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# BIOCHEMICAL AND CYTOTOXIC PROPERTIES OF THE ISOMERIC FORMS OF *N*,*N*′-BIS[*N*-2-CHLOROETHYL)-*N*-NITROSOCARBAMOYL] CYSTAMINE

Jean Jacques Farhi, Myriam Bennoun, Haim Tapiero, Ann L. Wang* and Kenneth D. Tew*†‡

Departement de Pharmacologie Cellulaire et Moleculaire, et de Pharmacocinetique, Institut de Cancerologie et d'Immunogenetique (INSERM U-50), Hopital Paul-Brousse, 94804-Villejuif, France

*Division of Medical Oncology and †Department of Biochemistry, Lombardi Cancer Research Center, Georgetown University Hospital, Washington, DC 20007, U.S.A.

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**Abstract**—Three isomeric forms of a cystamine-containing chloroethylnitrosourea, *N*,*N*′-bis[*N*-(2-chloroethyl)-*N*-nitrosocarbamoyl]cystamine (CNCC), have been identified and separated by high pressure liquid chromatography. Isomer S, 3,3′-bis[*N*-(2-chloroethyl)-*N*-nitrosocarbamoyl] ethyl disulfide, was significantly less cytotoxic than isomer C, 1,1′-bis [*N*-(2-chloroethyl)-*N*-nitrosocarbamoyl] ethyl disulfide, or isomer M, 1,3′-bis[*N*-(2-chloroethyl)-*N*-nitrosocarbamoyl] ethyl disulfide, in either a human Namalva lymphoblastoid or a rat Walker 256 carcinoma cell line. Both isomers S and C inhibited DNA synthesis at a 50 $\mu$M concentration. A structural analysis of the isomeric forms suggested that bioreduction of the disulfide bond would permit both isomers to produce isocyanate moieties which would carbamoylate intracellular proteins and depress nucleic acid synthesis. The reduced cytotoxic potential of isomer S is consistent with a prolongation in the half-life of production of alkylating carbonium species that lack the capacity to cross-link macromolecules. Overall, the relative position of the NH group within each of the nitrosourea isomers appears critical to the biological properties of the drug.

Among the alkylating agents used in anticancer chemotherapy, the chloroethylnitrosoureas have a broad spectrum of activity in human tumors, mainly against lymphomas, melanomas, gliomas and cancer of the gastrointestinal tract [1–3]. Therapeutic efficacy is dependent upon spontaneous decomposition at neutral pH to produce electrophilic alkylating (carbonium ion) and carbamoylating (isocyanate) species [4]. These intermediates have the potential to interact *in vivo* with cellular nucleophiles including nucleic acids and a wide variety of proteins [5, 6]. Because of the diversity of possible drug lesions, the precise mechanism by which nitrosoureas induce cytotoxicity has not been determined; however, the covalent modification of nuclear macromolecules with a resulting inhibition of nucleic acid synthesis has been considered critical [5]. Aspects of drug structure which influence uptake or metabolism will be important in determining potential activity.

A new nitrosourea, *N*,*N*′-bis[*N*-(2-chloroethyl)-*N*-nitrosocarbamoyl] cystamine (CNCC) (Fig. 1), was synthesized recently by Imbach *et al.* [7]. According to the positions of the two nitroso groups, three isomers could be separated when the drug was subjected to analysis by high pressure liquid chromatography (HPLC) (Fig. 2). Preliminary studies with the mixed isomers of CNCC (see Fig. 1) show that the drug has a high oncostatic activity against mouse solid tumors and against intracerebrally grafted L1210 cells [7]. Moreover murine toxicity varied with the isomer (Roger Bellon Laboratories, Paris, France, unpublished data). In the present study, we report the relationship between the biochemical properties of CNCC and their effects on cell growth and macromolecular synthesis.

## MATERIALS AND METHODS

*Cell cultures.* Namalva human lymphoblastoid B cells were grown in RPMI 1640 with 10% fetal calf serum (GIBCO, Grand Island, NY) and antibiotics. Murine erythroleukemia cells (MEL) were derived from a clone of Friend virus transformed 745A cells. MEL were grown in modified Eagle's spinner medium without calcium, containing 10 mM sodium phosphate, nonessential amino acids, and 10% fetal calf serum (Gibco Bio-Cult, Ltd., Paisley, Scotland) [8]. Walker 256 mammary carcinoma cells were maintained in Dulbecco's Minimum Essential Medium (MEM) with glucose (4.5 g/l) supplemented with 10% horse serum (MA Bioproducts, Walkerville, MD). Resistant (WR) cells had been developed through selective exposure to chlorambucil [9]. This resistance is maintained biannually by exposure to chlorambucil (20 $\mu$g/ml). All cell cultures were maintained in static suspension under 5% $CO_2$.

