Title
Chemical and metabolic reactions of chloroethylnitrosoureas

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CHEMICAL AND METABOLIC REACTIONS OF CHLOROETHYLNITROSOUREAS

by

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DISSERTATION

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in the

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of the

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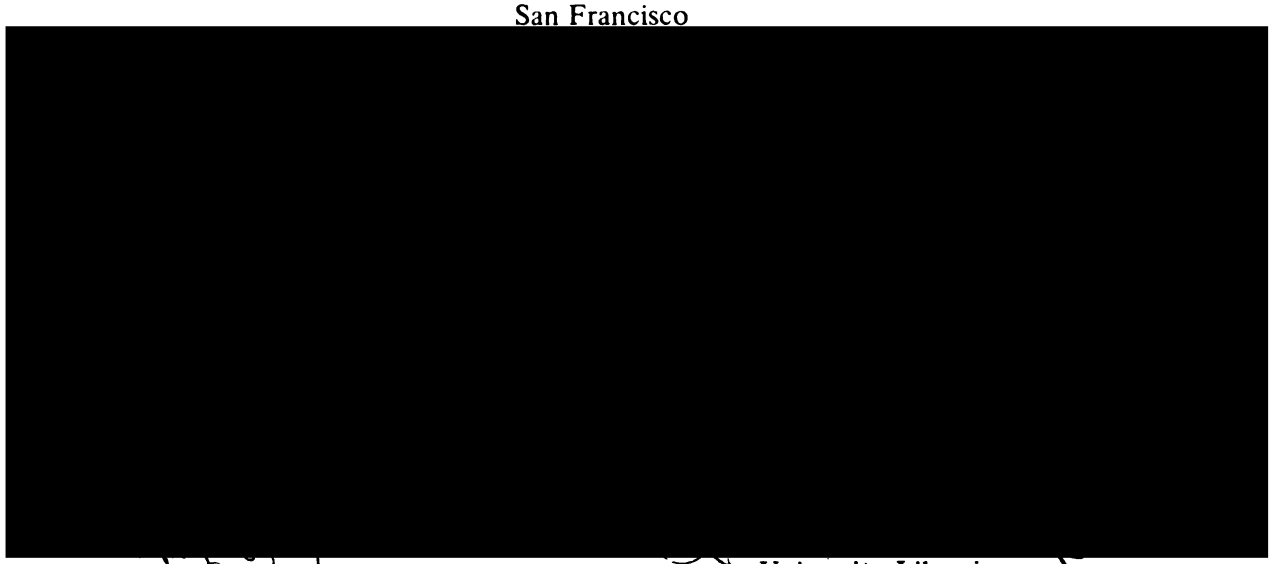
San Francisco



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Finally, I especially want to thank my parents for their love and encouragement.

Abstract

The chemical and enzymatic reactions of an antineoplastic agent BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) were investigated. The chemical degradation of BCNU in phosphate buffer at pH 7.4, 37°C was studied, the products were identified and quantitated, and the reaction sequence defined. This is the same reaction that leads to the generation of the alkylating species that is thought to be responsible for antitumor activity. Analytical techniques used in these studies involved gas chromatography, high performance liquid chromatography, and gas chromatography-mass spectrometry interfaced with a computer. This included the development of direct reaction mixture analytical techniques using chemical ionization mass spectrometry. A number of the chemical degradation products of BCNU including BCU (1,3-bis-(2-chloroethyl)urea), BHU (1,3-bis(2-hydroxyethyl)urea), CHU (1-(2-chloroethyl)-3-(2-hydroxyethyl)urea) and CAO (2-(2-chloroethylamino)-2-oxazoline) were synthesized and used to identify the chemical reaction products and as standard references in quantitative analysis.

Aqueous decomposition reactions were conducted using a mixture of BCNU and BCNU-d_8 labeled on the ethylene positions. The relative amounts of the deuterium present in the products indicate the reaction pathways leading to their formation. BCU was formed through a recombination mechanism which involved cleavage of BCNU molecule as the first step. CAO may be formed from BCNU directly and from the cyclization of BCU.

The half-life of chloroethylnitrosoureas was reduced in the presence of plasma protein. Albumin catalyzed the degradation of BCNU in a nonspecific manner through the formation of a BCNU-protein complex which reacts to give BCNU degradation products and albumin. The products of this reaction have also been identified and quantitated. The catalytic effect of albumin

is reversed by the presence of highly protein bond agents, such as salicylic acid. Studies with radioisotope labeled BCNU indicated high percentage of covalent binding of reaction products with albumin.

