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NITROSOUREA-INDUCED DNA SINGLE-STRAND BREAKS*

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Abstract—Reaction of DNA with nitrosoureas *in vitro* results in extensive formation of alkali labile sites. Two types of single-strand scission (SSS) processes may be distinguished by their different rates: (1) type I SSS which occurs relatively fast at high pH, and (2) type II SSS which is a much slower process. Neither of these processes is affected by free radical traps. Dimethyl sulfate, which is known to alkylate DNA bases but not phosphate residues, shows no type I SSS but does show extensive type II SSS. That the latter process involves alkylation of bases followed by the formation of apurinic sites was confirmed by using endonuclease VI, an enzyme specific for apurinic positions. Reactions of chloroethylnitrosoureas with DNA produces both type I and type II SSS. Aliphatic amines produced in the decomposition of alkyl nitrosoureas do not contribute significantly to the scission of apurinic sites via Schiff base formation. However, this process may be significant for aryl nitrosoureas. Ethyl nitrosourea (ENU), 1,3-bis(2-chloroethyl)nitrosourea (BCNU), and 3-cyclohexyl-1-(2-hydroxyethyl)-1-nitrosourea (CHNU) readily degrade poly A by phosphate alkylation, with rates that parallel their relative rates of decomposition. The relative rates of hydrolysis of triethylphosphate and β-hydroxyethyl diethyl phosphate parallel the type I SSS observed for ENU and CHNU with DNA. The type I SSS of DNA by these compounds appears to involve a similar phosphotriester formation and hydrolysis. The type I SSS is in accord with the observed extreme liability of β-hydroxyethyl diethyl phosphate which is attributed to participation of the OH group, and by the fact that methylation of the OH completely inhibits the type I SSS process.

The 2-haloethyl nitrosoureas, a group of antitumor agents including BCNU, BFNU and CCNU+, provide the most active drugs currently available for the treatment of cerebral neoplasms, and are also of proven clinical value in the treatment of Hodgkin's disease, Burkitt's lymphoma and other neoplasms [1, 2]. Nitrosoureas decompose under physiological conditions to react with biological macromolecules by two mechanisms: alkyation, which can be observed both with nucleic acids and proteins, and carbamoylation, which is observed with proteins but not nucleic acids [1, 2]. It has been suggested from structure–activity studies that the alkylating action is responsible for the therapeutic effects of nitrosoureas and that the carbamoylating activity leads to toxicity effects [3]. Alkylation, in addition to possible mispairing or miscoding effects, has been observed to lead to the production of interstrand cross-links [4, 5]. The extent of DNA cross-linking has been shown to correlate well with antileukemic activity [5].

Lesions produced in DNA, which lead to single-strand breaks, upon alkaline treatment, have been reported for both BCNU and CCNU in cell cultures [6–8]. However, the occurrence of single-strand breaks under physiological conditions without the use of high pH has not been demonstrated, nor has the mechanism of the scission process been investigated. DNA single-strand scission (SSS) and/or formation of alkali labile sites can occur primarily through three processes. First, the generation of highly reactive radicals (superoxide or hydroxyl) in the vicinity of the DNA molecule results in the formation of DNA strand breaks. Second, alkylation followed by depurination or depyrimidination results in labile apurinic sites. Three pathways are possible for the transformation of apurinic sites to single-strand breaks: (a) hydrolysis under alkaline conditions, (b) treatment with an appropriate amine, and (c) enzymatic action. Third, alkylation of the phosphate groups forms phosphate triesters which are susceptible to alkaline hydrolysis resulting in single-strand breaks. This paper considers these alternative pathways as they apply to the mechanism of action of nitrosoureas.

MATERIALS AND METHODS

Materials

Preparations of the nitrosoureas in this work have been described previously [5]. Triethyl phosphate was obtained from the Aldrich Chemical Co. (Milwaukee, WI) and was redistilled (b.p. 93–95°/12 mm). Ethidium bromide was purchased from the Sigma Chemical Co. (Saint Louis, MO). PM2-CCC DNA was obtained as described previously [9]; the calf thymus topoisomerase was prepared according to Herrick and Alberts [10]. Compounds not previously reported are described below.

Melting points were determined on a Fisher–Johns apparatus and are uncorrected. The i.r. spectra were

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+ Abbreviations: CCC, covalently closed circular; OC, open circular; SSS, single-strand scission; ENU, ethyl nitrosourea; BCNU, 1, 3-bis(2-chloroethyl)nitrosourea; CHNU, 3-cyclohexyl-1-(2-hydroxyethyl)-1-nitrosourea; CCNU, (1,2-chloroethyl)-3-cyclohexyl-1-nitrosourea; BFNU, 1,3-bis(2-fluoroethyl)-1-nitrosourea; and CNU, 2-chloroethylnitrosourea.

recorded on a Nicolet 7199 F.T. spectrophotometer, and only the principal sharply defined peaks are reported. The n.m.r. 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 a 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.

β-Chloroethyl diethyl phosphate. This compound was prepared according to the method of Bailey and Robinson [11], b.p. 136–137°/12 mm (lit. 144–145/18 mm).

