REACTION OF BCNU (1,3-BIS (2-CHLOROETHYL)-1-NITROSOUREA) WITH POLYCYTIDYLIC ACID. SUBSTITUTION OF THE CYTOSINE RING.

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SUMMARY. BCNU has been reacted with polycytidylic acid and two derivatives of CMP, 3-hydroxyethyl-CMP and 3,N^4-ethano-CMP, have been identified in the acid hydrolysate of the polymer. Their formation accounts for some of the reaction of BCNU with nucleic acids, and may be related to the mechanism of action of this compound.

The nitrosoureas, including streptozotocin, BCNU (1,3-Bis (2-chloroethyl)-1-nitrosourea), and related compounds are useful agents for the treatment of lymphomas and certain other malignancies. These compounds are not typical alkylating agents in a biological sense, but they do possess alkylating activity (1). In view of the presumed mechanism of action of the alkylating agents (2), the reactions of the nitrosoureas with nucleic acids and polynucleotides are accordingly of great interest. Different or unique reactions of this kind might help to explain the observed differences in biological activity between the nitrosoureas and the standard alkylating agents.

Methyl nitrosourea, a well-known carcinogen, is known to methylate nucleic acids (3-7), but the corresponding reactions of the therapeutic nitrosoureas do not appear to have been extensively investigated. Cheng et al. (8) have reported that CCNU (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea) reacts to a limited extent with poly C, poly G, or t-RNA, but the products of the reaction have not been identified.

In this paper, we wish to describe the reaction of BCNU with poly C and to report the structures of substituted cytidylic acids isolated from the hydrolyzed polymers.

MATERIALS AND METHODS. Crystalline BCNU (NSC-409962) was obtained from Dr. Robert Engle, Drug Research and Development, National Cancer Institute, Division of Cancer Treatment. BCNU labelled with ^{14}C in the chloroethyl groups (specific activity, 10 μCi/μmole) was also obtained through Dr. Engle.

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Polycytidylic acid was purchased from Miles Laboratories; bacterial alkaline phosphatase, from Worthington; and other reagents, from standard sources.

The reaction of poly C with labelled BCNU was followed by withdrawing 50 µl aliquots from 0.5 ml reaction mixtures at appropriate time intervals. These samples were applied to filter paper discs (9), washed free of acid soluble radioactivity, and counted in a Beckman scintillation counter. Residues from BCNU are acid soluble and background counts were, therefore, low. These were subtracted, and the extent of reaction was calculated as the number of BCNU molecules combined with 10^4 CMP units. Quench corrections were made by an internal standardization method.

Poly C which had reacted with BCNU was hydrolyzed with 1N HCl at 100° for 30 min., and substituted nucleotides were isolated by column chromatography on DEAE-Sephadex. They were then characterized by paper chromatography on Whatman #1 paper in: Solvent 1, methanol-conc. HCl-H_2O (8:1:1, v/v/v); Solvent 2, isobutyric acid-conc. NH_4OH-H_2O (66:1:33, v/v/v); and Solvent 3, isopropanol-conc. NH_4OH-H_2O (7:1:2, v/v/v). The ascending method was used with Solvent 1 and the descending method with Solvents 2 and 3; all papers were run for 16 hours.

Fractions from the DEAE-Sephadex column containing substituted cytidylic acids were further purified and separated from substituted uridylic acid, a decomposition product of the former as described below, by chromatography on Dowex 50 with H_2O as eluent. 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) on a Beckman DB-G spectrophotometer.

Nucleotides were converted to nucleosides by alkaline phosphatase treatment in order to facilitate mass spectrometric analysis. These were separated from salts and enzymes by passage through a small Dowex-1 column using H_2O as eluent. Approximately 0.3 mg of each nucleoside was converted to the trimethylsilyl derivative by treatment with 20 µl of bis(trimethylsilyl)-trifluoro acetamide for an hour at 37° in 20 µl of pyridine. Solvent and excess reagent were removed under vacuum and mass spectrometry of the residue was measured on a CEC 21-110 instrument. Samples were introduced directly on a probe, and spectra were determined at 225° and 70 eV.