The in vitro metabolism of BCNU was also investigated. BCNU metabolism by 100,000 x g rat liver microsomes produced BCU as its major metabolite. The formation of this metabolite was quantitated by HPLC. The metabolic pathway of the formation of BCU is different from that in chemical degradation. It was elucidated by a mixture of octadeuterated BCNU in the incubation with microsomal enzymes. The results suggested a denitrosation mechanism from BCNU to the metabolite BCU.

The formation of BCU was not significantly affected by the presence of oxygen or nitrogen. Rat liver microsomes prepared from phenobarbital pretreated rats had a greater activity on the metabolic reaction of BCNU. The biotransformation of BCNU to the less active BCU is clearly of importance in terms of BCNU chemotherapeutic efficacy.

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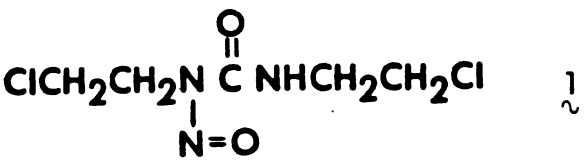
Chapter I. INTRODUCTION

Chloroethylnitrosoureas are used in cancer chemotherapy for the treatment of brain tumors[1-3] and in combination therapy for other malignant diseases[4]. The work discussed in this dissertation involves the chemical and metabolic reactions of chloroethylnitrosoureas with an emphasis on 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, 1), the most widely used member of this class. The goal of this research is to gain insight into the reactions of chloroethylnitroureas that occur in vivo. Chemical degradation in



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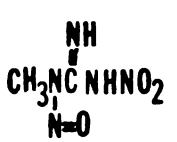
aqueous media, plasma protein mediated chemical reactions, and metabolic transformations have been studied and found to be important factors affecting the antitumor activity and pharmacokinetics of these agents. It is felt that an understanding of these factors will help in the design of chloroethylnitrosoureas with better cytotoxicity and improve the clinical use of similar drugs that are currently in use.

The history of this class of drugs is very long[5]; the National Cancer Institute (NCI) initiated drug development in the 1950's. The nitrosoureas are one of the first and most significant classes of anticancer agents that have evolved from this program. The first compound in this series was 1-methyl-3-nitro-1-nitrosoguanidine (MNNG, 2) which was synthesized in 1947 with no biological rationale in mind[6]. It was found to

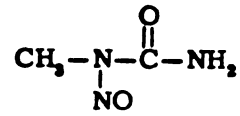


2

be somewhat active against murine leukemia L1210 during routine screening for therapeutic activity[7].

MNNG is used for generating diazomethane, a reagent in organic synthesis. Other compounds that act as progenitors of diazomethane were investigated.

Alkylnitrosoureas were found to be more active than alkynitrosoguanidines. Southern Research Institute developed 1-methyl-1-nitrosourea (MNU, 3) which was the first active compound of this class of drugs. Of



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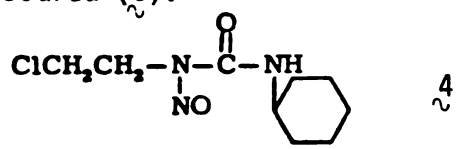
great interest at that time, was the fact that 1-methyl-1-nitrosourea was effective against L1210 leukemia when the tumor was implanted intracerebrally[8]. SRI received an NCI contract to synthesize and test other nitrosourea analogs and they found 2-Chloroethylnitrosoureas to have the highest anticancer activity. Most compounds of this class that are used clinically today are 1-(2-chloroethyl)-1-nitrosoureas. At present, there are three compounds of this class that are in non-investigational clinical use: i.e., BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea (1), CCNU (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (4), and MeCCNU (1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (5).



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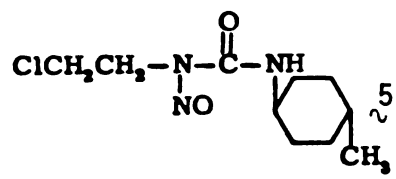

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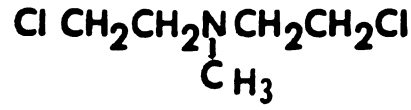
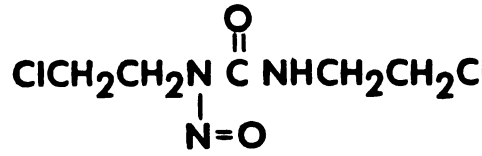
Chloroethylnitrosoureas are chemically unstable in aqueous solution[9] and thus in vivo. The parent compounds, which are not active, react in aqueous media to generate highly reactive species[10] that are capable of covalently binding with biological macromolecules[11]. The chloroethylnitrosoureas are classified as alkylating agents, although their mechanism of

action is not completely clear. A typical alkylating agent in cancer chemotherapy, such as a nitrogen mustard (6), has a bifunctional chloroethylamino moiety. There is some structural similarity between BCNU and nitrogen mustards, both have two $Cl\text{-}CH_2\text{-}CH_2\text{-}N$- groups, but the active species responsible for activity is totally different. The nitrogen