β-Hydroxyethyl diethyl phosphate. Ethyl ethylene phosphate [12] was allowed to solvolyze overnight in absolute ethanol. After solvent removal, the product β-hydroxyethyl diethyl phosphate is distilled in near quantitative yield, b.p. 97–99°/10^{-4} mm. Anal. Calc. for $C_6H_{15}PO_5$ (mol. wt + proton 199.0736, mol. wt − proton 197.0579): C, 36.36; H, 7.64. Found (199.0740, 197.0583 mass spectrum): C, 36.16; H, 7.56. P.m.r.[$CDCl_3$] δ 1.38 (t, 6H, CH_3); 3.34 (s, 1H, exchangeable); 3.72–4.32 (m, 8H, CH_2). I.r. ν_{max} (film) 3400 (OH; 1260 (P=O); 1030 (P—O) cm^{-1}.

Depurinated PM2-CCC DNA. To 400 μl of PM2-CCC DNA 8.0 A_{260} was added 25 μl of 1 M sodium acetate buffer, pH 3.05. The mixture was incubated at 37°. Two-μl aliquots were withdrawn and added to the standard assay solution (which was 20 mM phosphate, pH 11.8, 0.4 mM EDTA, and 0.5 γ/ml of ethidium); the fluorescence was measured and compared to that obtained after heating at 96°/3 min, followed by rapid cooling to 23° (see Methods).

Under these conditions, unreacted PM2-CCC DNA returns to register after heat denaturation because of topological constraints [9]. Depurinated PM2-CCC shows a decrease in fluorescence due to alkaline strand scission of the apurinic site in the assay medium. 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 depurination. As long as the initial fluorescence reading remains constant, DNA degradation other than depurination is negligible. Typically a 90–120 min incubation is necessary to introduce at least one apurinic site per molecule. After incubation, 50 μl of 1 M phosphate buffer, pH 7.2, is added to quench the reaction. The solution of apurinic PM2-CCC DNA may be stored for several days at 4°.

Endonuclease specific for apurinic sites of Escherichia coli (endonuclease VI). Endonuclease VI was purified according to Verly and Rassart [13] from *E. coli* BATCC 11303; after the phosphocellulose chromatography, the enzyme was stored in 0.15 M NaCl, 0.04 M sodium phosphate, pH 6.5, with an equal volume of glycerol and kept at −20°. For the experiments, this preparation was diluted with a suitable buffer.

Assay for endonuclease VI activity. The basis of the assay is that the enzyme cleaves apurinic PM2-CCC DNA and thereby converts it to linear DNA, which results in a change in ethidium fluorescence both before and after heat denaturation when measured at pH 8.0. The reaction solution consisted of PM2 DNA 1.0A_{260} units in potassium phosphate buffer, pH 8.0. A 10-μl aliquot of the enzyme was added, the reaction solution was incubated at 37° for 15 min, and the fluorescence of the resulting PM2-OC- DNA read, using the standard pH 8 ethidium assay. Conversion of PM2-CCC DNA to PM2-OC DNA by the endonuclease VI results in a characteristic 30 per cent increase in fluorescence, as a result of the release of topological constraints. After heat denaturation at 96°/4 min, when the PM2-OC DNA is converted into single strands, followed by rapid cooling to 23°, the fluorescence was read again. An active endonuclease VI fraction is revealed by loss of fluorescence after heat denaturation. The control for the assay consisted of a similar reaction substituting native PM2-CCC DNA.

Methods

Ethidium fluorescence assay for type I SSS of DNA. The fluorometric methods of measuring strand breakage of PM2-CCC DNA have been described [9, 14]. The conversion of PM2-CCC DNA to PM2-OC DNA results in a 30 per cent increase in fluorescence in the pH 11.8 ethidium assay solution (which was 20 mM potassium phosphate, pH 11.8, 0.4 mM EDTA and 0.5 μg/ml of ethidium bromide), owing to release of topological constraints.

The increase in fluorescence can be enhanced further by initially treating the PM2-CCC DNA with the calf thymus topoisomerase. Native PM2-CCC DNA contains negative supercoils. The topoisomerase, by acting as both an endonuclease and a ligase, removes the supercoils to relax the DNA. During this process the number of intercalation sites for ethidium (which itself unwinds the supercoiled PM2-CCC DNA) is decreased. The relaxation process can be monitored by a 25–30 per cent decrease in fluorescence. The conversion of relaxed PM2-CCC DNA to PM2-OC DNA now results in a 80–100 per cent increase in fluorescence.

A 300-μl sample containing PM2-CCC DNA 1.0 A_{260}, 50 mM sodium cacodylate buffer, pH 7.0, and 400 mM NaCl was incubated at 37° with the topoisomerase. The fluorescence was monitored by transferring 20-μl aliquots into 2 ml of the pH 11.8 assay solution. When a 25–30 per cent decrease in fluorescence had been observed (typically requiring a 30-min incubation), a 5 mM concentration of the desired drug was introduced and the fluorescence again monitored using 20-μl aliquots in 2 ml of the pH 11.8 assay solution. Readings must be taken immediately after addition of the aliquot so that apurinic site hydrolysis does not contribute to the observation of type I SSS.

