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MECHANISM OF ACTION OF THE NITROSOUREAS—III

REACTION OF BIS-CHLOROETHYL NITROSOUREA AND BIS-FLUOROETHYL NITROSOUREA WITH ADENOSINE

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Abstract—Previous papers in this series have provided evidence for the formation of haloethyl nucleoside derivatives from the interaction of the therapeutic nitrosoureas with cytidine and guanosine. Such derivatives could be important in explaining the cytotoxic action of bis-chloroethyl nitrosourea (BCNU), bis-fluoroethyl nitrosourea (BFNU), and related therapeutic agents. We now report the formation of 1-haloethyl adenosines from the reaction of BCNU and BFNU with adenosine. These 1-substituted haloethyl adenosines cyclize to form 1,N^6-ethanoadenosine: 1-chloroethyladenosine with a half-life of 20 min in neutral aqueous solution at 37°, and 1-fluoroethyladenosine with a half-life of 20 hr under the same conditions. 1-Hydroxyethyladenosine is also a major product of the reaction of either BCNU or BFNU with adenosine, but it is not formed from the hydrolysis of either 1-haloethyladenosine. Accordingly, a reaction mechanism involving a cyclized nitrosourea derivative is proposed to explain the formation of this and other hydroxyethyl nucleosides.

The nitrosoureas, including bis-chloroethyl nitrosourea (BCNU) and bis-fluorethyl nitrosourea (BFNU), are relatively new antitumor agents with some interesting pharmacological properties. As a group, they are related to the classical alkylating agents in that most of them contain at least one haloethyl group and possess alkylating activity [1, 2]. However, they are different from the classical agents in several important ways. Most significantly, from the mechanistic point of view, they may be useful in treating tumors resistant to the classical agents [3].

Although the mechanism of action of the nitrosoureas has not been established, Cheng *et al.* [4] demonstrated that they react with nucleic acids. The nature of this reaction was clarified by our early studies, leading to the suggestion that nucleosides react with haloethyl carbonium ions released from the nitrosourea [5–7]. The transfer of a haloethyl group to a nucleoside in a DNA strand could explain the crosslinking reactions observed by Kohn [8].

By analogy with the classical alkylating agents, the cytotoxicity of the nitrosoureas could well arise from such crosslinking reactions. Furthermore, differences in the crosslinked structures might explain differences between the pharmacological properties of alkylating agents and nitrosoureas. Accordingly, we have embarked on a detailed study of the nucleoside modifications produced by these agents.

In the first paper of this series, we showed that a reactive haloethyl nucleoside intermediate was formed from the reaction of cytidine and BFNU [9]. BFNU was used in these studies because the relative stability of the fluoroethyl nucleosides made them easier to identify then the less stable chloroethyl nucleosides which arise from BCNU. In the second paper of the series, we published evidence for the formation of 7-β-fluoroethylguanosine which, in addition to being a product of the reaction between guanosine and BFNU, is also an antitumor agent in its own right [10]. In this paper, we explore the reactions of BCNU and BFNU with adenosine.

MATERIALS AND METHODS

Crystalline BCNU and BFNU were kindly provided by Dr. Harry B. Wood, Jr. (Division of Cancer Treatment, Drug Research and Development, National Cancer Institute, Bethesda, MD). An additional sample of crystalline BFNU was the gift of Dr. Glynn P. Wheeler, Southern Research Institute, Birmingham, AL. Reagent grade 1-bromo-2-fluoroethane and 1-bromo-2-chloroethane were purchased from the Columbia Organic Chemical Co. (Columbia, SC), and the J. T. Baker Co. (Phillipsburg, NJ). Adenosine, 6-chloropurine riboside, ethanolamine and 6-hydroxyethyl adenine were purchased from the Sigma Co. (St. Louis, MO). All other materials were from standard sources.

Several derivatives of adenosine were synthesized by classical methods as chromatographic markers. 1-β-Hydroxyethyl adenosine was prepared from adenosine and ethylene oxide, according to the method of Windmueller and Kaplan [11]. Material purified by high pressure liquid chromatography had the correct ultraviolet spectrum for this compound. Further confirmation of structure was provided by the fact that 1-β-hydroxyethyl adenosine underwent a Dimroth rearrangement, under alkaline conditions, to 6-β-hydroxyethyl adenosine.