*Viability determinations.* Viability was estimated by trypan blue exclusion. For the Walker 256 cells, colony-forming ability was measured in 0.3% agar as previously described [10].

*Chemicals.* All chemicals were of analytical grade.

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‡ Address all correspondence to: Kenneth D. Tew, Ph.D., Division of Medical Oncology, Lombardi Cancer Research Center, 3800 Reservoir Rd., N.W., Washington, DC 20007.

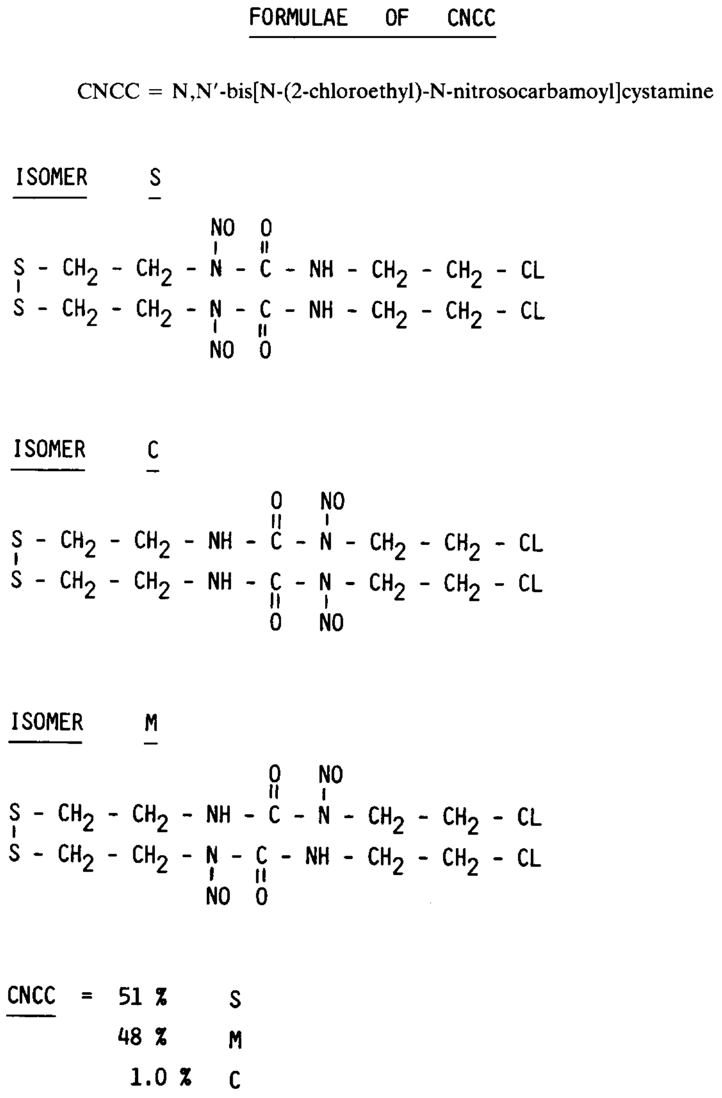

Fig. 1. Structures of the isomeric forms of CNCC.

The isomeric forms of CNCC were provided by Dr. J. L. Imbach and Roger Bellon Laboratories, Paris, France. These drugs were dissolved in either dimethyl sulfoxide (DMSO) or ethanol. Prior to each experiment, drug purity and concentration were monitored by HPLC.

*Incorporation studies.* Log phase Namalva cells were harvested by centrifugation and resuspended to a final concentration of 4 × $10^5$ cells/ml in RPMI 1640 containing 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) to adjust the pH to 7.5. Drug was added to the samples and incubated at 37° for various time periods. Cells were recentrifuged and resuspended in a prewarmed Hanks' balanced salt solution containing 2 μCi/ml [$^3$H]-thymidine (40–60 Ci/mmole), 2 μCi/ml [$^3$H]-uridine (40 Ci/mmole) or 0.5 μCi/ml [U-$^{14}$C]protein hydrolysate (50 mCi/milliatom) (Amersham, Buckinghamshire, England). After 20 min, 1 vol. of 10% cold trichloroacetic acid (TCA) was added to each sample. After 5 min on ice, the acid precipitable material was collected by filtration on Whatman glass fiber filters GF/B. Filters were rinsed with 20 ml TCA and 5 ml of ethanol and dried, and the radioactivity was counted in a liquid scintillation counter. Each sample was compared to a corresponding control without drug.

