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Mechanism of Action of 2-Haloethylnitrosoureas on DNA and its Relation to their Antileukemic Properties

J. WILLIAM LOWN, LARRY W. MCLAUGHLIN, AND YAU-MIN CHANG

Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada

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1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) and related 2-haloethylnitrosoureas covalently cross-link DNA under physiological conditions. The rate of the cross-linking increases with increasing pH in the range 4–10 and with the (G + C) content of natural DNAs. The reaction leads to stable interstrand cross-links by a two-step process and is strongly dependent on the 2-halogen in the nitrosourea where Cl ≫ Br > F ≫ I. Only one 2-haloethyl group is necessary for cross-linking, which is not observed when the halogen is replaced by –OH or $-OCH_3$. Promoting the acidity of the N^3–H group by appropriate aryl substitution increases the rate of cross-linking. The position of the halogen is critical since, while 1-(2-chloroethyl)-1-nitrosourea cross-links DNA efficiently, 1-(3-chloropropyl)-1-nitrosourea shows no reactivity. N^4-(2-Chloroethyl)-1-methylcytosine hydrochloride, very similar to a suggested intermediate in the cross-linking process, alkylates PM2-CCC-DNA readily. The modes of aqueous decomposition of nitrosoureas as they apply to alkylation and cross-linking are discussed. The results are in accord with formation of a haloethonium ion which forms a nitrogen half mustard intermediate with a DNA base then completes the cross-link. A correlation exists between the extent of DNA cross-linking and activity of the nitrosoureas against L1210 leukemia. Based on the results of this work, a new nitrosourea is designed and synthesized which shows more efficient cross-linking.

INTRODUCTION

2-Haloethylnitrosoureas including BCNU, CCNU, chlorozotocin, and the antibiotic streptozotocin are of proven clinical value in the treatment of CNS neoplasms, Hodgkin's disease, Burkitt's lymphoma, and others (*1–4*).[1] Nitrosoureas break down *in vivo* without enzymatic activation to form alkylating agents and isocyanates (*4*), and considerable evidence indicates they inhibit nucleic acid synthesis by alkylating DNA and RNA (*2*, *4*). Ludlum *et al.* isolated 3,N^4-ethanocytidine monophosphate from treatment of poly(C) with BCNU, in which it appeared that a β-chloroethyl group had been transferred from the drug (*5*, *6*). It was suggested that the intermediate chloroethylcytidine monophosphate would retain alkylating ability, and the formation of covalent interstrand cross-links was therefore anticipated.

We report observations on the nature of the alkylation and cross-linking of DNA by BCNU, CCNU, chlorozotocin, and related nitrosoureas employing the ethidium fluorescence assay which [as in previous problems involving inhibitors of nucleic acid synthesis (*7–12*)] provided detailed information on the course of the reactions. The

[1] Abbreviation used: CLC, covalently linked complementary; CCC, covalently closed circular; OC, open circular; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BBNU, 1,3-bis(2-bromoethyl)-1-nitrosourea; BFNU, 1,3-bis(2-fluoroethyl)-1-nitrosourea; BINU, 1,3-bis(2-iodoethyl)-1-nitrosourea; CCNU, (1,2-chloroethyl)-3-cyclohexyl-1-nitrosourea.

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study includes an examination of the factors determining the rate, the extent, and pH- and DNA base-dependence of the reactions and the correlation with antileukemic activity. During the course of our work the cross-linking of DNA by some 2-haloethyl-nitrosoureas *in vivo* detected by inhibition of alkali-induced strand separation was reported (*13*).

Based on the conclusions of the present study, the design and synthesis of a new nitrosourea which exhibits superior DNA cross-linking properties are described.

EXPERIMENTAL

Materials

Chlorozotocin was a gift from Dr. Gerald Goldenberg, Manitoba Institute of Cell Biology. 1-(2-Chloroethyl)-1-nitroso-3-phenylurea and 3-(2-adamantyl)-1-(2-chloroethyl)-1-nitrosourea were kindly supplied by Dr. Thomas Johnston, Kettering–Meyer Laboratory, Southern Research Institute. 1,3-Bis(2-fluoroethyl)-1-nitrosourea, 1,(2-chloroethyl)-3-(*cis*-2-chlorocyclohexyl)-1-nitrosourea, and 1-(2-chloroethyl)-3-(*trans*-2-chlorocyclohexyl)-1-nitrosourea were obtained from Dr. Harry B. Wood Jr., Division of Cancer Treatment, National Cancer Institute, Washington, D.C. Streptozotocin was purchased from Calbiochem. Compounds not previously known are described below. All other nitrosoureas were prepared by literature procedures (*1*, *14–16*). Ethidium bromide was purchased from Sigma, and λ-DNA (MW, 31×10^6) was obtained as described previously (*17*).

Melting points were determined on a Fisher–Johns apparatus and are uncorrected. The ir spectra were recorded on a Nicolet 7199 F.T. spectrophotometer, and only the principal, sharply defined peaks are reported. The nmr spectra were recorded on Varian A-60 and A-100 analytical spectrometers. The spectra were measured on approximately 10–15% (w/v) solutions in appropriate deuterated solvents with tetramethylsilane as standard. Line positions are reported in parts per million from the reference. Mass spectra were determined on an Associated Electrical Industries MS-9 double-focusing high-resolution mass spectrometer. The ionization energy, in general, was 70 eV. Peak measurements were made by comparison with perfluorotributylamine at a resolving power of 15 000. Kieselgel DF-5 (Camag, Switzerland) and Eastman Kodak precoated sheets were used for thin-layer chromatography. Microanalyses were carried out by Mrs. D. Mahlow of this department.

1-(2-Chloropropyl)-1-nitrosourea. Sodium cyanate (675 mg, 10.0 mmol) was added to 1.0 g (7.7 mmol) of 2-chloro-1-propylamine hydrochloride in 10 ml of water, and the mixture stirred mechanically overnight. After chilling the resulting precipitate was collected and recrystallized from chloroform:petrolem ether affording 1-(2-chloropropyl)urea: 800 mg (76% yield); mp, 88–90°C.

