluated more precisely later as comparative data on the mutagenic or carcinogenic activities of the various nitrosoureas accumulate.

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