Larger quantities of 6-β-hydroxyethyl adenosine were obtained from the reaction of 6-chloropurine riboside with ethanolamine. One hundred mg of 6-chloropurine riboside was dissolved in 3 ml acetonitrile, 200 μl ethanolamine was added, and the mixture was heated in a sealed tube with stirring for 1 hr at 55°. Acetonitrile was removed under vacuum and the residual material was extracted with water. This solution was

lyophilized to give 125 mg of solid residue. This material exhibited one major peak on high pressure liquid chromatography which had the same retention time and ultraviolet spectrum as the 6-hydroxyethyl adenosine obtained by rearrangement of 1-hydroxyethyl adenosine. Our spectrum of 6-hydroxyethyl adenosine was also the same as that given by Windmueller and Kaplan [11].

1-β-Fluoroethyl adenosine was prepared by incubating 100 mg adenosine in 1 ml dimethylsulfoxide (DMSO) with 100 μl of 1-bromo-2-fluoroethane at 37° for 4 days. At the end of this period, 5 ml of 0.05 M sodium formate buffer, pH 5, was added and the mixture was applied to a SP-Sephadex C-25 (1.5 × 60 cm) column. This column was eluted with sodium formate buffer at a flow rate of 0.7 ml/min and 10 min fractions were collected. Unchanged adenosine eluted as an early peak, and a later peak, containing mostly 1-fluoroethyl adenosine, was collected. This material was applied to an AG 50 × 8 (200–400 mesh, 0.5 × 5 cm) column in a cold room at 4°, washed free of salt, and eluted with 0.01 M ammonium hydroxide. After lyophilization, the product was purified further by high pressure liquid chromatography on a μ-Bondapak C_{18} column, as described below. This compound had an ultraviolet spectrum typical of a 1-substituted adenosine and cyclized slowly at room temperature to 1, N^6-ethanoadenosine.

1-Choloroethyl adenosine was synthesized in a similar manner from adenosine and 1-bromo-2-chloroethane. The late peak from SP-Sephadex C-25 chromatography was analyzed by high pressure liquid chromatography. The major product had an ultraviolet spectrum consistent with a 1-substituted adenosine and cyclized rapidly to 1, N^6-ethanoadenosine. 1-β-chloroethyl adenosine could not be isolated because it cyclized during lyophilization.

The compound, 1, N^6-ethanoadenosine, has not been described previously. It was synthesized by incubating 100 mg adenosine with 100 μl of 1-bromo-2-chloroethane in 1 ml DMSO at 38° for 4 days. At the end of this period, 5 ml of 0.05 M sodium formate buffer at pH 5 was added, and the reaction mixture was separated on SP-Sephadex C-25 as described above. Again, unreacted adenosine appeared as an early peak and product was collected as a later peak in fractions 50–60. The product was desalted on an AG 50 column, eluted with 0.01 M ammonium hydroxide, lyophilized, and purified further by high pressure liquid chromatography on a μ-Bondapak C_{18} column. One hundred and eighty O.D.U. of 1, N^6-ethanoadenosine were obtained which, upon hydrolysis with 0.01 N HCl at 100° for 1 hr, yielded a compound identified as 1, N^6-ethanoadenine. To confirm this structure, 1, N^6-ethanoadenine was synthesized by the same route used for the synthesis of N^6-chloroethyl 1, N^6-ethanoadenine by Johnston *et al.* [12].

1-β-Hydroxyethyl adenosine and 1-β-fluoroethyl adenosine were also hydrolyzed to the corresponding substituted adenines by treatment with dilute hydrochloric acid. Attempts to hydrolyze 1-β-chloroethyl adenosine again led to cyclization of that derivative.

Ultraviolet spectra were obtained on all derivatives in 0.1 N HCl, 0.1 M sodium cacodylate buffer, pH 7, and 0.1 N NaOH on a Beckman model 35 spectrophotometer.