Anal. Calcd. for $C_4H_9ClN_2O$ (MW, 136.0404): C, 35.15; H, 6.66; N, 20.52; Cl, 25.96. Found (136.0405, mass spectrum): C, 34.89; H, 6.54; N, 20.44; Cl, 25.93. Pmr [$(CD_3)_2SO$] δ 1.35 (d, 3H, CH_3); 3.2 (t, 2H, CH_2); 4.1 (m, 1H, CH); 5.6 (s, 2H, exchangeable); 6.4 (s, 1H, exchangeable). Ir ν_{max} ($CHCl_3$) 3400 (N–H), 1655 (C=O) cm^{-1}.

1-(2-Chloropropyl)urea (800 mg, 5.9 mmol) was nitrosated in 1 ml of 98% formic

acid at 0–5°C using 500 mg (7.2 mmol) of sodium nitrite. After stirring the mixture for 1 hr, 1 ml of cold water was added cautiously and stirring was continued for 30 min. The mixture was extracted with chloroform, washed with H_2O, and dried ($MgSO_4$), and the solvent was removed. The residual solid was recrystallized from ether/petroleum ether to give 1-(2-chloropropyl)-1-nitrosourea: 120 mg (12% yield); mp, 79–80°C.

Anal. Calcd. for $C_4H_8ClN_3O_2$ (MW, 165.0305): N, 25.38; Cl, 21.41. Found (165.0301, mass spectrum); N, 25.11; Cl, 21.20. Pmr ($CDCl_3$) δ 1.4 (d, 3H, CH_3); 3.8–4.4 (m, 3H, CH_2, CH); 5.0–7.0 (m, 2H, exchangeable). Ir ν_{max} ($CHCl_3$) 3400 (N–H), 1710 (C=O), 1535 (N=O) cm^{-1}.

1-(3-Chloropropyl)-1-nitrosourea. 3-Chloropropylurea (18) (100 mg, 0.7 mmol) was nitrosated in a similar fashion in 98% formic acid and afforded 1-(3-chloropropyl)-1-nitrosourea: 70 mg (85% yield); mp, 78–79°C.

Anal. Calcd. for $C_4H_9ClN_3O_2$ (MW, 165.0305): C, 29.01; H, 4.88; N, 25.38; Cl, 21.41. Found (165.0313, mass spectrum): C, 29.13; H, 4.83; N, 25.15; Cl, 21.51. Pmr ($CDCl_3$) δ 1.9 (m, 2H, CH_2); 3.4 (t, 2H, CH_2); 4.0 (t, 2H, CH_2); 5.1–7.0 (m, 2H, exchangeable). Ir ν_{max} ($CHCl_3$) 3380 (N–H), 1735 (C=O), 1480 (N=O) cm^{-1}.

3-Cyclohexyl-1-(2-hydroxyethyl)-1-nitrosourea. Cyclohexyl isocyanate (2.5 g, 20.0 mmol) was added to 1.2 g (20.0 mmol) of ethanolamine in toluene at ambient temperature. After 4 hr 2.9 g of crude 3-cyclohexyl-1-(2-hydroxyethyl)urea was collected. A 500-mg (2.2 mmol) portion of the urea was dissolved in 5 ml of 98% formic acid at 0–5°C, and 300 mg (4.0 mmol) of sodium nitrite were added slowly over a 30-min period maintaining a temperature of 0–5°C. After stirring for 30 min, 10 ml of cold water was added cautiously. The mixture was extracted with chloroform, the extract was washed with water and dried ($MgSO_4$), and the solvent was removed. The residue was recrystallized from chloroform/petroleum ether affording 3-cyclohexyl-1-(2-hydroxyethyl)-1-nitrosourea: 310 mg (54% yield), mp, 49–51°C.

Nitrosation of the less hindered amidic nitrogen occurs in 98% formic acid as observed by Johnston *et al.* (*1*) and has been confirmed by pmr studies of the nitrosoureas prepared in this work.

Anal. Calcd. for $C_9H_{17}N_3O_3$ (MW, 215.1270): C, 50.24; H, 7.98; N, 19.54. Found (215.1265, mass spectrum): C, 50.21; H, 8.00; N, 19.58. Pmr ($CDCl_3$) δ 1.2–2.2 (m, 10H, CH_2); 2.7 (s, 1H, exchangeable); 3.6 (t, 2H, CH_2); 3.85 (m, 1H, CH); 4.16 (t, 2H, CH_2); 6.9 (d, 1H, exchangeable). Ir ν_{max} ($CHCl_3$) 3490 (OH), 3370 (NH), 1705 (CO), 1480 (N=O) cm^{-1}.

3-Cyclohexyl-1-(2-methoxyethyl)-1-nitrosourea. Cyclohexyl isocyanate (3.0 g, 24.0 mmol) was added dropwise to 2.0 g (24.0 mmol) of 2-methoxyethylamine in benzene at room temperature. After stirring for 4 hr, 3.8 g of the crude 3-cyclohexyl-1-(2-methoxyethyl)urea was collected. A 500-mg portion of the urea was nitrosated by the same procedure described above giving 3-cyclohexyl-1-(2-methoxyethyl)-1-nitrosourea as a pale yellow oil which crystallized from petroleum ether upon chilling: 300 mg (52% yield); mp, 23°C.

Anal. Calcd. for $C_{10}H_{19}N_3O_3$ (MW, 229.1426): C, 52.42; H, 8.37; N, 18.34. Found (229.1426, mass spectrum): C, 52.74; H, 8.40; N, 18.34. Pmr ($CDCl_3$) δ 1.2–2.2 (m, 10H, CH_2); 3.3 (s, 3H, CH_3)/3.4 (t, 2H, CH_2); 3.7–4.1 (m, 1H, CH); 4.1 (t, 2H, CH_2); 6.9 (d, 1H, exchangeable). Ir ν_{max} ($CHCl_3$) 3350 (NH), 1735 (C=O), 1490 (N=O) cm^{-1}.

