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EVALUATION OF THE ROLE OF ISOCYANATES IN THE ACTION OF THERAPEUTIC NITROSOUREAS

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Abstract—The half-lives of chloroethyl and cyclohexyl isocyanate have been determined in tissue culture medium, and the isocyanate concentration produced during the breakdown of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) has been calculated. L1210 or HeLa cells exposed either to the parent nitrosourea or to an equivalent constant isocyanate concentration show no deficiency in the repair of gamma-irradiation damage as measured by DNA strand separation in alkali. Viability studies indicate that the isocyanates play a minor role in the overall cytotoxicity of the nitrosoureas.

The production of organic isocyanates during the breakdown of the therapeutic nitrosoureas BCNU,* CCNU and methyl CCNU [1] has been considered to be one of the factors which determines nitrosourea toxicity. Nitrosoureas with high carbamylating activity in an assay *in vitro* have lower therapeutic indices *in vivo* [2]. This view has been strengthened by the observation of Schein *et al.* [3] that nitrosoureas which do not form functional isocyanates show reduced marrow toxicity in mice. Although the relationship between isocyanate formation and nitrosourea toxicity appears convincing, the functional role of these substances in the overall action of the nitrosoureas is not clear. In this respect, Wheeler and Bowdon [4] have demonstrated that BCNU and its related chloroethyl isocyanate inhibit *in vitro* DNA synthesis, and Kann *et al.* [5] have shown that chloroethyl isocyanate inhibits both the maturation of nuclear RNA and the repair of X-irradiation-induced DNA damage in L1210 cells [6].

Recently, we have pursued some earlier observations from this laboratory which were at variance with a functional role of the isocyanate component of the nitrosoureas. The half-lives of chloroethyl isocyanate and cyclohexyl isocyanate in aqueous media have been determined, and cells have been exposed to the isocyanates at concentrations present during the breakdown of the parent nitrosoureas. Under these conditions, we have been unable to detect any effect of the isocyanates on the rate or extent of repair of gamma-irradiation-induced DNA damage and have demonstrated that under these conditions the isocyanates are relatively noncytotoxic agents.

* Abbreviations used: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; methyl CCNU, 1-(2-chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea; RPMI 1630, Roswell Park Memorial Institute Medium 1630; and saline–EDTA, 75 mM NaCl–24 mM EDTA, pH 7.4.

MATERIALS AND METHODS

Materials. BCNU (NSC 409962), CCNU (NSC 79037) and Chlorozotocin (NSC 178248) were obtained from the Drug Procurement Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD. [Methyl-^{14}C]-thymidine (50 mCi/m-mole) was purchased from New England Nuclear, Boston, MA. Chloroethyl isocyanate and cyclohexyl isocyanate were from Eastman Organic Chemicals, Rochester, NY. Tissue culture materials were purchased from Grand Island Biologicals Co., Grand Island, NY.

Determination of isocyanate half-life. Isocyanate half-life was determined by the method of Brown and Wold [7], which measures the u.v. absorbance of the *N*-alkyl-*N'*-benzylurea formed by the reaction of the alkyl isocyanate with benzylamine in chloroform. Two modifications to the method were made. First, redistilled benzylamine, dissolved in chloroform, was extracted into 0.1 N HCl, the acid solution was made alkaline with sodium hydroxide and the benzylamine was extracted back into chloroform. This procedure reduced the background u.v. absorbance in the method by freeing the benzylamine of u.v. absorbing material not extractable from chloroform into dilute acid. In the second modification, anhydrous acetonitrile was substituted for anhydrous acetone as the solvent for the isocyanates.

Exposure of cells to nitrosoureas and isocyanates. L1210 cells were grown in RPMI 1630 medium supplemented with 10% fetal calf serum, penicillin (110 units/ml) and streptomycin (100 μg-ml). The doubling time was 12 hr. HeLa S3 cells were maintained as monolayers in Basal Medium (Eagle) supplemented with 10% fetal calf serum, penicillin (110 units/ml) and streptomycin (100 μg/ml) in an atmosphere of 5% CO_2. Cells were grown in the presence of [methyl-^{14}C]-thymidine (0.1 μCi/ml, 2×10^{-6} M) for two to three generations, washed free of residual isotope and incubated in isotope-free medium for 3 hr before use.