High pressure liquid chromatography was performed on a modular apparatus consisting of a Milton Roy 5000-PSI mini pump, a Laboratory Data Control model 709 pulse damper, a Water Associates μ-Bondapak C_{18} column, a Perkin Elmer LC 55 u.v. detector, and a Perkin Elmer Sigma 10 Data System. A μ-Bondapak C_{18} analytical column (4 mm × 30 cm) was used to demonstrate the presence of nucleoside derivatives in nitrosourea–nucleoside reaction mixtures while a larger, preparative column (7 mm × 30 cm) was used for purifying marker material. All columns were run at room temperature. The somewhat elaborate isocratic and gradient elution system, described in the footnote to Table 1, insured adequate separation of early peaks and reasonably prompt elution of late peaks.

RESULTS

The development of high pressure liquid chromatographic separations on μ-Bondapak C_{18} columns has been the key to the successful study of the nucleoside derivatives formed by nitrosoureas. Retention times for adenosine derivatives are shown in Table 1. Good

Table 1. High pressure liquid chromatography of adenosine and its derivatives*

Compound	Time
1-β-Hydroxyethyladenosine	16
1,N^6-ethanoadenosine	19
1-β-Fluoroethyladenosine	33
1-β-Chloroethyladenosine	47
Adenosine	48

* Column: μ-Bondapak C_{18}, 4 mm × 30 cm. Solvent: 0.05 M KH_2PO_4, pH 4.5, for 10 min; gradient, 0.05 M KH_2PO_4 with 10% CH_3CN into 0.05 M KH_2PO_4, 20 ml each, for 50 min; 0.05 M KH_2PO_4 with 10% CH_3CN for 10 min; gradient, 0.05 M KH_2PO_4 with 40% CH_3CN into 0.05 M KH_2PO_4 with 10% CH_3CN, 10 ml each, for 20 min. Flow rate: 0.8 ml/min. Detector: u.v./260 nm. Temperature: ambient.

Table 2. Ultraviolet spectra of adenosine derivatives

Compound	Acid		Neutral		Base	
	Max	Min	Max	Min	Max	Min
1-β-Fluoroethyladenosine	260	235	260	234	260	236
1-β-Chloroethyladenosine	259	234	259	234	259	235
1-β-Hydroxyethyladenosine	258	234	259	234	259	235
1,N^6-ethanoadenosine	262	235	262	235	269	239
1-β-Fluoroethyladenine	260	222	264	238	270	242
1, N^6-ethanoadenine	262	234	265	242	274	245

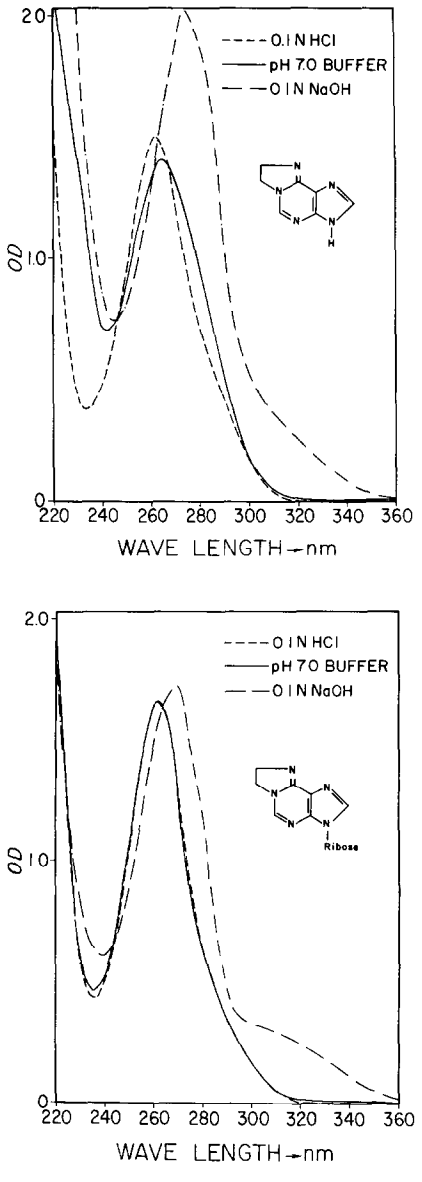

Fig. 1. Ultraviolet spectra of 1,N^6-ethanoadene (top panel) and 1,N^6-ethanoadenosine (lower panel).