Ethidium fluorescence assay for type II SSS of DNA. After the fluorescence reading had been taken to determine type I SSS, the pH 11.8 assay solution containing the 20-μl aliquot of reaction mixture was incubated at 37°. At designated times, the solution was re-equilibrated to 22° for the fluorescence reading.

Detection of apurinic sites. A 300-μl solution containing 5 mM drug, 50 mM sodium cacodylate, pH 7.0, and relaxed PM2-CCC DNA 1.0 A_{260} was allowed to react for 120 min while monitoring for type I SSS.

Twenty μl of the apurinic endonuclease solution was then added (the amount was determined by previous experiments with low pH depurinated PM2-CCC). The fluorescence was then monitored, as described in part 1 of Methods. The per cent of fluorescence increase with respect to the fluorescence at time 0 min was corrected for dilution by the enzyme solution.

Reaction of apurinic DNA with amines. A 200-μl solution containing apurinic PM2 CCC DNA 1.0 A_{260}, 50 mM potassium phosphate, pH 7.2 and 5 mM of the appropriate amine was incubated at 37°. Twenty-μl aliquots were withdrawn and added to the pH 11.8 assay solution and the fluorescence reading was taken immediately.

Detection of phosphate alkylation by RNA degradation. A 140-μl solution containing 4 mg/ml of poly A (Sigma, mol. wt 139,000), 150 mM sodium cacodylate buffer, pH 7.0, and 150 mM of the desired nitrosourea was incubated from 1 to 8 hr. The reactions were quenched in ice and dialyzed against 50 mM potassium phosphate, pH 7.2, 1000 mM NaCl, and 1 mM EDTA in triply distilled water at 4° for 36 hr. The dialysate was then diluted with the dialysis solution to 1.0 A_{260}, and the sedimentation velocity was determined on a Beckman analytical ultracentrifuge.

Phosphotriester hydrolysis. Gas–liquid chromatographic analysis of the triesters was performed on a Hewlett–Packard model 5830 A temperature programmable research chromatograph equipped with a flame ionization detector. To a 1-ml solution containing 200 mM potassium phosphate, pH 11.8, and 0.5% dioxane, as an internal control, was added the appropriate triester to a concentration of 20 mM. Al-μl aliquot was injected immediately after thorough mixing, onto a 6-ft stainless steel column containing a support of polyphenyl ether. The column temperature was maintained at 150° for 4 min at which time it was heated at 20°/min until a temperature of 200° had been reached. This temperature was maintained for 20 min or until all volatiles had been swept from the column. Additional 1-μl aliquots were taken from the solutions during the next 6 hr. The following retention times were observed: ethanol, 0.9 min; dioxane, 1.9 min; triethyl phosphate, 7.8 min; β-chloroethyl diethyl phosphate, 14.4 min; β-hydroxyethyl diethyl phosphate, 16.7 min.

Attempted conversion of β-chloroethyl diethyl phosphate to β-hydroxyethyl diethyl phosphate. A 200-μl solution containing 10% dioxane, 100 mM potassium phosphate, pH 7.2, and 25 mM β-chloroethyl diethyl phosphate was incubated at 37°. At 1-hr intervals, a 10-μl aliquot was transferred to a 250-μl solution containing 200 mM potassium phosphate, pH 11.8, and a 1-μl aliquot of this solution was injected onto the polyphenyl ether column, as described above. After a 3-hr incubation, a 1-μl sample of the pH 7.2 reaction mixture was injected onto the column.

RESULTS AND DISCUSSION

Extensive formation of alkali labile sites which result in single-strand breaks in DNA is observed after treatment of relaxed PM2-CCC DNA with nitrosoureas. The lesions produced in the DNA have much greater stability when the pH 7.2 ethidium assay is employed, while under alkaline assay conditions, pH 11.8, they are readily cleaved. The different rates of production of

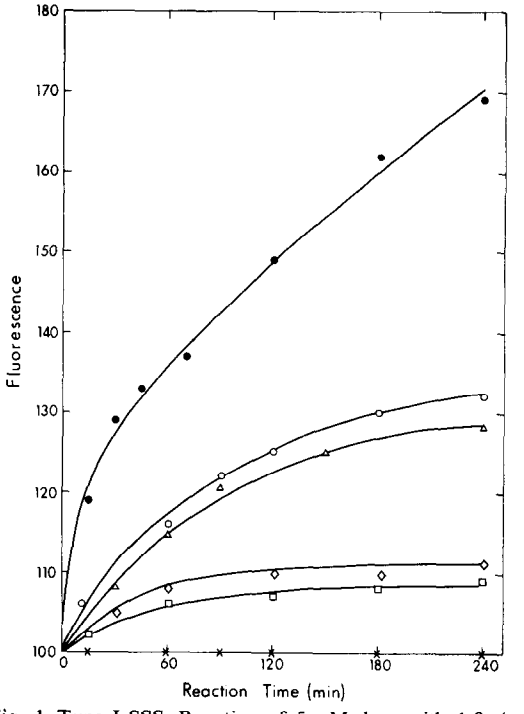

Fig. 1. Type I SSS. Reaction of 5 mM drug with 1.0 A_{260} relaxed PM2-CCC DNA, pH 7.0, 37°. Fluorescence values were obtained within 30 sec of the addition of a 20-μl aliquot to the pH 11.8 assay solution at 22°. Type II SSS was not observed at 22°. Key: (●——●) CHNU; (○——○) chlorozotocin; (△——△) BCNU; (□——□) ENU; (◇——◇) BFNU; and (×——×) dimethyl sulfate or 3-cyclohexyl-1-(2-methoxyethyl)-1-nitrosourea or control (containing relaxed PM2-CCC DNA with no drug).