*HPLC analysis.* CNCC isomers were diluted in the mobile phase: iso-octane–dichloromethane–methanol (79.8:18.7:1.5, by vol.) and eluted on a Radial-pak B liquid chromatography cartridge, 10 μm (Waters Associates, Inc., Milford, MA), using a RCM100 radial compression module, a WISP injector 710B, a dual model 6000A pump, a M720 system controller, and a M730 data module (Waters). Drugs were detected at 245 nm with a SF770 spectroflow monitor (Schoeffel Instruments, Kratos Inc., NJ).

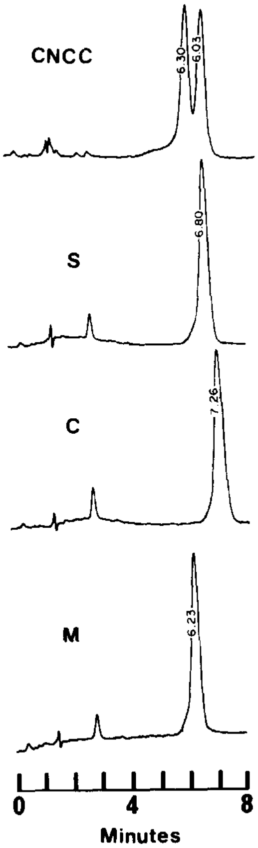

Fig. 2. Elution profiles of CNCC and its isomers by high pressure liquid chromatography. Aliquots of 1 $\mu$g dissolved in the mobile phase were injected in the HPLC column. Ultraviolet absorption was monitored as described in Materials and Methods.

## RESULTS

The isomeric forms of CNCC were separated by HPLC, yielding retention times ($R_t$) that permitted separation of the two major isomers S ($R_t$ 6.80 min) and M ($R_t$ 6.23 min). Thus CNCC, composed of 51% S and 48% M, gave two distinct peaks. Isomer C ($R_t$ 7.26 min) was also separable as a single isomer but did not give an additional peak when CNCC was separated, presumably because of the low levels present in the mixture (1%).

Comparative cytoxicities of CNCC and its three isomers were explored in a number of cell systems. Figure 3 shows drug effects on Walker 256 rat carcinoma cells which are characterized as sensitive (WS) or resistant (WR) to nitrogen mustards, but lack collateral resistance to nitrosoureas [10]. Isomers C and M produced similar suppressions in cell survival with IC$_{50}$ values of approximately 5 $\mu$M. Isomer S was less cytotoxic with IC$_{50}$ values of greater than 50 $\mu$M for each cell line. CNCC toxicity was found to be between these values. The patterns of cytotoxicity were the same in WS and WR showing that the nitrosoureas were effective in circumventing the mustard resistance properties of WR cells. Further cytotoxicity studies using Namalva cells were designed to compare isomers S and C with CNCC. Inhibition of cell growth (Fig. 4) showed that isomer S was less cytotoxic than C and that CNCC was a composite of the two.

Although the mechanism of action of these compounds is still unknown, we have assumed that the first step may be related to the ease with which they were incorporated into the cells. To support this assumption, cells were exposed to 50 $\mu$M drug for different times, after which drugs were removed and cells were allowed to grow for 72 hr. Under these