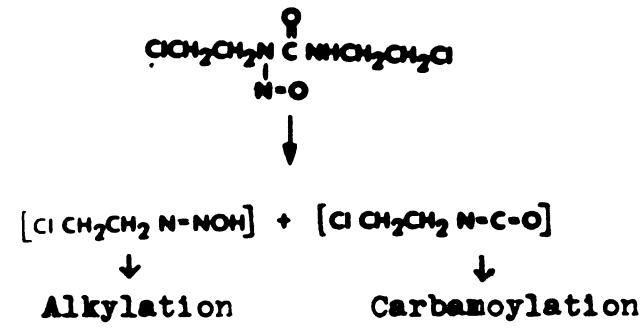
6 Nitrogen mustard



1 BCNU

mustards react by cyclization to an aziridine intermediate whereas this is not the case with nitrosoureas, since the nucleophilicity of the urea nitrogen is greatly decreased by the adjacent carbonyl group[12]. The chloroethylnitrosoureas decompose in aqueous solution into chloroethylazohydroxide (or chloroethyldiazonium ion) and an alkylisocyanate[13]; both of these species are the chemically reactive intermediates. Chloroethylazohydroxide

Scheme I



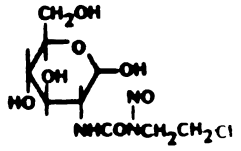
undergoes chloroethylation reaction and can alkylate nucleic acids and proteins, whereas alkylisocyanates react with free amino groups of protein amino acids to form ureas; this later reaction is referred to as carbamoylating activity[11,14]. After chloroethylazohydroxide reacts with an amino or a sulfhydryl group, it generates a 2-chloroethylamino or 2-chloroethylsulfide function, which can then cyclize to give aziridium or

episulfonium ion intermediates and react with another nucleophile. This is analogous to the bifunctional activity of nitrogen mustard.

BCNU is reported to form interstrand or intrastrand cross links in DNA by ethylene bridge formation [15-17]. Evidence for several mechanisms has been presented for the cytotoxicity of nitrosoureas: 1) inhibition of DNA synthesis by the inhibition of nucleotidyltransferase[18], 2) inhibition of DNA synthesis by the inhibition of DNA polymerase II[19], 3) regulation of ribosomal RNA synthesis and processing to inhibit protein synthesis and thus inhibit cell growth [4,20,21] DNA strand breaks[22,23] and cross-linkage [15-17], 5) inhibition of repair of DNA strand breaks[24-28]. It is not surprising to find that a wide range of biological effects are produced by these agents since the reactive intermediates formed from chloroethylnitrosoureas are highly reactive. The overall cytotoxicity could be a summation of all these effects, or one dominant interaction could account for cytotoxic activity, and work continues in this area to clarify this point. Another consequence of the high reactivity of chloroethylnitrosourea is the low selectivity with which these agents distinguish between normal and tumor cells. Nitrosoureas affect all rapidly dividing cells, and as a consequence, bone marrow toxicity is the major adverse effect and is dose-limiting of cancer chemotherapy[1,29].

One important feature of chloroethylnitrosoureas, such as BCNU, CCNU, MeCCNU, is their high lipophilicity[8]. This property enables these compounds to cross the blood brain barrier and kill the tumor cells present in the brain. BCNU and CCNU are widely used in clinical treatment and are highly active drugs. BCNU is the single most effective agent now being used in the treatment of CNS tumors[30]. A continuing effort is being made to develop new chloroethylnitrosoureas. Chlorozotocin, 2-(3-(2-chloroethyl)-3-nitrosoureido)-D-glucopyranose (7), is a chloroethyl analog of a naturally

occurring nitrosourea, streptozotocin. Chlorozotocin is active against murine L1210 leukemia and displays reduced bone marrow toxicity at 10% lethal dose[31]. Streptozotocin is an antibiotic derived from streptomyces acromogenes. It has been particularly useful in treating functional, malignant pancreatic islet-cell tumors[32], but was reported to be diabetogenic[33]. Chlorozotocin does not have this side effect. Other derivatives have been synthesized in an effort to reduce bone marrow toxicity such as GCNU, a tetraacetyl derivative of chlorozotocin (8). It has an increase in life span (ILS) greater than 100% at a LD_{10} dose without a leukopenia side effect[34]. Placement of cytotoxic group on C-1 of glucose gives another analog GANU, (1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea (9)[35]. It demonstrated minimum myelosuppression and had significant