1-(2-Chloroethyl)-3-(p-nitrophenyl)-1-nitrosourea. A mixture of 1.0 g (7.0 mmol) of *p*-nitroaniline and 2 g (20.0 mmol) of chloroethyl isocyanate in 50 ml of chloroform was heated under reflux for 10 hr. The mixture was cooled, and the volume was reduced *in vacuo*. Crystallization proceeded overnight to give 1-(2-chloroethyl)-3-(*p*-nitrophenyl)urea; 300 mg (18% yield); mp, 131–133°C.

Anal. Calcd. for $C_9H_{10}ClN_3O_3$ (MW, 243.0411): C, 44.77; H, 4.16; N, 17.29; Cl, 14.59k. Found (243.0406, mass spectrum): C, 44.62; H, 4.23; N, 17.32; Cl, 13.78. Pmr [$(CD_3)_2SO$] δ 3.5 (t, 2H, CH_2); 3.7 (t, 2H, CH_2); 6.45 (t, 1H, exchangeable); 7.65, 8.15 (A_2B_2, 4H, ArH); 9.4 (s, 1H, exchangeable). Ir ν_{max} ($CHCl_3$) 3320 (N—H), 1625 (C=O), 1560, 1330 (NO_2) cm^{-1}.

A 100-mg (0.4 mmol) portion of 1-(2-chloroethyl)-3-(*p*-nitrophenyl)urea was nitrosated in 2 ml of 98% formic acid using 60 mg (0.9 mmol) of sodium nitrite. The precipitated 1-(2-chloroethyl)-3-(*p*-nitrophenyl)-1-nitrosourea was collected as an off-white solid: 65 mg (58% yield); mp, 124–124.5°C.

Anal. Calcd. for $C_9H_9ClN_4O_4$ (MW, 272.0312): C, 39.73; H, 3.34; N, 20.60; Cl, 13.03. Found (272.0316, mass spectrum): C, 39.80; H, 3.33; N, 20.54; Cl, 13.17. Pmr [$(CD_3)_2SO$] δ 3.7 (t, 3H, CH_2); 4.2 (t, 2H, CH_2); 8.05, 8.3 (A_2B_2, 4H, ArH); 11.3 (s, 1H, exchangeable). Ir ν_{max} ($CHCl_3$) 3320 (NH), 1740 (C=O), 1480 (NO), 1560, 1350 (NO_2) cm^{-1}.

1-{2-[(2-Chloroethyl)thio]ethyl}-3-cyclohexyl-1-nitrosourea. Triethylamine (300 mg, 3.0 mmol) was added to 500 mg (2.9 mmol) of *S*-(2-chloroethyl)thioethylamine hydrochloride (*19*) at 0–5°C in chloroform solution. Cyclohexylisocyanate (350 mg, 2.9 mmol) was added dropwise to this mixture, and stirring was continued at room temperature for 18 hr. The chloroform was removed *in vacuo*, and the residual white solid was suspended in cold water to remove hydrochloride salts and filtered. The 1-{2-[(2-chloroethyl)thio]ethyl}-3-cyclohexylurea was purified by recrystallization from chloroform/petroleum ether: 450 mg (63% yield); mp, 122–124°C.

Anal. Calcd. for $C_{11}H_{21}ClN_2OS$ (MW, 228.1296, M-36): C, 50.04; H, 8.03; N, 10.61; Cl, 13.43; S, 12.12. Found (228.1296, M-36, mass spectrum): C, 50.04; H, 8.02; N, 10.68; Cl, 13.45; S, 12.15. Pmr ($CDCl_3$) δ 0.9–2.1 (m, 10H, CH_2); 2.7 (t, 2H, CH_2); 2.9 (t, 2H, CH_2); 3.4 (t, 2H, CH_2); 3.6 (t, 2H, CH_2); 3.3–3.7 (m, 1H, CH); 4.1–4.9 (m, 2H, exchangeable). Ir ν_{max} ($CHCl_3$) 3300 (NH), 1620 (C=O) cm^{-1}. A 100-mg (0.4 mmol) portion of the urea was nitrosated in 2 ml of 98% formic acid using 200 mg (2.9 mmol) of sodium nitrite as described above affording 1-{2-[(2-chloroethyl)thio]ethyl}-3-cyclohexyl-1-nitrosourea as a yellow oil: 65 mg (59% yield).

Anal. Calcd. for $C_{11}H_{20}ClN_3O_2S$ (MW, 293.0964): C, 44.96; H, 6.87; N, 14.30; Cl, 12.06; S, 10.91. Found (293.0958, mass spectrum): C, 44.72; H, 6.76; N, 14.15; Cl, 12.33; S, 11.11. Pmr ($CDCl_3$) δ 1.0–2.2 (m, 10H, CH_2); 2.6 (t, 2H, CH_2); 2.9 (t, 2H, CH_2); 3.6 (t, 2H, CH_2); 4.0 (t, 2H, CH_2); 3.7–4.1 (m, 1H, CH); 6.8 (d, 1H, exchangeable). Ir ν_{max} ($CHCl_3$) 3350 (NH), 1725 (C=O), 1525 (N=O) cm^{-1}.

N-Nitroso-2-oxazolidinone. This was prepared according to the method of Newman and Kutner (*20*): 380 mg (68% yield); mp, 48–50°C (lit. mp, 50–53°C).

N^4-(2-Chloroethyl)-1-methylcytosine hydrochloride. This was prepared according to the method of Ueda and Fox (*21*). It exhibited double mp at 160 and 272–275°C (lit. mp, 163–164 and 271–273°C).

3,N^4-Ethano-1-methylcytosine hydrochloride. This was prepared according to the

method of Ueda and Fox (*21*) by heating a small amount of the above chloroethyl derivative on a heating block for a few minutes at 170°C: mp, 272–275°C (lit. mp, 271–273°C).