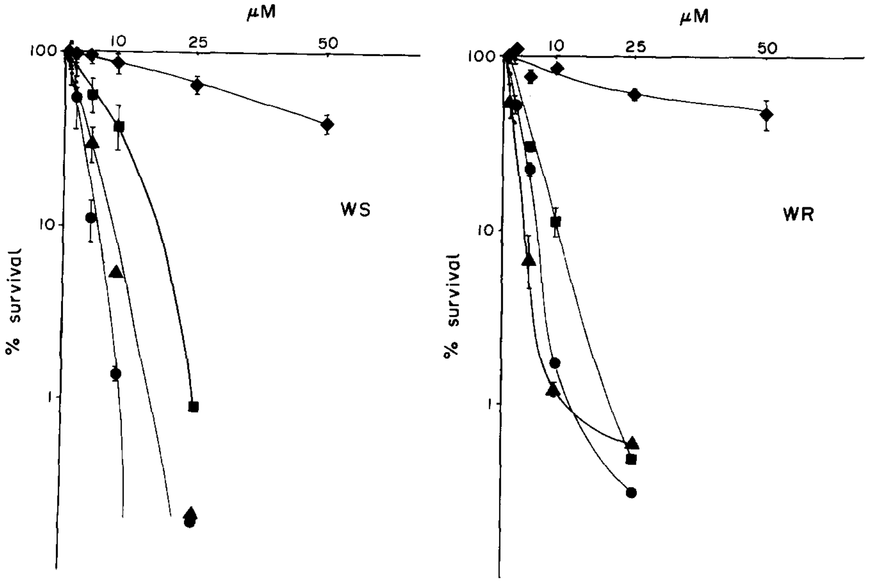

Fig. 3. Cytotoxicity of CNCC and its isomers in Walker 256 rat carcinoma cells as judged by a soft agar colony-forming assay. Wild-type Walker sensitive cells (WS) or Walker resistant cells (WR) were exposed to micromolar concentrations (abscissa) of CNCC (■——■) or its isomers C (●——●), S (◆——◆) or M (▲——▲) for 2 hr, and the drug was diluted out of the medium prior to plating. Log survival (ordinate) was expressed as percentage of control.

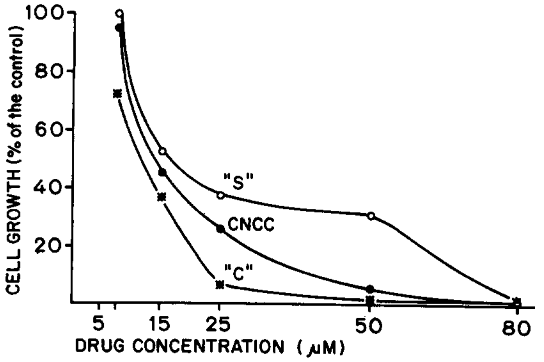

Fig. 4. Cytotoxic effect of CNCC and its isomers. Human lymphoblastoid cells (Namalva) seeded at 4 × $10^5$/ml were grown in medium supplemented with various concentrations of CNCC (●——●), S (○——○) and C (*——*) isomers (described in Fig. 1). Counts of trypan-blue-excluding cells were performed after 72 hr in triplicate and compared to control culture. All counts were expressed as a percentage of control cell growth.

conditions, growth inhibition was related to the time of cell exposure; however, this effect was obtained after a shorter time with isomer C than with isomer S. After the 2-hr exposure, cell growth was highly inhibited with both isomers (Fig. 5). If the cytotoxic activity were related to the inhibition of macromolecular synthesis, one would expect to find a higher inhibition with the C rather than with the S isomer. The incorporation of appropriate precursors into DNA, RNA and protein of intact cells was unexpectedly lowered when cells were exposed to the S rather than to the C isomer (Fig. 6). In the presence of C but not of S, protein synthesis was inhibited for the first 20 min but recovered thereafter (Fig. 6). According to these results, we assumed that other variables such as hydrolysis of intact drug to inactive metabolites were involved in drug efficacy. To test this assumption, the drug was incubated at 37° in the growth medium. Following the incubation time, cells were added to the medium and grown for 72 hr. Under these conditions, growth inhibition was inversely proportional to the time of incubation.

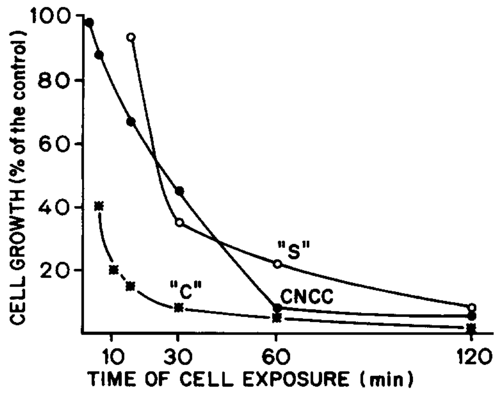

Fig. 5. Cytotoxic effect of CNCC and its isomers with respect to drug exposure time. See legend of Fig. 4 for experimental details.