Nitrosoureas and isocyanates in anhydrous acetone were added to the cultures with the final acetone concentration never exceeding 0.5% (v/v). Control cultures received acetone alone. An approximately constant concentration of isocyanate in the cultures was achieved by the addition of 1 μl of isocyanate solution in anhydrous acetone each minute through a PE 10 cannula from a repeating dispenser with a 50-μl syringe (Hamilton Co., Reno, NV) while the cultures were shaking (100 oscillations/min) at 37°. The concentration of the isocyanate solutions used was calculated by assigning a half-life of 60 min to BCNU and CCNU at 37° and half-lives of 17 and 183 sec for chloroethyl and cyclohexyl isocyanate respectively. Exposure to chemical agents was terminated by washing the cells in cold medium. Cultures were irradiated in cold medium (0–4°) by a ^{60}Co gamma source at a dose rate of 200 rad/min. For the determination of immediate radiation damage, L1210 cultures were diluted with 9 vol. of cold saline–EDTA, while HeLa monolayers were washed with cold saline–EDTA. Recovery from irradiation damage was determined in cells maintained in medium and incubated at 37° for 60 min after irradiation. At the end of the repair period, the cultures were washed with cold saline–EDTA. Cultures were kept on ice until the cells were harvested. HeLa monolayers were detached in 0.25% trypsin at 4° for 15 min, and cells were suspended in saline–EDTA at 0° at a cell density of 1×10^6/ml.

DNA strand separation in alkali. A detailed description of the application of the method of Rydberg [8] is described elsewhere [9].

Cell viability studies. L1210 cells in log phase (0.5×10^6 cells/ml) were exposed to BCNU, CCNU, chloroethyl isocyanate and cyclohexyl isocyanate dissolved in anhydrous acetone. Chlorozotocin was dissolved in 1 mM sodium acetate buffer, pH 5.0. Appropriate vehicle-treated cultures were assayed. The dilute agar colony method of Chu and Fischer [10] was used. The final composition of the growth medium was 0.1% agar, 20% fetal calf serum in RPMI 1630 with penicillin (110 units/ml), and streptomycin (100 μg/ml). Colonies were enumerated after 10–14 days of growth at 37°. The cloning efficiency of vehicle-treated L1210 was 90–95 per cent.

RESULTS

Half-life of chloroethyl and cyclohexyl isocyanate. The breakdown of chloroethyl and cyclohexyl isocyanate in aqueous media at 37° followed first-order kinetics (Fig. 1). Chloroethyl isocyanate showed a short half-life (17 sec), being the same in either 0.08 M Tris–HCl buffer, pH 7.7, or RPMI 1630 with 10% fetal calf serum. The half-life of cyclohexyl isocyanate was much longer and increased in RPMI 1630 with 10% fetal calf serum (183 sec) compared to its half-life (115 sec) in 0.08 Tris–HCl buffer, pH 7.7.

Calculation of isocyanate concentrations during incubation of nitrosoureas. The half-lives of BCNU and CCNU in RPMI 1630 with 10% fetal calf serum were found to be 53 and 64 min respectively (unpublished observations). Published half-lives for

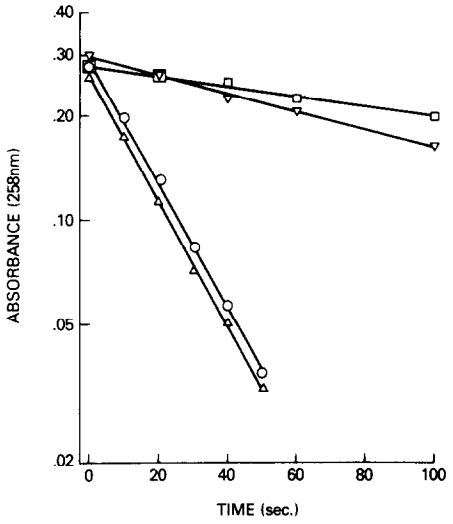

Fig. 1. Rate of decomposition of chloroethyl and cyclohexyl isocyanates. Key: chloroethyl isocyanate in 0.07 M Tris–HCl buffer, pH 7.7 (△), and complete RPMI 1630 medium (○); cyclohexyl isocyanate in 0.07 M Tris–HCl buffer, pH 7.7 (▽), and complete RPMI 1630 medium (□).