Conversion of N^4-(2-chloroethyl)-1-methylcytosine hydrochloride to 3,N^4-ethano-1-methylcytosine hydrochloride. The pmr (D_2O) of the chloroethyl derivative shows a sharp resonance for the methylene protons at δ 3.61 (s, 4H). Under the same conditions the cyclized product exhibits a close A_2B_2 pattern centered at δ 4.25 (m, 4H). When a solution of N^4-(2-chloroethyl)-1-methylcytosine hydrochloride is heated in the pmr probe at 40°C, a slow disappearance of the singlet at δ 3.61 and the appearance of a multiplet at δ 4.25 can be observed.

Methods

Fluorescence assay for determining CLC sequences in DNA produced by nitrosoureas. All measurements were performed on a G. K. Turner and Associates Model 430 spectrofluorometer equipped with a cooling fan to minimize fluctuations in the xenon lamp source. Wavelength calibration was performed as described in the manual for the instrument. One-centimeter-square cuvettes were used. The excitation wavelength was 525 nm and the emission wavelength was 600 nm. The 100× scale of medium sensitivity was generally used, and water was circulated between the cell compartment and a thermally regulated bath at 22°C. The fluorometric method of detecting CLC sequences in λ-DNA has been described (*8*, *22*). Cross-linking of DNA creates a nucleation site which allows renaturation of λ-DNA after heat denaturation (96°C/3 min) and rapid cooling and thus provides intercalation sites for ethidium. That this assay procedure detects the formation of CLC-DNA as a result of a chemical cross-linking event has been confirmed by experiments with the enzyme S_1-endonuclease (*8*, *9*). This enzyme specifically cleaves single-stranded DNA and is essentially inactive on duplex DNA and therefore distinguishes DNA which is renaturable by virtue of a chemical cross-link and DNA which separates into single strands on heating. A 20-μl aliquot was taken at intervals from the reaction mixture (50 m*M* potassium phosphate, pH 7.2; 1.4 A_{260} units of λ-DNA; 5 m*M* nitrosourea; total volume, 200 μl) at 37 and/or 50°C and added to the standard assay mixture (which was 20 m*M* potassium phosphate, pH 11.8, 0.4 m*M* EDTA, and 0.5 μg/ml of ethidium). The fluorescence after the heating and cooling cycle compared with control times 100 gives the percentage of CLC-DNA in a sample. For a standard set of conditions (i.e., type and concentration of DNA, pH, ionic strength, and the temperature), the accuracy of the CLC assay is determined by the precision of the fluorescence readings. Overall accuracy of the CLC assay is estimated at ±2%.

Stability of interstrand cross-links. A 300-μl sample containing 1.12 A_{260} units of λ-DNA, 40 m*M* potassium phosphate, pH 8.6, and 10 m*M* BCNU was incubated at 50°C for 3 hr, and the extent of DNA cross-linking was measured. The sample was dialyzed against 0.15 *M* NaCl and 0.015 *M* sodium citrate (known to reverse the cross-links of carzinophillin) (*11*, *23*) at 4°C for 15 hr. The sample was then incubated at 37°C for 48 hr, and the extent of cross-linking was measured again.

Two-step nature of cross-linking of λ-DNA by nitrosoureas. A 400-μl sample

containing 1.4 A_{260} of λ-DNA solution at pH 7.2 was prepared with BCNU or CCNU with the concentrations used above and incubated at 37°C for a period corresponding to two half-lives of decomposition of the nitrosourea (*24*). The reactions were quenched in ice and dialyzed against 20 m*M* potassium phosphate, pH 7.2, at 4°C for 15 hr to remove unreacted nitrosourea. The dialysate was incubated at either 37 or 50°C and assayed for cross-linking. The control consisted of nitrosourea and λ-DNA at 0°C with dialysis as described, incubation at 37 or 50°C, and assaying for DNA cross-links.

Dependence of cross-linking of natural DNAs by nitrosoureas on the (G + C) content. A 200-μl sample containing 50 m*M* potassium phosphate, pH 8.6, 10 m*M* nitrosourea, and 10% acetonitrile was incubated at 37°C with 1.6 A_{260} units of *Clostridium perfringens* DNA (30% G + C; MW, 7.80×10^6), calf thymus DNA (40% G + C; MW, 3.47×10^6), or *E. coli* DNA (50% G + C; MW, 0.87×10^6). (The MWs were determined by sedimentation velocities.) Assuming a Poisson's distribution of the cross-links and assuming that one link is sufficient to permit spontaneous renaturation, the average number of cross-links per molecule (m) was determined from $m = \ln(1/P_0)$ [where P_0 is the fraction of molecules not cross-linked (*25*)].

Decomposition of 1,3-bis(2-haloethyl)-1-nitrosoureas. The decompositions were carried out at pH 7.2, 37°C. One milliliter of a 40 m*M* nitrosourea solution was allowed to decompose in a sealed tube for 24 hr. The solutions were then cooled to 4°C, the sealed tube was opened, and immediate gas chromatographic (GC), analysis of the solution was undertaken.

GC analyses were performed on a Hewlett–Packard Model 5830 A temperature programmable research chromatograph equipped with a flame ionization detector. Samples were injected onto a 2-m, 6.5-mm-o.d. column of 10% Carbowax on Chromosorb W. The column was heated at 90°C for 4 min after injection; a heating rate of 20°C/min was then maintained until the column temperature reached 120°C; this temperature was maintained until all volatile products had been swept from the column. Identification of acetaldehyde and haloethanol was done using retention times of authentic samples.

Fluorescence determination of alkylation of PM2-CCC-DNA by nitrosoureas: A 20-μl aliquot was taken at intervals from the reaction mixture [50 m*M* potassium phosphate, pH 7.2, 1.5 A_{260} units of PM2-CCC-DNA (90% CCC), 5 m*M* nitrosourea in a total volume of 200 μl at 37°C] and added to the standard assay mixture (which was 20 m*M* potassium phosphate, pH 11.8, 0.4 m*M* EDTA, and 0.5 μg/ml of ethidium). The fluorescence after heating at 96°C/3 min followed by rapid cooling was compared with the initial value.