DNA single-strand breaks observed in the alkaline assay solution, after treatment with nitrosoureas, suggest that at least two mechanisms are operative. There is an extremely fast reaction resulting in single-strand breaks which occurs immediately after the addition of an aliquot of nitrosourea-treated DNA to a 20 mM phosphate buffer, pH 11.8, at 22°. The extent of this type I single-strand scission increases with the time of reaction between DNA and drug (Fig. 1). A second slower production of single-strand breaks is observed when the nitrosourea-treated DNA is allowed to incubate at 37° and pH 11.8. This type II process can be observed to occur for 90–120 min after addition to the pH 11.8 buffer (Fig. 2). Controls run with untreated DNA indicate its stability to the high pH conditions for 90 min, as well as the observation that reaction of the alkylating agent with DNA is quenched upon dilution 1: 100 in the assay solution. The ethidium bromide was not observed to affect the rate of type II SSS.

Neither of the two processes was affected by the presence of enzymatic radical trapping agents, such as superoxide dismutase and catalase. Chemical radical traps such as isopropyl alcohol and sodium benzoate also had no effect on the scission phenomenon. No strand scission is observed when a pH 7.2 assay solution is used. These observations rule out a radical process, similar to that which has been observed for drugs such as bleomycin and the anthracylines in the presence of reducing agents [15, 16], to account for the observed DNA degradation in the case of the nitrosoureas.

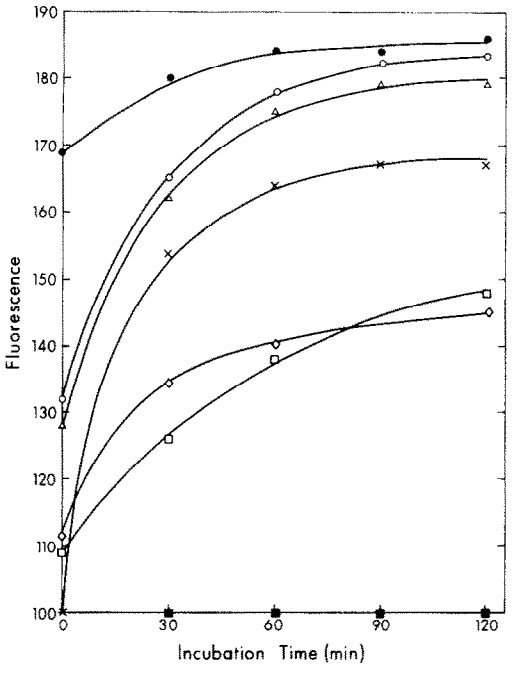

Fig. 2. Type II SSS. Incubation of the 20-μl aliquot (taken after 240 min of the reactions described in the legend to Fig. 1) at 37° and pH 11.8. Assay solutions were cooled to 22° before fluorescence reading. Key: (●——●) CHNU; (○——○) chlorozotocin; (△——△) BCNU; (□——□) ENU; (◇——◇) BFNU; (×——×) dimethyl sulfate; and (■——■) control (20 μl or relaxed PM2-CCC DNA with no drug).

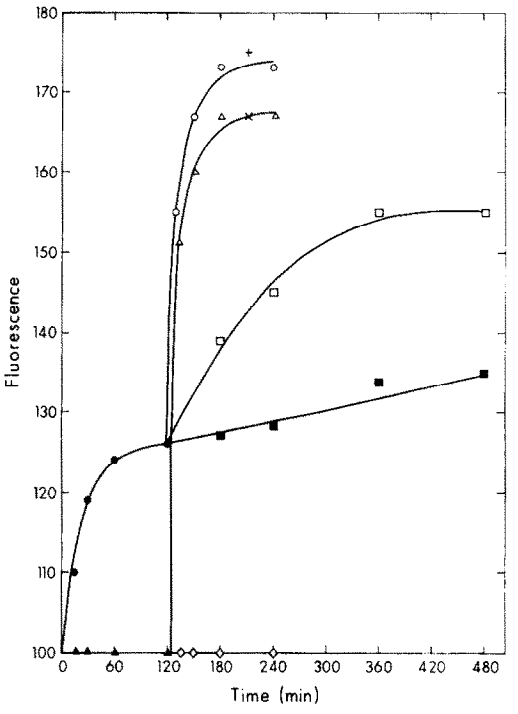

Fig. 3. Reaction of 5 mM drug with 1.0 A_{260} relaxed PM2-CCC DNA, pH 7.0, 37°. Measurement of type I SSS for (●——●) CNU, followed after 120 min of reaction with: (○——○) endonuclease VI; (+) 90-min incubation at 37°, pH 11.8; (□——□) 5 mM aniline; and (■——■) 5 mM cyclohexylamine. Measurement of type I SSS for (▲——▲) dimethyl sulfate followed after 120 min of reaction with: (△——△) endonuclease VI; (×) 90-min incubation at 37° pH 11.8; (◇——◇) control (relaxed PM2-CCC DNA with endonuclease VI).