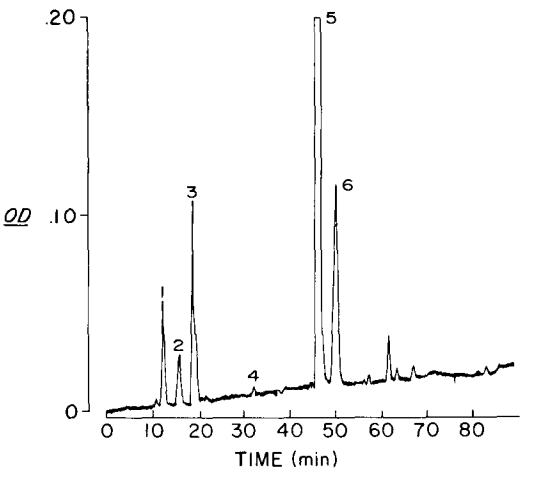

Fig. 2. High pressure liquid chromatogram showing the formation of various adenosine derivatives from adenosine and BFNU. Adenosine and BFNU were incubated together, and the mixture was analyzed directly on a μ-Bondapak C_{18} column as described in the text. Peak 1, unknown; peak 2, 1-β-hydroxyethyladenosine; peak 3, 1,N^6-ethanoadenosine; peak 4, 1-β-fluoroethyladenosine; peak 5, adenosine; and peak 6, BFNU residue.

resolution was achieved for all of the principal products with the exception of 1-β-chloroethyladenosine; small amounts of this compound were overshadowed by large amounts of adenosine. However, the presence of 1-β-chloroethyladenosine could be demonstrated after a preliminary separation on SP-Sephadex C-25.

Ultraviolet spectra of the adenosine derivatives are shown in Table 2. Spectra of the 1-substituted compounds are all rather similar, while the spectrum of 1, N^6-ethanoadenosine is unique. Its spectrum and that of its hydrolysis product, 1, N^6-ethanoadenine, are shown in Fig. 1.

Once the marker compounds had been synthesized and characterized, it was relatively simple to demonstrate their formation by the nitrosoureas. Initially, studies were performed with BFNU because of the greater stability of the fluoroethyl nucleosides. In a typical experiment, 10 mg BFNU was incubated with 5 mg adenosine in 0.05 M sodium cacodylate buffer at pH 6 for 24 hr at 37°. The reaction mixture was then analyzed directly on a μ-Bondapak C_{18} column. The total reaction with adenosine, calculated from the peak areas at 260 nm, was about 2.5 per cent under these conditions.

As shown in Fig. 2, we were able to identify three derivatives from the reaction of BFNU and adenosine. In order of elution, and expressed as per cent of total adenosine substituted, these were: 1-β-hydroxyethyladenosine, 0.3 per cent; 1, N^6-ethanoadenosine, 1.1 per cent; and 1-β-fluoroethyladenosine, 0.15 per cent. The presence of the latter derivative provides evidence for the transfer of a fluoroethyl carbonium ion to adenosine. Since fluoroethyladenosine is a reactive intermediate, however, it is found in relatively small amounts.

The stability of 1-fluoroethyladenosine was studied in separate experiments. Incubation in sodium cacodylate buffer at pH 7 resulted in cyclization to 1, N^6-ethanoadenosine with a half-life of approximately 28 hr at 37°. Significantly, no hydroxyethyladenosine was formed, suggesting that it may arise by another mechanism, such as that proposed below.

1-Fluoroethyladenosine did not react with nitrobenzyl pyridine, which indicates that the compound has relatively little ability to alkylate other molecules. Presumably, as in the case of 3-fluoroethylcytidine [9], it reacts preferentially with the neighboring amino group. However, a group on the complementary strand of DNA may be in a position to compete successfully with the intra-molecular reaction to form interstrand crosslinks. Of course, interstrand crosslinking reactions may involve another haloethyl nucleoside, or another reaction mechanism altogether.