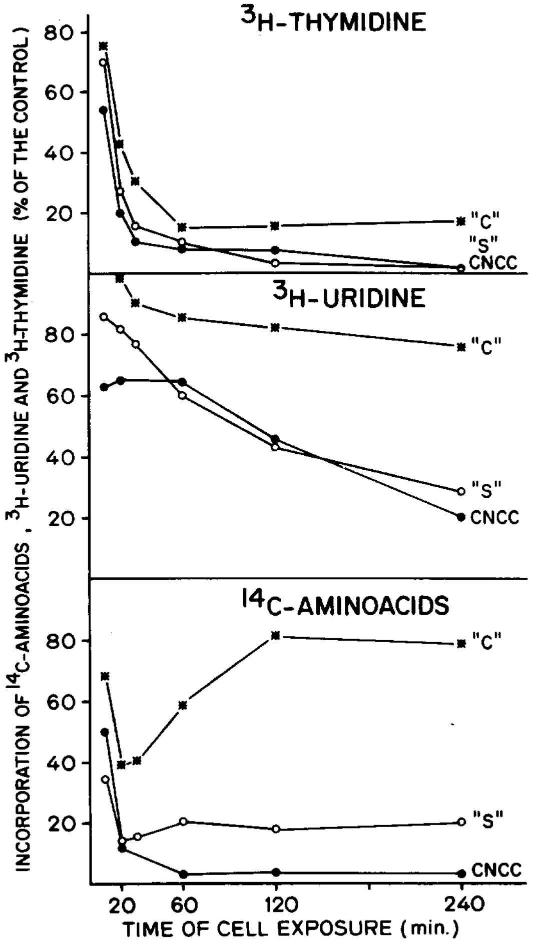

Fig. 6. Inhibitory effect of CNCC and of its isomers on DNA, RNA and protein synthesis. Exponentially growing Namalva cells (4 × $10^5$/ml) were exposed to 50 μM drug in growth medium at 37°. After various times of exposure, cells were pulse-labeled for 20 min with the appropriate precursor as described in Materials and Methods. Results are expressed as a percentage of incorporation in untreated cells.

When C and S isomers were incubated for 60 min, cell growth was inhibited to 50 and 15% respectively (Fig. 7).

Metabolism of nitrosoureas to alkylating and carbamoylating species has been shown to be critical to the activity of this class of drug [4]. To test the hypothesis that intracellular compartmentalization of CNCC in its unmetabolized form is critical to its activity, CNCC was incubated in growth medium for different time periods, after which cells were added to this medium and pulse-labeled with [$^3$H]-thymidine or [$^3$H]-uridine. The incorporation of these precursors into DNA and RNA varied with both the concentration and time of drug incubation (Fig. 8). DNA synthesis was reduced by 50% when 50 and 200 μM CNCC were incubated for 40 and 150 min respectively (Fig. 8A) whereas at the same concentrations, a 50% inhibition of RNA synthesis was achieved in 65 min and 110 min (Fig. 8B). This suggested that the cytotoxicity of CNCC was a composite

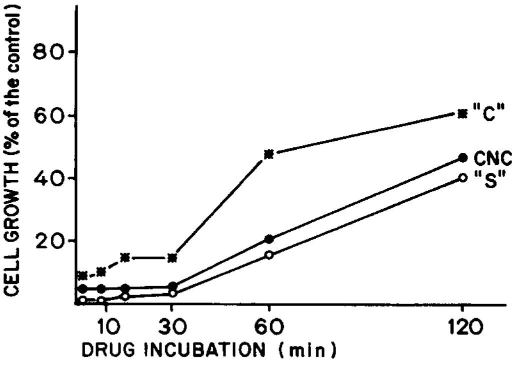

Fig. 7. Comparative cytotoxicity of hydrolyzed CNCC and isomers. Prior to cell exposure, 50 μM drug were incubated at 37° in growth medium. Following the incubation time, $4 \times 10^5$ cells/ml were seeded in this medium and allowed to grown for 72 hr at 37° in a $CO_2$ incubator. Cells were counted as previously described, and counts were expressed as a percentage of control cell growth.

of cellular drug uptake as the parent molecule and subsequent effects on nucleic acid synthesis.

## DISCUSSION

A number of amino acid derivatives of chloroethylnitrosoureas have been synthesised [11] with the aim of increasing the chance of selective uptake of the drug into target tissues. Synthesis of CNCC, a cystamine derivative of the basic nitrosourea structure [7], has produced three isomeric forms that differ in their biological effects. A consideration of the decomposition of each isomer provides some insight into these differential responses. The proposed general mechanism of spontaneous decomposition at physiological pH [4, 12] involves the formation of a chloroethyl carbonium moiety via hydride shift [4, 13] and proton abstraction from N-3 in the conventional nitrosourea structure.