the nitrosoureas at 37° in similar media are 53 min for CCNU [2] and range from 43 to 76 min for BCNU [11, 12]. On the basis of this information, the half-lives of BCNU and CCNU were nominally taken to be 60 min at 37° in RPMI 1630 with 10% fetal calf serum. The sequence of reactions—breakdown of nitrosourea to form an isocyanate and the hydrolysis of the isocyanate—can be treated as a consecutive reaction. The concentration of isocyanate at time t is given by: $k_1/k_2 - k_1 \cdot (A_0) \cdot (e^{-k_1 t} - e^{k_2 t})$ where k_1 and k_2 are the rate constants of the breakdown of nitrosourea and isocyanate, respectively, and A_0 is the initial concentration of the nitrosourea. Figure 2 depicts the calculated isocyanate concentration present during the breakdown of BCNU and CCNU (initial concentration 50 μM) in RPMI 1630 with 10% fetal calf serum at 37°. Chloroethyl isocyanate derived from 50 μM BCNU is present at an approximately constant level of about 0.2 μM over the first hour, while cyclohexyl isocyanate released from 50 μM CCNU requires about 10 min to reach

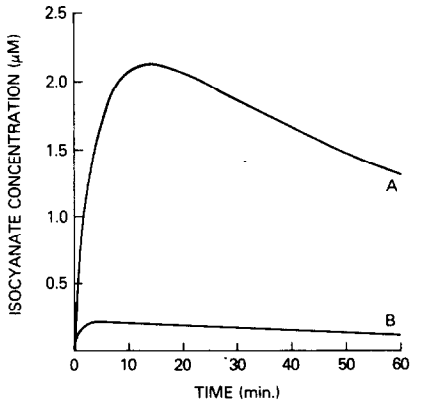

Fig. 2. Time course of the concentration of cyclohexyl and chloroethyl isocyanates during the breakdown of 50 μM CCNU (A) and 50 μm BCNU (B) in complete RPMI 1630 medium at 37°.

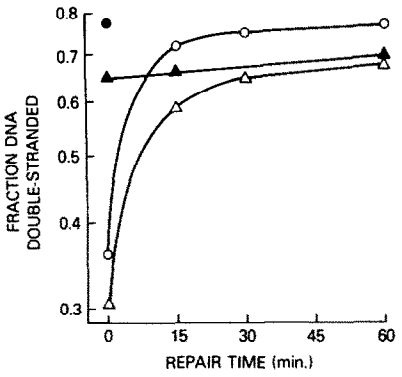

Fig. 3. Time course of repair of gamma-irradiation damage in L1210 cells as measured by DNA strand separation in alkali. Cells were exposed to 0.5% (v/v) acetone (circles) or 50 μM BCNU (triangles) for 60 min and received either no irradiation (closed symbols) or 300 rad irradiation (open symbols).

a peak of approximately 2 μM and then declines linearly to 1.5 μM at 60 min. In exposing cell cultures to the isocyanates at levels corresponding to an initial concentration of nitrosourea of 50 μM, we have chosen 0.2 μM to represent the level of chloroethyl isocyanate and 2 μM to represent the level of cyclohexyl isocyanate.

Repair of gamma-irradiation damage in the presence of nitrosoureas. We have been unable to detect any effect of exposure of L1210 or HeLa cells to nitrosoureas on the rate or extent of repair of gamma-irradiation damage.

The method of Rydberg [8] measures the fraction of DNA which becomes completely strand separated in alkali such that, on neutralization, no renaturation occurs. Figure 3 shows data from a typical experiment which measures the rate and extent of repair of gamma-irradiation damage. L1210 cells exposed to 50 μM BCNU were washed and either received 300 rad irradiation or were nonirradiated. After the irradiation the cultures were incubated at 37° and were analyzed at various times for the fraction of DNA irreparably strand separated in alkali. At the end of the initial treatment period, the fraction of renaturable DNA of acetone-treated cells was 0.78, whereas that of the BCNU-treated cells was 0.65, indicating that this method detects a small number of single-strand breaks induced by BCNU treatment alone. Exposure of the cultures to 300 rad irradiation produces a large decrease in the fraction of renaturable DNA, with the effect of BCNU plus irradiation being approximately additive. Incubation of the irradiated cultures at 37° leads to a rapid increase in the fraction of renaturable DNA in both vehicle-treated and BCNU-treated cultures. The time courses are very similar and the final fraction of renaturable DNA approaches that found in cultures that were not irradiated but went through a period of repair incubation. Essentially similar results were obtained when this type of experiment was repeated with 50 μM CCNU with L1210 cells or 100 μM BCNU or CCNU with HeLa cells.