The reaction of BCNU with adenosine produced rather similar results. When 10 mg BCNU was incubated with 5 mg adenosine for 24 hr in 0.05 M sodium cacodylate buffer, pH 6, at 37°, approximately the same overall substitution of adenosine was observed.

An aliquot of the reaction mixture was analyzed by high pressure liquid chromatography as shown in Fig. 3, top panel.

Again hydroxyethyladenosine, with an appearance time of 16 min, accounts for 0.5 per cent of the total peak area at 260 nm, while ethanoadenosine at 19 min accounts for 0.9 per cent of the total. β-Chloroethyladenosine was hidden under the adenosine peak in this chromatogram. However, it could be demonstrated by high pressure liquid chromatography after a preliminary separation was made in SP-Sephadex C-25, as shown in Fig. 3, bottom panel. Also evident in both panels of this figure are several smaller, unidentified nucleoside peaks.

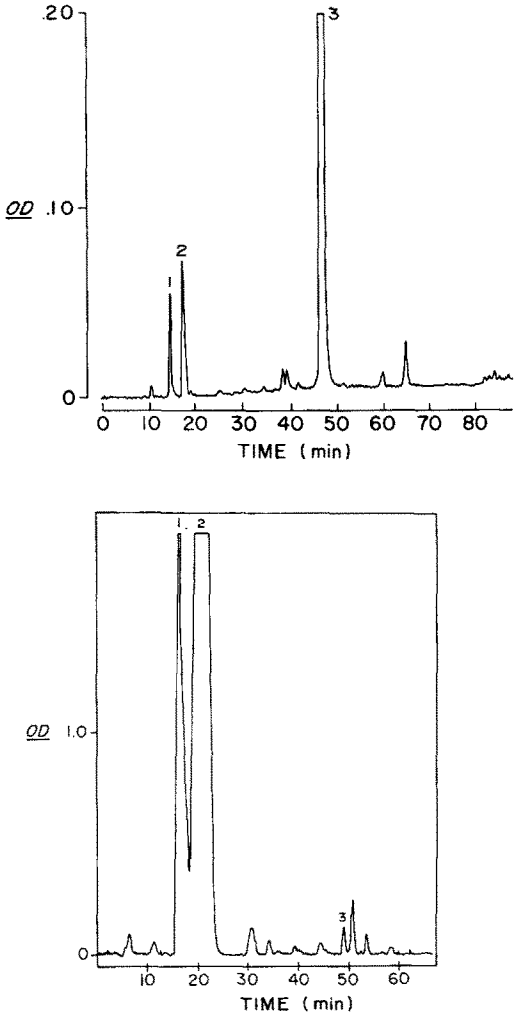

Fig. 3. Top panel: high pressure liquid chromatogram showing the formation of adenosine derivatives from adenosine and BCNU. Adenosine and BCNU were incubated together and analyzed as described above. Peak 1, 1-β-hydroxyethyladenosine; peak 2, 1, N^6-ethanoadenosine; and peak 3, adenosine. Bottom panel: high pressure liquid chromatogram showing the presence of 1-β-chloroethyladenosine after incubation of adenosine with BCNU and preliminary separation on SP-Sephadex C-25. Peak 1, 1-β-hydroxyethyladenosine; peak 2, 1, N^6-ethanoadenosine; and peak 3, 1-β-chloroethyladenosine.

Studies on the stability of chloroethyladenosine revealed that it cyclizes in cacodylate buffer at pH 7 and 37° with a half-life of approximately 20 min. Thus, the distribution of products obtained at pH 7 would be different from the distribution seen in incubations at pH 6, which was chosen to stabilize the haloethyl intermediates. This cyclization reaction was useful in establishing the identity of the small 1-β-chloroethyladenosine peak shown in Fig. 3, lower panel; this material cyclized to ethanoadenosine on standing. Ethanoadenosine was again the only product of cyclization, and no hydroxyethyladenosine was produced by hydrolysis.

Thus, the major site of adenosine substitution with either BFNU or BCNU is the 1-position. Substitution in this position by a haloethyl carbonium ion leads to ethanoadenosine formation. However, it appears that hydroxyethyladenosine is produced directly by some other mechanism.