Type II SSS, enzymatic or alkaline catalyzed. Nitrosoureas have been observed to alkylate the bases of nucleic acids [2]. The cytosine and guanine residues are reported to be most extensively alkylated [2]. A number of modified nucleosides have been isolated after treatment of synthetic polynucleotides with BCNU, which indicates that a β-chloroethyl or β-hydroxyethyl alkylating moiety has been transferred to the base [17, 18]. The facile loss of alkylated bases from the modified DNA polymer to produce apurinic sites is well documented and accounts for the depurination and depyrimidination observed in the present study [19, 20].

To determine the contribution of the depurination-depyrimidination strand scission pathway to the overall degradation of nitrosourea-treated DNA, the effects of dimethyl sulfate were first studied. Dimethyl sulfate is known to alkylate DNA extensively, with the principal sites of attack occurring at guanine-N-7 and adenine-N-3 [19–21], with no oxygen alkylation of the bases or phosphates [22]. It has also been observed that N-3 alkylated adenine and N-7 alkylated guanine residues are readily lost to yield apurinic sites [23], which, while stable under neutral conditions, are subject to alkaline hydrolysis and the formation of DNA single-strand breaks [24]. While dimethyl sulfate-treated relaxed PM2-CCC DNA showed none of the type I SSS, extensive type II SSS was observed upon incubation for 90 min at 37° and pH 11.8 (Fig. 3).

Confirmation that the type II SSS observed for dimethyl sulfate was due to production of apurinic sites and subsequent hydrolysis was obtained, using an apurinic site specific endonuclease. Endonuclease VI, first isolated by Verly and Rassart [13], recognizes apurinic sites and hydrolyzes the DNA sugar backbone at such points. Treatment of relaxed PM2-CCC DNA with dimethyl sulfate for a period of 2 hr was followed by treatment with the endonuclease VI. An immediate and extensive production of single-strand breaks was observed, indicating apurinic sites produced by dimethyl sulfate (Fig. 3). Treatment of supercoiled or relaxed PM2-CCC DNA with the endonuclease was run as a control to show that the enzyme had no effect on the native DNA. Additionally, it was observed that the rate of the type II SSS process is comparable with the rate of hydrolysis of apurinic DNA (generated under low pH conditions [25, 26]) at 37° and pH 11.8 (Fig. 4).

A similar study was then undertaken for 2-chloroethylnitrosourea (CNU). CNU was chosen for two reasons. First, the half-life for CNU at 37° and pH 7.2 is approximately 9 min,* which compares favorably with the 10-min half-life observed for dimethyl sulfate under similar conditions [27]. Second addition to an alkylating moiety, CNU produces sodium cyanate in cacodylate buffer. The N-3 substituted nitrosoureas decompose to form isocyanates which then hydrolyze to amines. It has been observed that amines react with apurinic

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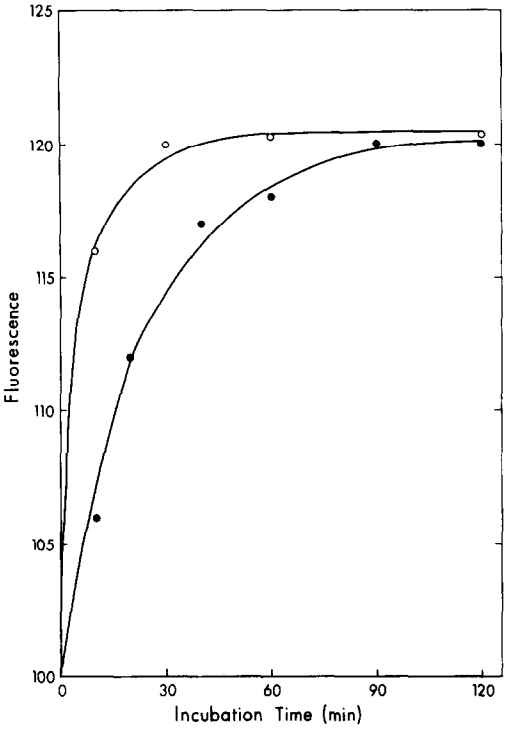

Fig. 4. Alkaline catalyzed strand scission. Twenty μl of 1.0 A_{260} apurinic PM2-CCC DNA [generated by: (●——●) low pH; (○——○) treatment with dimethyl sulfate] was incubated in 2 ml of the pH 11.8 assay solution at 37°. Solutions were cooled to 22° before fluorescence readings.

sites [28, 29] a process which will be discussed shortly. The use of CNU removed the possibility of this competing pathway during the initial study.

After reaction of CNU with relaxed PM2-CCC DNA for a period of 120 min, significant type I SSS was observed (Fig. 3). Incubation of the reaction mixture for 90 min at pH 11.8 and 37° indicated that a considerable amount of type II, SSS had also taken place. Confirmation that the type II SSS observed for CNU was due to the formation of apurinic sites was again obtained using endonuclease VI (Fig. 3).