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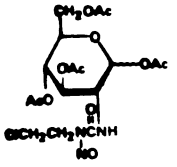

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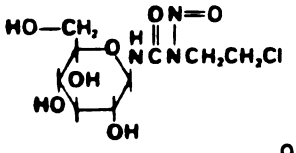

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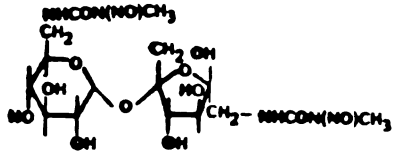
activity against L1210 in mice. Some sucrose derivatives have been synthesized based on a finding[36] that sucrose penetrates the cell membranes of tumor cells but not the normal brain cells. Only methylnitrosoureas have been made due to the inherent difficulties of the synthesis. The analogs 6,6'-dideoxy-6,6'-di(3-methyl-3-nitrosoureido)sucrose (10) and 1,6,6'-trideoxy - 1,6,6'-tri(3-methyl-3-nitrosoureido)sucrose (11) showed antitumor activity against both L1210 leukemia and ependymoblastoma brain tumor in mice[37]. A pyrimidine analog, ACNU (1-(4-amino-2-chloroethyl)-3-nitrosourea (12), is highly active against murine L1210 leukemia, but shows little reduction in bone marrow toxicity[38,39]. Presently this compound is undergoing clinical trial in Japan[40]. Some methylnitrosourea derivatives of



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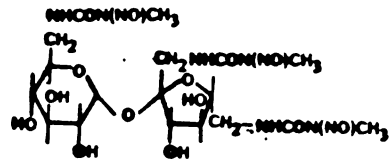

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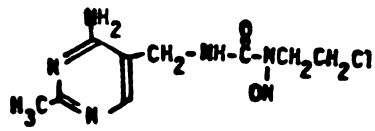

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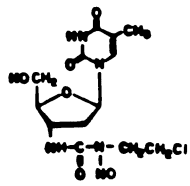
3'-amino and 5'-amino analogs of thymidine have been synthesized and also tested for antitumor activity. Two analogs, 3'-(3-(2-chloroethyl)-3-nitrosoureido)-3'-deoxythymidine (13) and 3'-(3-methyl-3-nitrosoureido)-3'-deoxythymidine (14)[41], inhibited L1210 cell growth in culture more effectively than BCNU.



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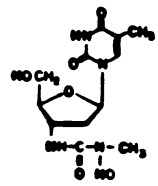

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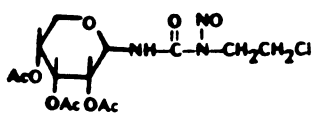
Ribose-containing nitrosoureas 15 and 16 displayed activity against Friend leukemia and L1210, respectively. Clinical evaluation of 16 has begun because of its superior therapeutic index (TI∿4)[42,43].



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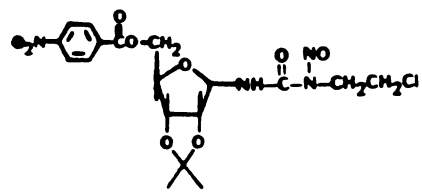

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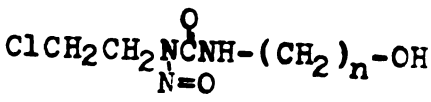
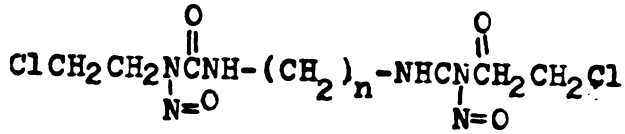
Fiebig and coworkers investigated the antitumor activity of three water soluble and six bifunctional analogs (18, 19) of BCNU. The water



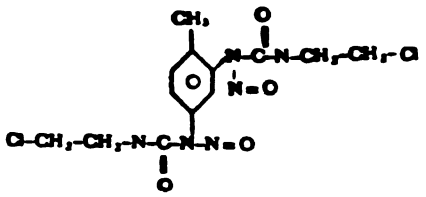
(n=2,3,4)

17



(n=2,3,4,5,6)

18



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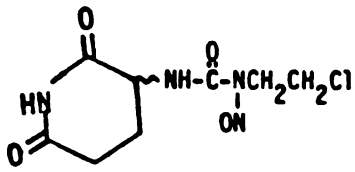
soluble 1-(2-hydroxyethyl)-3-(2-chloroethyl)-nitrosourea (17) showed a higher activity than BCNU in subcutaneously inoculated Walker carcinoma 256. Treatment of intracerebral inoculated Walker carcinoma 256 with the bifunctional analogs and 1-(2-hydroxyethyl)-3-(2-chloroethyl)-3-nitrosourea resulted in a marked increase in life span over untreated rats; however, no significant differences were found in comparison with BCNU[44].