The possibility that gamma-irradiation repair might be affected by the presence of nitrosourea during the irradiation or during the repair period was investigated. The data depicted in Fig. 4 are from a series of experiments with BCNU (50 μM) and L1210 cells. In panel A, the cells were exposed prior to irradiation; in panel B, there was continued exposure, from an initial pretreatment, irradiation and the 60-min repair period; in panel C, the cultures were made 50 μM in BCNU while at 0° immediately after irradiation, and then received 60 min incubation at 37°. There is no evidence that the period during which cells are exposed to BCNU has any influence on the extent of gamma-irradiation repair: the fraction of renaturable DNA in irradiated cells almost returns to the value found in nonirradiated cells during the 60-min repair period. The data in Fig. 4 indicate that doubling the period of exposure to BCNU (before and after irradiation) causes a larger decrease in the fraction of renaturable DNA in treated cells. A similar experiment with HeLa cells exposed to 100 μM BCNU also failed to indicate any effect of the timing of nitrosourea exposure on the repair of gamma-irradiation damage.

Repair of gamma-irradiation damage in the presence of isocyanates. The experimental approach was similar to that used in studying the effect of the nitrosoureas except that an approximately constant isocyanate concentration was maintained by multiple additions of the isocyanate to the cultures over a 50-min period at 37°. In Fig. 5, we show the effect of exposure of L1210 cells to cyclohexyl isocyanate at two levels: 2 μM, which corresponds to an initial CCNU concentration of 50 μM, and 4 μM, corres-

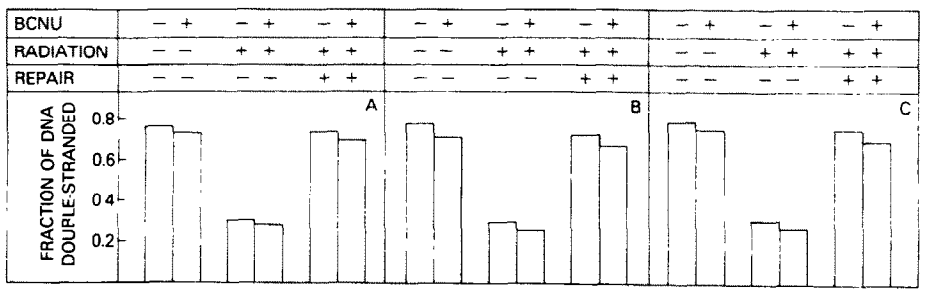

Fig. 4. Effect of exposure of L1210 cells to BCNU (50 μM) on the repair of gamma-irradiation damage as measured by DNA strand separation in alkali. Panel A: cells were pretreated for 60 min at 37° before irradiation (300 rad). Panel B: cells were exposed to BCNU throughout pretreatment, irradiation and a 60-min repair period at 37°. Panel C: cells were exposed to BCNU during a 60-min repair period.

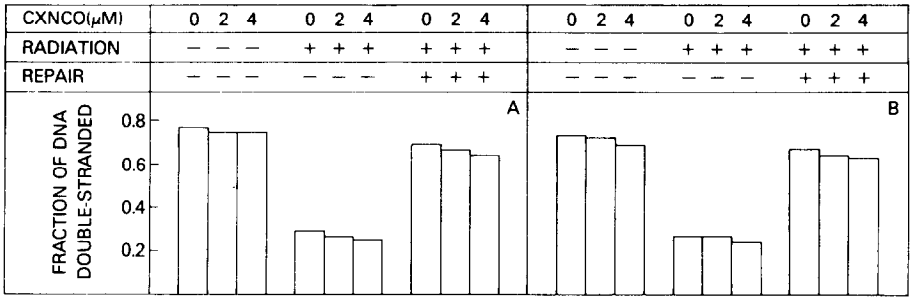

Fig. 5. Effect of exposure of L1210 cells to constant cyclohexyl isocyanate concentrations (CXNCO) on the repair of gamma-irradiation damage (300 rad) as measured by DNA strand separation in alkali. Cells exposed to acetone (0.5%, v/v), 2 μM or 4 μM cyclohexyl isocyanate before (A) or after irradiation (B) were allowed to repair during 60 min at 37°.