DISCUSSION

The findings reported above support the suggestion that haloethyl nitrosoureas generate haloethyl carbonium ions and that they form reactive haloethyl nucleoside intermediates. Combining data from the first two papers of this series with the results reported here, we have evidence for the formation of 3-haloethyl cytidine, 7-haloethyl guanosine, and 1-haloethyl adenosine. In addition, there is chromatographic evidence for the formation of several minor derivatives.

It seems likely that one or more of these haloethyl nucleosides is involved in the formation of an interstrand crosslink. By analogy with the classical alkylating agents, such a crosslink could well be cytotoxic. In contrast to nitrogen mustard where such a crosslink would involve a bridge of five atoms, the crosslink here would involve only two carbon atoms. Thus, the geometry and perhaps the biological significance of the crosslinks would be different.

The formation of the tricyclic nucleoside, 1, N^6-ethanoadenosine, is also potentially significant. This compound, which is similar to the fluorescent 9-methyl,1, N^6-ethenoadenine described by Kochetkov *et al.* [13], has an intra-molecular bridge involving two base-pairing positions. Thus, the informational content of a nucleic acid which contained this modification might well be altered. If the modification occurred in a critical region of DNA, it would probably be cytotoxic.

There is also evidence in this and earlier papers that nucleoside modifications may be induced by some additional mechanism besides the transfer of a haloethyl carbonium ion. Thus, we have identified hydroxyethyl derivatives of cytidine, guanosine and adenosine which do not appear to arise from the hydrolysis of the corresponding haloethyl nucleoside. This situation is shown in Fig. 4. A question arises as to the nature of the reactive species which causes the formation of hydroxyethyl derivatives.

Recently, Michejda and Koepke [14] have reported the formation of the compound shown as I in Fig. 5. Compound I is formed when β-tosylethyl methylnitrosamine is heated in methylene chloride, and is relatively stable in aqueous solution. However, it reacts readily with a variety of nucleophiles. By analogy, compound II might well be formed from the nitrosoureas as a reactive intermediate. Reaction with a nucleoside at

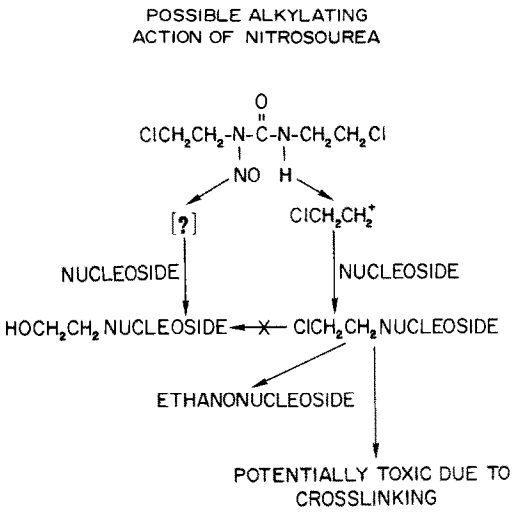

Fig. 4. Scheme for the reaction of BCNU with a nucleoside. Ethano compounds can be formed from either adenosine or cytidine.

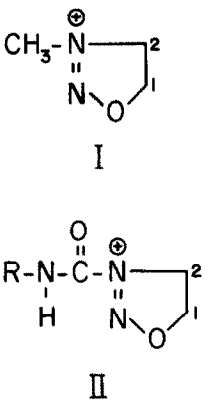

Fig. 5. Structures of the compound isolated by Michejda and Koepke (I) and an analogous structure (II) postulated as an intermediate in the reactions of haloethyl nitrosoureas.

carbon atom 2, followed by a decomposition reaction, would lead directly to a hydroxyethyl nucleoside. Attack at carbon atom 1, however, would probably lead back to a nitrosourea which contained a nucleoside rather than a halogen attached to the ethyl group. Such an intermediate would be of interest because it too could lead to a crosslinking reaction.

In either case, it appears that the interstrand crosslink will involve a 2-carbon bridge, and evidence for such a structure is being pursued actively.

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