Under these conditions unreacted PM2-CCC-DNA returns to register after heat denaturation because of topological constraints. Alkylated PM2-CCC-DNA shows a decrease in fluorescence because of thermally induced depurination followed by alkaline strand scission of the apurinic site in the assay medium (*26*). The ratio of the decrease in fluorescence (after the heating and cooling cycle) to that of the control is a measure of the extent of alkylation. In a control experiment it was shown that none of the components interfered with the ethidium fluorescence. Provided no single-strand cleavage of PM2-CCC-DNA is observed (detected by the characteristic rise in fluorescence before the heating/cooling cycle), this technique can be used to measure levels of alkylation not readily observed using λ-DNA.

RESULTS AND DISCUSSION

Bis(2-haloethyl)nitrosoureas cross-link DNA under physiological conditions [e.g., at pH 7.2 and 37°C, BCNU cross-links 42% of λ-DNA in 6 hr (Fig. 1)]. Although the same reactions occurred more slowly at 37°C, the cross-linking was generally performed at 50°C so as to obtain convenient rates for the study. The rate of cross-linking increases with increasing pH in the range 4–10 (data not shown) in accord with the suggested mechanisms of decomposition to produce alkylating species (*24*, *27*, *28*)

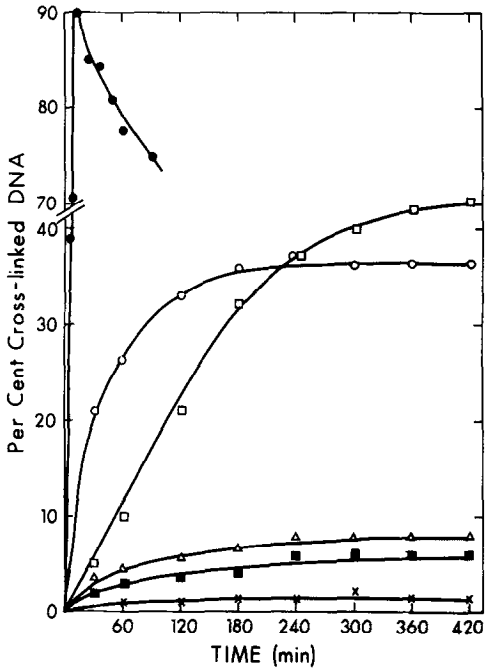


FIG. 1. Covalent interstrand cross-linking of λ-DNA by 2-haloethylnitrosoureas. Reactions were performed at 37°C in a total volume of 200 μl buffered at pH 7.2 wiyh 20 m*M* potassium phosphate and contained 1.4 A_{260} units of λ-DNA and 5 m*M* nitrosourea. (×——×), BINU; (■——■), BFNU; (△——△), BBNU; (□——□), BCNU; (○——○), 1-(2-chloroethyl)-1-nitroso-3-phenylurea; (●——●), 1-{2-[(2-chloroethyl)thio]ethyl}-3-cyclohexyl-1-nitrosourea.

(see Fig. 5). The cross-linking increases with the (G + C) content of natural DNAs (average number of cross-links/nucleotide × 10^5:(G + C) content: 1.4:30%; 4.2:40%; 9.1:50%) in accord with previous work with synthetic polynucleotides (*6*, *29*). The interstrand DNA links are stable for at least 48 hr at 37°C under conditions which reverse those produced by carzinophillin (*11*, *23*), thus suggesting two covalent bonds. The formation of interstrand links by BCNU and CCNU is clearly a two-step reaction. The second alkylation to complete the cross-link occurred at 37°C for as long as 4.5 hr after removal of the free nitrosourea by dialysis. If the incubation temperature was raised to 50°C, a corresponding increase in the rate of cross-linking after removal of free drug was observed (Fig. 2). This suggests the initial alkylation (on G or C) requires a low activation energy and that the second bond is formed by a slower alkylation of higher activation energy.

As indicated earlier, Ludlum *et al.* isolated 3,N^4-ethanocytidine monophosphate and the 3-(2-hydroxyethyl)cytidine monophosphate from treatment of poly(C) with BCNU, from which it appeared that a β-chloroethyl group had been transferred from the drug to the base (*5*, *6*). The isolation of these compounds suggests two intermediates: N^4-(2-chloroethyl)cytidine which could then react to form either (*1*) the intramolecular alkylation product (similar to the type isolated by Ludlum) and observed in the case of N^4-(2-chloroethyl)-1-methylcytosine hydrochloride (*21*) or (*2*) an interstrand link; 3-(2-

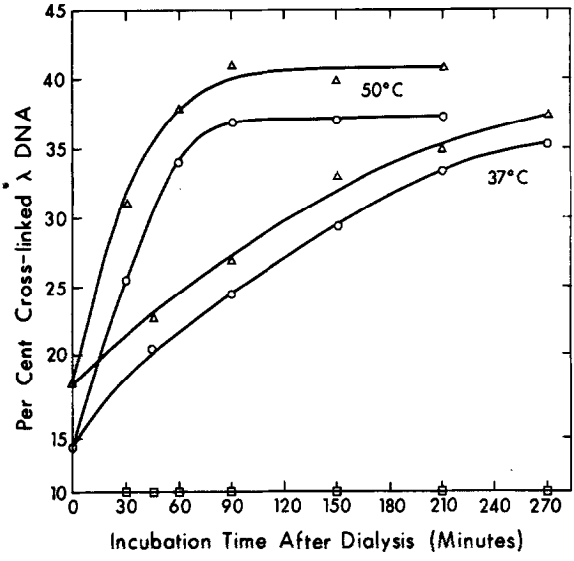


FIG. 2. Stepwise nature of interstrand cross-linking of λ-DNA by 2-haloethylnitrosoureas at 37 and 50°C in 400 μl at pH 7.2 with 20 m*M* potassium phosphate and 1.4 A_{260} units of λ-DNA and 5 m*M* nitrosourea. Reactions were quenched (0°C) and dialyzed against pH 7.2 buffer, and the dialysate was reincubated at 37 or 50°C. (O——O), BCNU; (△——△), CCNU; (□——□), control. Treatment of λ-DNA with 5 m*M* BCNU at 0°C, dialysis to remove free nitrosourea, and incubation at 37 or 50°C.

chloroethyl)cytidine could preferentially hydrolyze to the N^3-(2-hydroxyethyl)cytidine. Alkylation by 2-chloroethylamides is not presently known, nor has the cyclization of 3-(2-chloroethyl)cytidine to 3,N^4-ethanocytidine been demonstrated.