Amine catalyzed SSS. It is clear that apurinic sites lead to strand breaks under enzymatic treatment or alkaline hydrolysis. The third possibility involves the reaction of apurinic sites with amines. It has been reported that the reaction of apurinic acid with an aromatic amine in the presence of aqueous formic acid results in DNA chain scission [28, 29]. We have observed that at pH 7.2 there is significant reaction between aromatic amines and apurinic acid leading to strand scission, while aliphatic amines show little or no reaction (Fig. 4). The difference presumably reflects the relative stabilities of the Schiff's bases formed [30]. This was substantiated by observing the differences in ability to cause strand scission by an aromatic amine containing either an electron withdrawing substitute or an electron donating substituent (Fig. 5).

An investigation was then initiated to determine if hydrolysis of apurinic sites by amines was a contributing pathway in the degradation of nitrosourea-treated DNA. CNU was reacted with relaxed PM2-CCC DNA for a period of 120 min. While type I SSS was observed, no amine was present in the reaction mixture. After 2 hr of reaction, an equivalent concentration of either cyclohexylamine or aniline was added to the nitrosourea–DNA mixture. While aniline caused a significant further increase in single-strand breaks during the following 5 hr, cyclohexylamine showed only a small additional effect upon DNA degradation (Fig. 3).

Reactions of amines with apurinic sites is unlikely to be a contributing pathway for nitrosoureas which produce aliphatic amines from isocyanate hydrolysis. Specifically, the type I cleavage phenomenon does not reflect amine-apurinic site reaction in the case of BCNU, CCNU, CHNU or similar derivatives. However, it may be significant in the case of aryl substituted

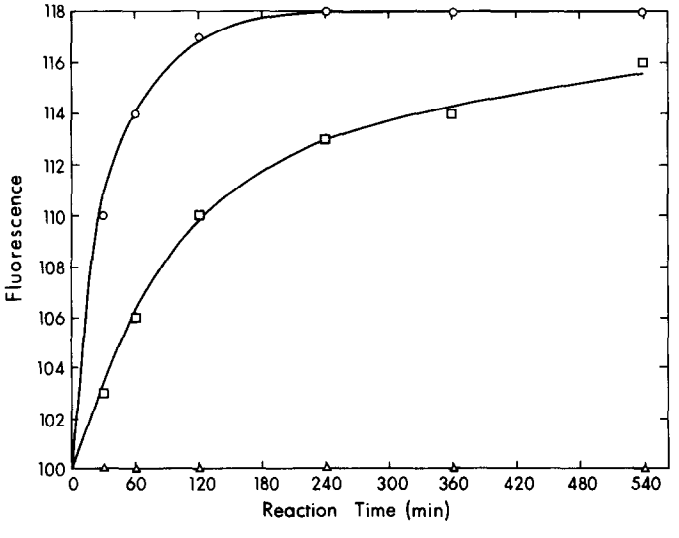

Fig. 5. Reaction of apurinic PM2-CCC DNA incubated at 37°. Conditions: pH 7.2 with 5 mM (○——○) *p*-methoxyaniline; (□——□) aniline; and (△——△) *p*-nitroaniline or cyclohexylamine or control. Fluorescence values were obtained within 30 sec of the addition of the 20-μl aliquot to the pH 11.8 assay solution.

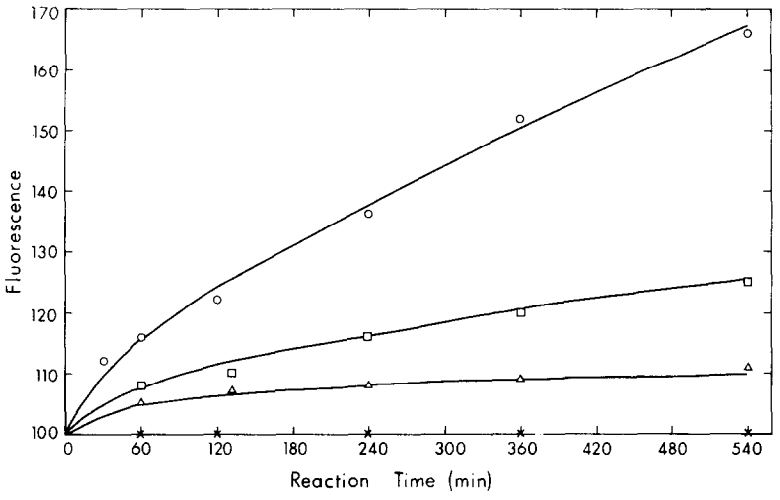

Fig. 6. Type I SSS. Reaction of 5 mM drug with 1.0 A_{260} relaxed PM2-CCC DNA, as described in the legend to Fig. 1. Key: (○——○) 1-(2-chloroethyl)-3-(*p*-methoxy-phenyl)-1-nitrosourea; (□——□) 1-(2-chloroethyl)-3-phenyl-1-nitrosourea; (△——△) 1-(2-chloroethyl)-3-(*p*-nitrophenyl)-1-nitrosourea; and (×——×) control, *p*-methoxy aniline.

nitrosoureas The relative extent of this contribution to the type I process was measured using three aryl derivatives: 1-(2-chloroethyl)-3-(*p*-methoxyphenyl)-1-nitrosourea, 1-(2-chloroethyl)-3-phenyl-1-nitrosourea and 1-(2-chloroethyl)-3-(*p*-nitrophenyl)-1-nitrosourea. An ethidium bromide fluorescence assay for the ability of the three derivatives to initiate type I SSS is shown in Fig. 6. The analog producing the electron rich aryl amine and thus the more stable Schiff's base shows extensive type I SSS. The derivative with the electron withdrawing substituent on the aryl moiety shows little DNA strand scission, while the unsubstituted aryl derivative is intermediate in reactivity.