RESULTS AND DISCUSSION. The labelling of poly C with [^{14}C]BCNU is shown in Fig. 1 under the conditions given in the figure legend. The reaction is clearly temperature-dependent, and ultimately reaches a level of about one nucleotide labelled per thousand. This degree of labelling is similar to

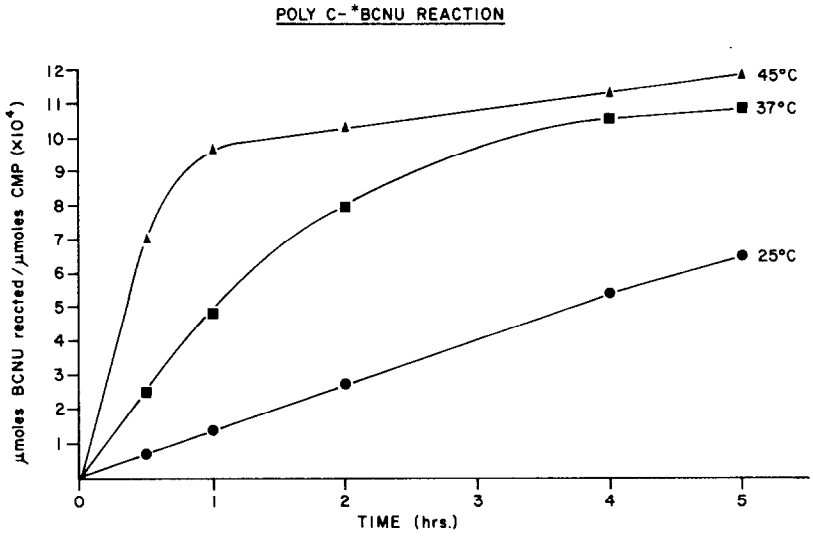

Figure 1. Reaction of Poly C with BCNU. [^{14}C]BCNU, 3.5 µCi/µmole, in 25 µl of ethanol was added to 0.475 ml of poly C in pH 7 buffer solution. Final concentrations: poly C, 0.05 mg/ml; BCNU, 0.61 mg/ml; 0.05 M Na cacodylate buffer; 0.1 M NaCl. Aliquots were withdrawn at the indicated times and analyzed as described in the text.

that observed by Cheng et al. (8) for the reaction between poly C and ethylene-labelled CCNU (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea). By contrast, the level of substitution observed with a classical alkylating agent, methyl methanesulfafonate (10) is approximately ten times as great. However, it is hard to generalize on the relative reactivity of BCNU and methyl methanesulfonate because this depends on many solvent and structural parameters. The significance of the substitution reaction, of course, also depends on the nature of the products.

This was determined by repeating the reaction at higher concentrations; poly C, 15 mg, was reacted with BCNU, 15 mg, for 24 hours at 37° in 1 ml of 0.05 M Na cacodylate buffer, pH 7. The polymer was then hydrolyzed as described above, evaporated to dryness, redissolved in 20 ml of 0.05 M trimethylammonium bicarbonate (TEAB), pH 8.5, and applied to a 1 x 20 cm column of A-25, DEAE-Sephadex. The column was eluted with 30 ml of 0.05 M TEAB, followed by a 0.1 M TEAB. Two derivative peaks (which were labelled if [^{14}C]BCNU was used) appeared ahead of CMP: Peak 1, after 60 ml of 0.1 M TEAB and peak 2 after an additional 60 ml of the same eluent.