An assay for DNA alkylation by a similar chloroethyl derivative, N^4-(2-chloroethyl)-1-methylcytosine hydrochloride (*21*) in pH 7.2 buffer at 37°C using PM2-CCC-DNA, demonstrated that DNA alkylation by this chloroethyl derivative occurs at approximately the same rate as does cross-linking by 2-chloroethylnitrosoureas (Fig. 3). The intramolecular alkylation of N^4-(2-chloroethyl)-1-methylcytosine hydrochloride can be followed in D_2O at 40°C by pmr (see Experimental) as well as by refluxing in pyridine (*21*). Figure 3 also contrasts the behavior of BCNU with the other nitrosoureas. BCNU shows an initial drop in fluorescence attributed to alkylation and thermally induced cleavage of the DNA followed by the characteristic rise of fluorescence at the onset of cross-linking.

The cross-linking with bis(2-haloethyl)nitrosoureas shows a strong halogen dependence: Cl, 37%; Br, 7%; F, 6%; I < 2% (Table 1). However, only one 2-haloethyl group

is necessary for cross-linking, in accord with the proposed decomposition to give a haloethonium intermediate (*24*, *27*, *28*). For example, the antitumor agent chlorozotocin cross-links DNA very efficiently, whereas the related streptozotocin, lacking a 2-haloethyl function, does not. Cross-linking is not observed when the halogen is replaced by $-OH$ or $-OCH_3$. The extent of cross-linking is unrelated to the nature of the N^3

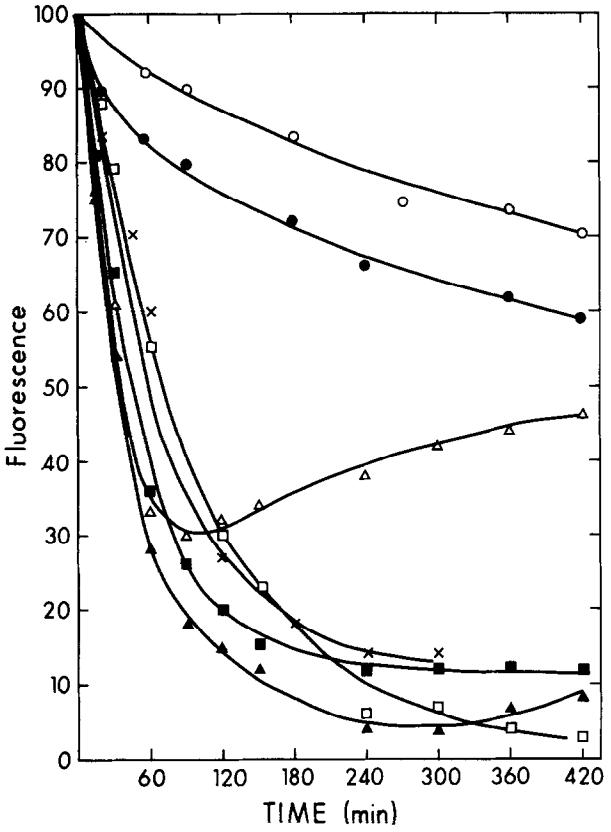


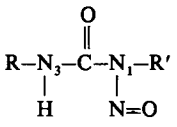
FIG. 3. Alkylation and thermally induced depurination and alkaline strand scission of PM2-DNA by 2-haloethylnitrosoureas at 37°C, pH 7.2, 50 m*M* potassium phosphate with 1.5 A_{260} units of PM2-DNA (90% CCC) and 5 m*M* nitrosourea in a total volume of 200 μl. (□——□), BINU; (▲——▲), BBNU; (×——×), *N*-nitroso-2-oxazolidinone; (△——△), BCNU; (■——■), BFNU; (○——○), N^4-(2-chloroethyl)-1-methylcytosine; (●——●), 10 m*M* N^4-(2-chloroethyl)-1-methylcytosine. Fluorescence values are those after the heat denaturation and cooling cycle.

substituents, provided the N carries at least one hydrogen. Aryl-N^3 substitution substantially increases the rate but not the extent of cross-linking by increasing the acidity of N^3–H (Fig. 1). This is in agreement with the results of the pH study. Substitution of the N^3-aryl moiety by *p*-methoxy or *p*-nitro groups did not significantly affect the rate of cross-linking. As expected, no interstrand cross-linking was observed in the case of a disubstituted N^3 [1-(2-chloroethyl)-3,3-dimethyl-1-nitrosourea].

Modification of the position of the halogen has a marked effect on observations concerning cross-linking. While 1-(2-chloroethyl)-1-nitrosourea is an efficient cross-linking agent, 1-(3-chloropropyl)-1-nitrosourea has no ability to induce covalent interstrand