ponding to 100 μM CCNU. Figure 5 (panel A) shows that prior exposure to cyclohexyl isocyanate for 50 min has no detectable effect on the repair of gamma-irradiation damage. The fraction of renaturable DNA, which falls immediately after gamma irradiation, returns to about 90 per cent of control values during the 50 min at 0°. Similarly, in Fig. 5 (panel B), exposure to cyclohexyl isocyanate (2 or 4 μM) during the repair period is without effect on the relative extent of repair.

Viability of L1210 cells exposed to nitrosoureas and isocyanates. L1210 cells were exposed to the nitrosoureas and isocyanates in a manner identical to that employed in the repair studies. The concentration scales on the abscissa of Fig. 6 have been constructed to allow direct comparison of the lethality of the nitrosourea and its derived isocyanate. Thus, exposure of cells to a continuous chloroethyl isocyanate concentration of 0.2 μM is the anticipated result of an initial exposure to 50 μM BCNU. Similarly, continuous exposure to 2 μM cyclohexyl isocyanate relates to an initial CCNU concentration of 50 μM.

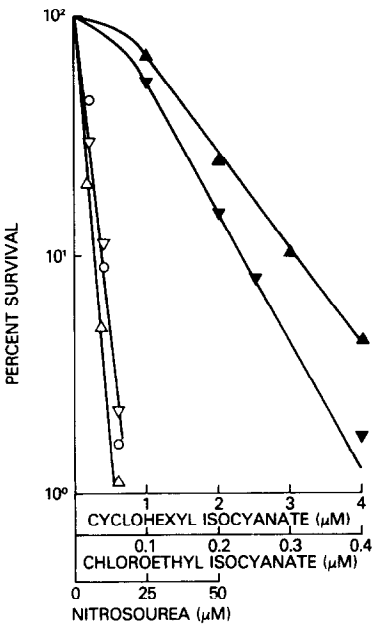

Fig. 6. Survival (clonegenicity) of L1210 cells following a 50-min exposure to nitrosoureas and isocyanates. Nitrosourea concentrations are initial concentrations. Isocyanate concentrations are those maintained approximately constant during the exposure period. Key: BCNU (△), CCNU (▽), Chlorozotocin (○), chloroethyl isocyanate (▲), and cyclohexyl isocyanate (▼).

The three nitrosoureas, BCNU, CCNU and chlorozotocin, are similar in their reduction of clonegenicity; a two-log kill being effected by a 1 hr exposure to about 15 μM nitrosourea. The isocyanates, when added in small increments and calculated to relate to a similar range of nitrosourea exposures, were relatively noncytotoxic; isocyanate exposures equivalent to approximately 100–150 μM nitrosourea were required to effect a two-log kill.

DISCUSSION

In an experimental approach to assess the role of the nitrosourea-derived isocyanates, it is clearly important to determine the isocyanate concentration present during nitrosourea breakdown. In determining the half-lives of chloroethyl and cyclohexyl isocyanate, we departed from the procedure of Brown and Wold [7] in substituting acetonitrile for acetone as the solvent for the isocyanates. We observed anomalous decomposition rates in the presence of acetone which we attribute to the formation of an imine through condensation between benzylamine and acetone. The two isocyanates, chloroethyl and cyclohexyl isocyanate, have widely different half-lives in aqueous media and also differ in respect to the effect of the presence of protein. The long half-life of cyclohexyl isocyanate is probably related to the hydrophobic nature of the cyclohexyl ring, while its increased half-life in media containing protein may reflect extra protection from aqueous decomposition through protein binding. In view of its long half-life, appropriate methodology should allow the detection of cyclohexyl isocyanate in tissues and body fluids.