Type I SSS. It has been shown previously by Singer *et al.* [31, 32] that, when DNA is treated with ethyl nitrosourea, 65 per cent of the alkylation events occur on the phosphate residues. The resulting phosphotriesters, while known to be stable under neutral conditions [33], have been observed to undergo base catalyzed hydrolysis [34, 35]. Based on these observations, we initiated a study to determine if the nitrosoureas of interest in this work alkylated phosphate residues of nucleic acids. The obvious differences in the type I SSSobserved for an ethylating agent, a chloroethylating agent and a hydroxyethylating agent (Fig. 1) dictated the three types of compounds that should be studied.

Bannon and Verly [33] have observed that ethyl and methyl phosphotriesters of DNA are stable under neutral conditions, while Shooter [35] has reported that ehtyl phosphotriesters hydrolyze only very slowly in 0.1 N NaOH. RNA internucleotide linkages are much less stable and the glycosidic linkages much more stable than those in DNA. phosphotriesters of ribonucleotides are unstable over the entire pH range, presumably due to participation in the hydrolysis step by the β-hydroxyl group on the sugar moiety [36]. This property has permitted observation of RNA degradation by alkylating agents to be used as a diagnostic test for phosphotriester formation [37, 38]. Alkylation of the base residues of RNA produces a much more stable system than in DNA and, therefore, depurination of alkylated bases followed by hydrolytic cleavage of the apurinic site is less likely to contribute to RNA degradation. Hence, it appeared that the best analytical method for the measurement of phosphate alkylation in nucleic acids in-

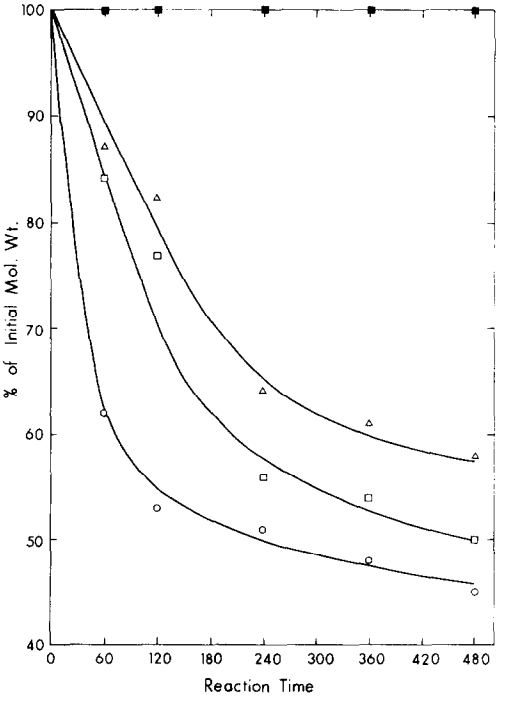

Fig. 7. Decreased in poly A molecular weight during reaction of 150 mM drug with 4 mg/ml of poly A (Sigma), pH 7.0, 37°. Key: (○——○) ENU; (□——□) BCNU; (△——△) CHNU; and (■——■) control, poly A (Sigma), pH 7.0, and 37°.

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volved monitoring molecular weight changes in RNA after treatment with the alkylating agent.

Reaction of poly A (Sigma, mol. wt 139,000) with the three compounds of interest, ENU, BCNU and CHNU, at 37° and pH 7.0, followed by molecular weight analysis using sedimentation velocity on a Beckman analytical ultracentrifuge, resulted in the curves observed in Fig. 7. The rate of RNA degradation parallels the rates of decomposition of the three nitrosoureas at pH 7.0 and 37°. The half-lives under these conditions have been measured as ENU 16.3 min, BCNU 61.4 min, and CHNU 186.2 min.* The extent of RNA degradation by the three compounds is approximately the same but whether or not this reflects the extent of phosphate alkylation cannot be stated with certainty. To determine if the type I SSS observed for these compounds results from hydrolysis of the phosphotriesters formed, the nature and the fate of the triesters resulting from the three different alkylating agents were examined.

Svenson *et al.* [34] have observed that the methyl phosphotriester of the thymidylyl-(3′-5′)-thymidine dinucleotide has a half-life of 2.3 hr in 0.1 N sodium hydroxide at 37°. Shooter [35] has reported the slow rates of alkaline hydrolysis of ethyl phosphotriesters in DNA while, as stated above, Bannon and Verly [23] have observed that methyl and ethyl DNA phosphotriesters are stable under neutral conditions.

No work has been reported for the stability of chloroethyl phosphotriesters which will be discussed shortly.