TABLE 1

COVALENT CROSS-LINKING OF λ-DNA BY 2-HALOETHYLNITROSOUREAS AND CORRELATION WITH ACTIVITY AGAINST LEUKEMIA L1210



R	R′	DNA cross-linking (%)[a]	Activity against Leukemia L1210[b] (ip injection, 10^5 cells) log kill[c]	Cures (%)	Ref.
H–	$-CH_3$	0	—	10	*32*
H–	$-CH_2CH_3$	0	Inactive	—	*33*
H–	$-CH_2CH_2Cl$	36	5	40	
H–	$-CH_2CH(CH_3)Cl$	8	—	—	
$ClCH(CH_3)CH_2$–	$-CH_2CH(CH_3)Cl$[d]	—	5	20	
cyclo-C_6H_{11}–	$-CH_2CH(CH_3)Cl$[d]	—	5	0	
H–	$-CH_2CH_2CH_2Cl$	0	—	—	
C_6H_5–	$-CH_2CH_2CH_2Cl$[d]	—	2	0	
FCH_2CH_2–	$-CH_2CH_2F$	6	6	50–60	
$ClCH_2CH_2$–	$-CH_2CH_2Cl$	37	6	0–100	
$BrCH_2CH_2$–	$-CH_2CH_2Br$	7	5	20	
ICH_2CH_2–	$-CH_2CH_2I$	<2	Inactive	0	
cyclo-C_6H_{11}	$-CH_2CH_2F$	6	6	70–90	
cyclo-C_6H_{11}	$-CH_2CH_2Cl$	37	5	80–100	
cyclo-C_6H_{11}	$-CH_2CH_2Br$	8	6	30	
cyclo-C_6H_{11}	$-CH_2CH_2OH$	0	—	—	
cyclo-C_6H_{11}	$-CH_2CH_2OCH_3$	0	—	—	
cis-2-Cl-cyclo-C_6H_{11}	$-CH_2CH_2Cl$	41	—	30–90	
trans-2-Cl-cyclo-C_6H_{11}	$-CH_2CH_2Cl$	41	—	80–100	
Phenyl	$-CH_2CH_2Cl$	36	3	0	
p-Nitrophenyl	$-CH_2CH_2Cl$	37	—	—	
p-Methoxyphenyl	$-CH_2CH_2Cl$	37	5	0	
2-Adamantyl	$-CH_2CH_2Cl$	42		≥50	*34*
cyclo-C_6H_{11}	$-CH_2CH_2SCH_2CH_2Cl$	90[e]	—	—	
Streptozotocin		0	Inactive[f]	—	*35*
Chlorozotocin		41	6	90	*35*
$(CH_3)_2NC(=O)N(NO)CH_2Cl$		0	Inactive	—	14

[a] At 50°, pH 7.2 after 2 hr of reaction.

[b] Antileukemic data from Ref. (*1*) unless otherwise stated for single-dose ip drug injection.

[c] Reduction of an inoculum of 10^5 cells to 10^2 cells is a 3 log kill (*1*).

[d] These compounds were not assayed for DNA cross-linking in this study; however, activity is shown for comparison of 2-chloropropyl and 3-chloropropyl derivatives.

[e] Assayed at 37°C since cross-linking rate is too fast to quantitate at 50°C.

[f] Shows on a qd 1–9 schedule (*36*).

cross-links. Additionally, if the carbon skeleton of the 2-chloroethyl group is substituted with a methyl to form 1-(2-chloropropyl)-1-nitrosourea, substantial inhibition of cross-linking is observed (Table 1).

While the N^4-cytidine-O^6-guanosine positions of opposing hydrogen bonded bases have been suggested as possible sites for the cross-link (*13*), it is conceivable that

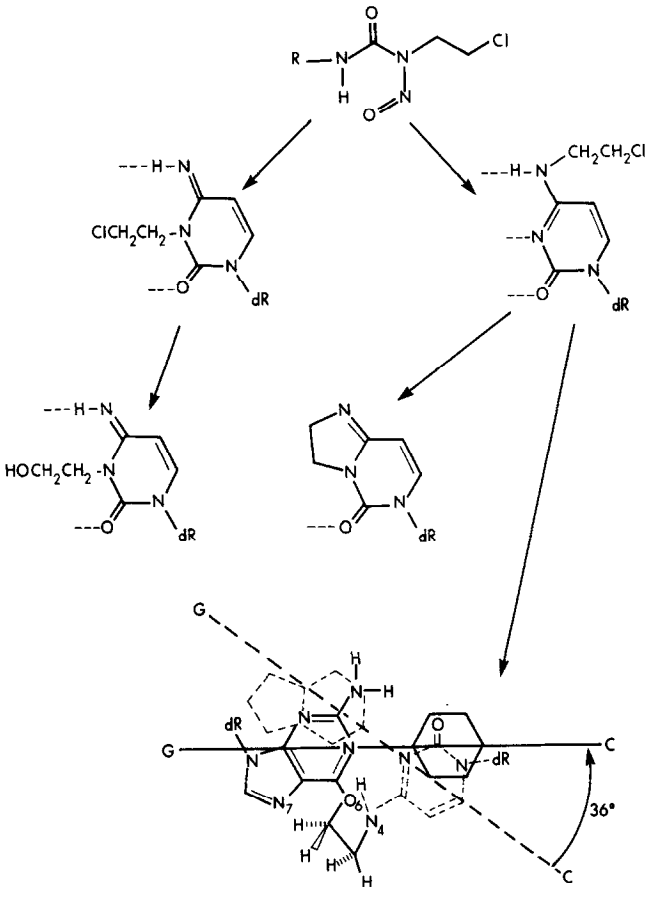


FIG. 4. Pathways illustrating the decomposition of 2-chloroethylnitrosoureas and subsequent alkylation of N^3 of cytidine followed by preferential hydrolysis, or alkylation of N^4 of cytidine followed by labilization of the chlorine and intramolecular alkylation or cross-linking to, e.g., O^6 of guanosine.

adjacent G–C pairs may also be cross-linked through these positions (Fig. 4). Examination of space-filling models shows that the 36° rotation between two adjacent G–C pairs in helical DNA allows the N^4-position of cytidine of one base pair to approach the O^6-position in guanosine of the adjacent G–C base pair such that a two-carbon link between these positions imparts minimal distortion of the helix. Thus initial alkylation of the N^4 position of cytidine may be followed by nucleophilic attack by O^6 or N^7 of guanosine to complete an interstrand cross-link accounting for the observed dependence of the frequency of the latter on (G + C) content of the DNA.

While the nitrogen half mustard intermediate (Fig. 4) explains the relative differences

in the abilities of 2-chloroethylnitrosourea and 2-fluoroethylnitrosourea derivatives to form interstrand cross-links, as well as the inability of the 3-chloropropyl and 2-chloropropyl derivatives to show substantial cross-linking, it does not explain the results obtained for 2-bromoethyl and 2-iodoethyl analogs. On the basis of a previous study with aromatic nitrogen mustards using the *p*-nitrobenzylpyridine test for alkylating activity (*30*) (admittedly artificial and far-removed from the reactions with DNA), one might expect the bromo and iodo analogs to display greater cross-linking abilities than the corresponding chloro and fluoro nitrogen mustard intermediates.