By treating the breakdown of the nitrosoureas and their isocyanates as consecutive reactions, the isocyanate concentrations have been deduced. We acknowledge that assigning half-lives of 60 min to BCNU and CCNU breakdown may introduce a small error into the calculation of the isocyanate concentrations; over the half-life range of 50 to 70 min for the nitrosoureas, one encounters a ±10 per cent difference in the calculated isocyanate concentration. Figure 2 shows the calculated isocyanate

concentration derived from an initial nitrosourea concentration of 50 μM during 60 min at 37° in RPMI 1630 with 10% fetal calf serum. In attempting to duplicate these isocyanate concentrations, we were unable to develop a reliable continuous infusion that would have been preferable to the intermittent method of addition to which we resorted.

Our search for an effect of the nitrosoureas and their isocyanates on the rate or extent of repair of gamma-irradiation-induced DNA damage has involved the use of two cell lines with dissimilar sensitivities to the nitrosoureas with exposure at two concentrations before, after, and during gamma irradiation. The data we have presented are a representative portion of our observations which, in total, show no effects of either the nitrosoureas or continuous exposure to their related isocyanates on repair of DNA damage.

The viability studies in L1210 cells reveal a large difference between the cytotoxicity of the parent nitrosoureas and their isocyanates. The concentration scale factors in Fig. 6 allow a direct comparison of the effect of the nitrosoureas and isocyanates. Thus, exposure of cells to an initial BCNU concentration of 50 μM is approximately equivalent to a continuous exposure of the cells to 0.2 μM chloroethyl isocyanate. Exposure of cells to a nitrosourea concentration of 15 μM for 50 min reduces clonability 100-fold, while the equivalent continuous exposure to the corresponding isocyanate concentration (approximately 0.05 μM chlorethyl isocyanate or approximately 0.5 μM cyclohexyl isocyanate) is without effect. The greater cytotoxicity of BCNU compared to CCNU, and of 2-chloroethyl isocyanate compared to cyclohexyl isocyanate, may be related to the generation of the potential alkylating agent 2-chloroethylamine during the breakdown of BCNU and 2-chloroethyl isocyanate. Chlorozotocin, which was included in the viability studies as an example of a nitrosourea that produces very small amounts of isocyanate, was indistinguishable from CCNU in its cytotoxicity in L1210 cells.

Although the literature contains a number of observations which indicate that the isocyanates may contribute to the overall effect of the parent nitrosoureas, the role of the isocyanates has been treated largely as a speculative issue. The ability of the nitrosourea-derived isocyanates to carbamylate proteins [13, 14] and the observations of Brown and Wold [7] on the active-site-specific reaction of alkyl isocyanates on serine proteases encouraged the idea that the nitrosourea-derived isocyanates could similarly have some specific point of action within the cell. The possibility that this site of action could involve proteins related to nucleic acid metabolism gained credence from early observations of Wheeler and Bowdon [4, 15] and from data showing that treatment of L1210 cells with chloroethyl isocyanate inhibited the maturation process of pre-ribosomal RNA [5] and inhibited the repair of X-irradiation-induced DNA damage in L1210 cells [4]. These experiments to determine the effect of chloroethyl isocyanate at the molecular level have involved exposure of isolated enzyme systems or cells in tissue culture to isocyanate concentrations equal to biologically relevant concentrations of the parent nitrosourea. In view of the very short half-life of alkyl isocyanates (especially chloroethyl isocyanate), these exposures bear little resemblance to the isocyanate exposure that is encountered during the breakdown of the nitrosourea.

Further interest in the isocyanates has evolved from the rational design of chlorozotocin [16], a nitrosourea which combines the therapeutic chloroethyl component of the haloethyl nitrosoureas with the 2-deoxy D-glucose substituent of streptozotocin. Chemical breakdown of chlorozotocin releases an isocyanate which probably undergoes preferential internal carbamylation and is unavailable to enter into carbamylation reactions with cellular proteins. It has been proposed that the decreased marrow toxicity of chlorozotocin in mice is related to its very low carbamylating activity [17].

The significance of our present results hinges on the relevance of the continuous exposure of cells in tissue culture to concentrations of isocyanates generated during the decomposition of the nitrosoureas. We believe that this approach, while more relevant than a single large addition of isocyanate, can probably be further refined through our development of methods to determine intracellular isocyanate levels in cells exposed to nitrosoureas.

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