Fractions containing these derivatives were pooled, concentrated, and freed of TEAB by repeated evaporation from 50% ethanol. They were then characterized by paper chromatography as shown in Table 1. Both derivatives

TABLE 1

CHROMATOGRAPHIC PROPERTIES OF DERIVATIVES

COMPOUND	ASSIGNED STRUCTURE	R_{UMP} in SOLVENT 1	SOLVENT 2	SOLVENT 3
Peak 1	3-hydroxyethyl CMP	1.08	2.19	3.53
Peak 2	$3,N^4$ - ethano- CMP	1.01	2.49	3.47
Decomposition Product	3-hydroxyethyl UMP	1.28	1.72	2.78

TABLE 2

ULTRAVIOLET SPECTRA OF DERIVATIVES

COMPOUND	ASSIGNED STRUCTURE	pH 2 λ min.	λ max.	pH 7 λ min.	λ max.	pH 12 λ min.	λ max.
Peak 1	3-hydroxyethyl CMP	245	281	245	280	245	267 224
Peak 2	$3,N^4$ - ethano- CMP	241	286	251	286	255	282 228
Decomposition Product	3-hydroxyethyl UMP	234	263	235	263	236	263

contained a common contaminant which had the UV spectrum of a 3-substituted UMP. Chromatographically pure material from peaks 1 and 2 had the UV spectra shown in Table 2. After alkaline treatment for one hour at 100°, both derivatives assumed the properties of 3-substituted UMP.

The structures shown in Fig. 2 were assigned to the two derivatives by the following arguments. Peak 1 had the UV spectrum of a 3-substituted CMP. Mass spectrometry of the corresponding silylated nucleoside revealed a mixture of the tetrakis (trimethylsilyl) and pentrakis (trimethylsilyl) derivatives with molecular weights of 575 and 647 respectively. The addition of five trimethylsilyl groups to the adduct under conditions where four are added to cytidine suggested that the new alkyl substituent contains a functional group which can be silylated. The molecular weight of the tetrakis (trimethylsilyl) derivative indicates that 45 mass units

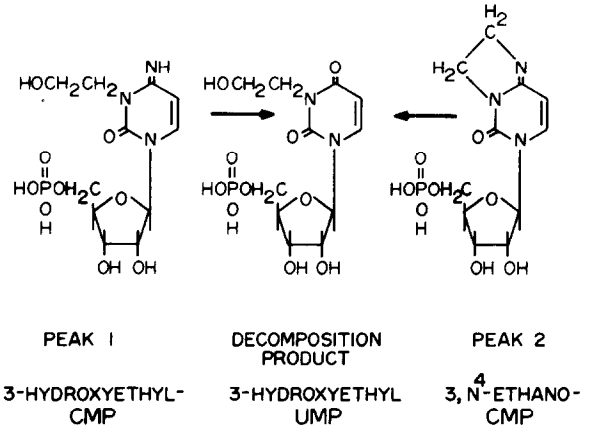

Figure 2. Structures of two substituted cytidylic acids isolated by DEAE-Sephadex chromatography from an HCl hydrolysate of poly C reacted with BCNU. Both compounds decompose to 3-hydroxyethyl UMP.

have been added to cytidine, corresponding to the addition of one hydroxyethyl group. Thus, we conclude that peak 1 is 3-hydroxyethyl CMP and its degradation product is 3-hydroxyethyl UMP as shown in Fig. 2.

The mass spectrum of the silylated nucleoside from peak 2 contained M^+ and M-15 peaks corresponding to a molecular weight of 485. This molecule appeared to carry one less trimethylsilyl (TMS) group than tetrakis (trimethylsilyl) cytidine, and had a molecular weight 26 mass units above that of tris (trimethylsilyl) cytidine. The occurrence of a B+1 peak at m/e 137 and a B+1+TMS peak at m/e 210 confirm the fact that 26 extra mass units have been attached to the cytosine ring (11). On the other hand, the UV spectrum of peak 2 does not correspond to any mono-substituted CMP. This, combined with the fact that the compound decomposes to 3-hydroxyethyl UMP, suggests that it is substituted in both the 3 position and the extranuclear amino group. The cyclized structure proposed satisfies both the mass and UV spectral characteristics and is related to that of peak 1.

Further work will be required to establish the intermediate steps in the formation of the cyclized derivative. One possibility is that a β chloroethyl group is transferred from BCNU to CMP to form a transient 3-β-chloroethyl CMP. Such a derivative would retain the ability to alkylate and might help to explain some of the unusual properties of BCNU.

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