Since the overall rates of decomposition of the 1,3-bis(2-haloethyl)-1-nitrosoureas are identical [at least for Cl and F (*24*)], it appeared possible that the differences in cross-

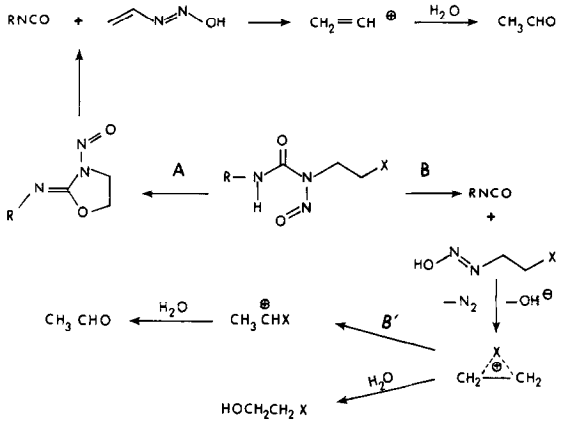


FIG. 5. Proposed decomposition pathways of 2-haloethylnitrosoureas.

linking ability arose from different preferred modes of decomposition, rather than due to an intrinsic difference in reactivity of the species formed.

It has been suggested (*24*, *27*, *28*) that the decomposition of 2-haloethylnitrosoureas occurs by two major pathways (Fig. 5), yielding as major products acetaldehyde and 2-haloethanol. After decomposition of the four 1,3-bis(2-haloethyl)-1-nitrosourea derivatives at pH 7.2, 37°C for 24 hr, the mixtures were assayed by gas-chromatographic analysis for acetaldehyde and 2-haloethanol. The ratio of 2-haloethanol to acetaldehyde was as follows: BFNU, 4.55; BCNU, 2.38; BBNU, 0.36; BINU, 0.0. The first two results are in agreement with previous work (*14*, *28*). While it cannot be stated with certainty if acetaldehyde is preferentially produced by pathway A or pathway B′ (Fig. 5), the results suggest that the bromo and iodo analogs preferentially decompose to form vinyl carbonium ions and/or acetaldehyde and little of a 2-halo alkylating species to initiate cross-linking.

Since both pathways of decomposition lead to alkylating species and the rates of total decomposition are comparable, then the alkylating ability of the 1,3-bis(2-haloethyl)-1-nitrosoureas (as opposed to their cross-linking ability) should be comparable. Reaction of the 1,3-bis(2-haloethyl)-1-nitrosoureas with PM2-CCC-DNA (where heat induced depurination of alkylated basis is followed by alkaline strand scission) gives a direct measure of the extent of total alkylation. It may be seen from Fig. 3 that the rates of total alkylation of the DNA by all four halogen compounds are closely comparable. In

addition, the rates of alkylation of DNA are comparable to that produced by *N*-nitroso-2-oxazolidinone (*20*), a derivative very similar to the intermediate which has been suggested to be produced through decomposition of 2-halonitrosoureas by pathway A (Fig. 5). Although it is recognized that the antitumor action of nitrosoureas may involve several different types of chemical reactions on nucleic acids, nevertheless the correlation that exists between the extent of interstrand cross-linking of DNA and the antileukemic properties of the 2-haloethylnitrosoureas (Table 1) encouraged us to design an agent which might display superior cross-linking properties.

The synthesis of this compound, 1-{2-[(2-chloroethyl)thio]ethyl}-3-cyclohexyl-1-nitrosourea (Fig. 6), incorporated four design features: (a) The compound should inhibit decomposition by pathway A (Fig. 5), thus more efficiently producing a cross-linking species; (b) the intermediate carbonium ion should be stabilized by the sulfur atom in much the same way that the chlorine atom has been suggested (*27*) to stabilize the chloroethyl carbonium ion produced from chloroethylnitrosoureas; (c) cross-linking should not be as dependent on the initial site of attack since the second alkylation is the

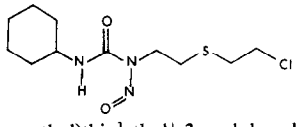


FIG. 6. 1-{2-[(2-Chloroethyl)thio]ethyl}-3-cyclohexyl-1-nitrosourea.

result of the sulfur half mustard portion of the molecule; (d) the distance between alkylation sites should be greater than the restrictive two-carbon link provided by the chloroethylnitrosoureas.

Ethidium bromide assay for the ability of 1-{2-[(2-chloroethyl)thio]ethyl}-3-cyclohexyl-1-nitrosourea to form interstrand cross-links (Fig. 1) indicates that it has a faster rate and a greater extent of cross-linking when compared with chloroethylnitrosoureas. The observed decrease in fluorescence after 12 min of reaction is due to depurination. While the chloroethylnitrosoureas most probably alkylate initially as a result of a stabilized carbonium ion followed by labilization of the chlorine and a further alkylation, this sulfur half mustard nitrosourea has the ability to alkylate in a similar manner or by initial alkylation through the sulfur half mustard followed by decomposition of the nitrosourea and a further alkylation. This compound gives a T/C (test to control) of $\approx$ 120 against L 1210 lymphoid leukemia.

The results serve to delineate the chemistry of alkylation and of covalent interstrand cross-linking of DNA by 2-haloethylnitrosoureas.

Based on results concerning the effective cross-linking of DNA by chloroethylnitrosoureas, we have undertaken a study of the effects upon DNA of chloroethyl alkylating species from other sources. Preliminary studies have shown that 5-[3-(2-chloroethyl)-1-triazenyl]imidazole-4-carboxamide, which has been suggested as a source of chloroethyl carbonium ion (*31*), forms 31% cross-linked DNA in 90 min under physiological conditions.

ACKNOWLEDGMENTS

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