Hydroxyethyl phosphotriesters of DNA have been reported to result in strand scission under neutral conditions [39, 40]. However, there is some disagreement concerning DNA strand scission after hydroxyethylation [27]. Mikhailov and Smrt [41] have observed that the β-hydroxyethyl phosphotriester of the deoxyuridylyl-(3′-5′)-uridine dinucleotide prepared recently is stable at pH 7.5 and 40° but will readily undergo base catalyzed hydrolysis in aqueous ammonia at 20° to yield a mixture of nucleotide products.

Phosphotriester model studies. While the enthalpy of hydrolysis of β-hydroxyethyl dimethyl phosphate has been reported to be only slightly greater than that observed for trimethyl phosphate [42, 43], there have been observations which indicate that some β-hydroxyethyl phosphates hydrolyze very rapidly under alkaline conditions [44, 45].

To determine the stability of the DNA phosphotriesters formed by ethyl, chloroethyl and hydroxyethyl alkylating agents under the alkaline assay conditions used to observe type I SSS, three model compounds were prepared. Triethyl phosphate, β-chloroethyl diethyl phosphate and β-hydroxyethyl diethyl phosphate were subjected to the alkaline conditions of the assay (pH 11.8, ambient temperature) and the extent of hydrolysis was measured using g.l. chromatography.

Both triethyl phosphate and β-chloroethyl diethyl phosphate were stable under the high pH conditions, with negligible hydrolysis after 6 hr at ambient temperature. A trace amount of ethanol could be identified in the hydrolysis mixture but this accounted for less than 5 per cent of the volatiles for each compound. However, in the case of the β-hydroxyethyl diethyl phosphate, the results were significantly different. An aliquot of the hydrolysis mixture was injected into the gas–liquid chromatograph within 30 sec after the addition of the phosphate to the pH 11.8 solution. The chromatograph indicated that the β-hydroxyethyl diethyl phosphate had hydrolyzed completely. Ethanol accounted for 95 per cent of the volatile products swept from the column within 20 min of sample injections.

Whether or not the rapid hydrolysis is the result of a concerted S_N2 mechanism [46], an addition elimination mechanisms [47] or a cyclic pentacoordinate intermediate which has been suggested for some β-hydroxyethyl phosphodiesters [48], as well as ribonucleotides [49], is not within the scope of this paper.

The extreme lability of this model β-hydroxyethyl phosphotriester accounts for the rapid type I SSS observed for CHNU (Fig. 1), while conversely the stability of triethyl phosphate to the alkaline assay conditions accounts for the very low type I SSS observed for ENU. While both compounds alkylate the internucleotide phosphate groups extensivley, the fate of the triesters is significantly different in alkaline solution. Further evidence as to the necessity of the hydroxyl function can be obtained by comparing the results obtained for 3-cyclohexyl-1-(2-methoxyethyl)-1-nitrosourea. By methylating the hydroxyl function, near total inhibition of type I SSS was observed (Fig. 1).

The stability of the β-chloroethyl diethyl phosphate in the pH 11.8 solution does not agree with the observed significant type I SSS noted for chlorozotocin and BCNU (Fig. 1). It appeared that the most likely route to type I SSS by chloroethylating agents would involve some hydrolysis of the chlorine to produce the labile hydroxyethyl derivative. Previous work [17, 18] has shown that hydroxyethylated bases can be isolated after treatment of synthetic polynucleotides with BCNU. To detect this possible pathway, a solution of β-chloroethyl diethyl phosphate, pH 7.2, was incubated at 37°. At 1-hr intervals, aliquots of this reaction mixture were transferred to a pH 11.8 buffered solution which was then chromatographed. No change in the concentration of β-chloroethyl diethyl phosphate was observed in a 3-hr period. After the incubation, a sample of the reaction mixture at pH 7.2 was chromatographed. No β-hydroxyethyl diethyl phosphate was detected.

While the model β-chloroethyl compound did not appear to follow the postulated hydrolytic pathway, this does not preclude the possibility that some hydrolysis of the chlorine occurs during transport or decomposition of chloroethyl nitrosoureas. Clearly the work by Ludlum *et al.* [17, 18] suggests chloride hydrolysis at some stage of the reaction of BCNU with nucleic acids.

In agreement with this hypothesis is the observation that BFNU exhibits significantly less type I SSS (Fig. 1). One would expect less hydrolysis to produce hydroxy ethyl alkylating agents in this case.

In conclusion, there is considerable evidence that the nitrosoureas react primarily so as to alkylate DNA and to form interstrand cross-links. These processes are accompanied by single-strand breakage of the DNA. The present work indicates the latter process can occur by two distinct pathways: (1) a relatively rapid reaction involving deoxyribosephosphate triesters and subsequent hydrolysis, and (2) a relatively slower base alkylation followed by depurination and hydrolysis of the apurinic site either enzymatically or by reaction with an

amine. The isolation of β-hydroxyethyl substituted pyrimidine moieties from nitrosourea-treated DNA and the observed efficient DNA scission by CHNU which contains a β-hydroxyethyl substituent suggested that the therapeutic properties of this class of nitrosoureas should be examined. CHNU is currently undergoing testing *in vivo* for antileukemic activity.

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