In addition to this general mechanism of decomposition, isomers S, M and C can undergo a preliminary bioreductive step of the disulfide bond producing a thiol derivative [14]. This thiol derivative could interact intramolecularly with the carbamate carbonyl group to yield a thiazoline-like structural intermediate which could readily transfer a proton to give the 2-chlorodiazohydroxide with isomer C and half of isomer M and subsequently the chloroethyl carbonium moiety. With isomer S the possible mechanism of decomposition cannot produce the chloroethyl carbonium moiety via this route, and previous studies have shown that this may prolong the half-life of the drug [14].

Chlorine is capable of acting as a leaving group from the chloroethyl carbonium moiety, and this has been proposed as a mechanism by which this group can cross-link macromolecules [15]. Sulfur does not possess electrophilic properties like those of chlorine, and thus a possible thiol ethyl group, formed upon decomposition of isomer S, will not possess the bifunctionality of the chloroethyl carbonium ion formed from isomers C and M. Other studies using a lambda phage system have shown that isomer S does not cross-link DNA in either the presence or absence of reducing agents. Conversely, isomers C and M cross-link DNA under either normal or reducing conditions.* The fact that isomer S exhibits significant *in vitro* alkylating activity with respect to its interaction with 4-(*p*-nitrobenzyl) pyridine (1.45 times that of chlorozotocin*) would be consistent with the monofunctional nature of the proposed thiol ethyl decomposition product. In addition, the observed cytotoxic properties of isomer S in comparison with isomers C and M would be consistent with the concept that macromolecular cross-linkage of DNA is more toxic than monoadduct formation [16].

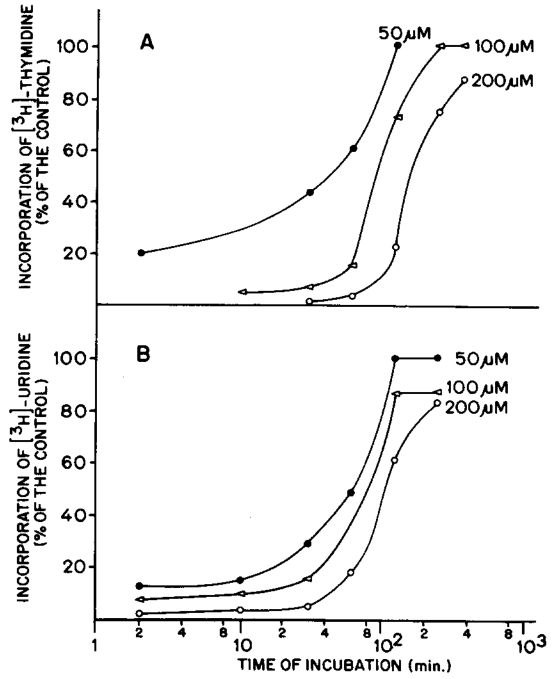

Fig. 8. Comparative effect of hydrolyzed CNCC on DNA and RNA synthesis. Various concentrations of CNCC were incubated at 37° in growth medium. Following the incubation time, $4 \times 10^5$ cells were resuspended in 1 ml of the above media containing either 2 μCi [$^3$H]-thymidine (A) or [$^3$H]-uridine (B). After a 20-min incubation at 37°, cells were washed with ice-cold Hanks' balanced salt solution and TCA precipitated. Results were expressed as a percentage of incorporation in untreated cells.

Inhibition of RNA and protein synthesis was less affected by isomer C than by isomer S and the mixed CNCC, although DNA synthesis was depressed to similar extents by all three. Although inhibition of nucleic acid synthesis was dose dependent with respect to CNCC, a direct correlation between macromolecular synthesis inhibition and cytotoxicity was not always apparent. Both isomers S and C produce isocyanate species which, for other nitrosoureas, have been shown to be capable of interacting covalently with a large number of intracellular proteins, including, but not restricted to, glutathione reductase [17], DNA ligase [6] and many histones

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* J. W. Lown, R. R. Koganty, K. D. Tew and J. L. Imbach, manuscript submitted for publication.

and non-histones [18, 19]. Other studies indicate that glutathione reductase is inhibited by treatment of Walker 256 carcinoma cells with either isomer S or C.* Thus, the similarity between the DNA synthesis inhibition patterns of isomers S and C may be a result of the carbamoylation of enzymes involved in the synthetic pathways. Such drug-induced lesions may be less critical to the cytotoxic effects of the drug than the carbonium ions, since the cells may be more capable of restoring enzyme activity by protein synthesis and turnover.

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