This research is primarily directed towards the study of agents that are active in the treatment of intracerebral tumors. A prerequisite for compounds to exert biological activity against these tumors is the ability to cross the blood brain barrier. Hansch, Smith, Engle and Wood[45] used the octanol-water partition coefficient to study the relationship between lipophilicity and activity against intracerebrally inoculated L1210 leukemia cells. Montgomery, Mayo and Hansch[46] carried out a similar study with subcutaneous Lewis lung carcinoma. They found that there is an optimum, octanol-water partition coefficient of log P = 0.83 (0.2 to 1.34). The log P value for BCNU is 1.53, CCNU is 2.83 and MeCCNU is 3.30[45]. A recently developed analog that has a log P value within optimal range is PCNU (20), log P equal to 0.37. Clinical trials of this agent were initiated in 1979.



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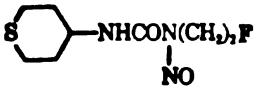
The primary concern of this dissertation is to study the reactions of BCNU and CCNU, two drugs that are used commonly for the treatment of

brain tumors. These agents must cross the blood brain barrier and get into the tumor cells. It is necessary for the parent compounds to remain intact in order to pass the blood brain barrier and then decompose near the site of action within the tumor cells. Pharmacokinetic profiles of these drugs in patients show a two-phase curve, a rapid decline followed by a slowly decreasing second phase[47]. The clearance of chloroethylnitrosoureas from biological fluids is a crucial factor affecting their efficacy. The reactions of these agents which occur during circulation and metabolism in the liver and other organs will account for the deactivation or toxicity of the drugs. Wide interindividual variations have been observed in the pharmacokinetic parameters for plasma clearance in patients. The chemico-biological interactions causing these variations, possibly related to the unusual lipophilicity and chemical reactivity of the parent compound, are unknown. Since the parent drugs are not active, it is also important to investigate the chemical reactions of these agents in aqueous media at physiological pH and temperature.

Chapter II. LITERATURE REVIEW

The first nitrosourea tested as an antitumor agent, 1-methyl-1-nitrosourea, was found to be active against leukemia L1210 tumor cells and especially intracerebrally inoculated L1210 cells. This observation stimulated further studies and many N-alkyl-N-nitrosourea congeners have been synthesized and evaluated for anticancer activity. Most of the early synthetic work was carried out by Southern Research Institute[48-51]. N-Nitrosoureas, RNHCON(NO)R', and N,N"-dinitrosobiureas, R'N(NO)CON-CON(NO)R', were synthesized and evaluated in 1963. BCNU was found to be the most active member of this series[48]. Continued structure-activity studies emphasized N-1 haloethyl substituents, with various N-3 substituents including cycloaliphatic, aromatic and heterocyclic groups. Screening of this series of compounds indicated that the most active nitrosoureas against both ip and ic inoculated L1210 mouse leukemia were 1-(2-(chloro or fluoro) ethyl)-1-nitrosoureas substituted in the 3-position by a 2-(chloro or fluoro)ethyl or cycloaliphatic group with a few exceptions[49]. Nitroso derivatives of biurets, biureas, and carboximides were also synthesized and tested for activity against intraperitoneally inoculated L1210 leukemia[50]. Some of the compounds significantly increased the life span of leukemic mice, but the highest % ILS (average percent increase in life span of treated animal over control animal (100(T/C-1)) was 88 for 1-methyl-1-nitrosobiuret, which was still much less than the % ILS, 184, for BCNU. Additional congeners of BCNU were synthesized in 1971, with special emphasis on alicyclic and heterocyclic analogs of CCNU[51]. The most effective compound against ip and ic inoculated murine leukemia L1210 was found to be the isomeric mixture of 1-(2-chloroethyl)-3-(2-fluoroethyl)-1-nitrosourea and 1-(2-fluoroethyl)-3-(2-chloroethyl)-1-nitrosourea in terms of the chemotherapeutic indices ED_{50}/LD_{10}. 1-(2-fluoroethyl)-1-nitroso-3-

(tetrahydro-2H-thiopyran-4-yl)urea S,S-dioxide (21), 1-(2-chloroethyl)-1-nitroso-3-(tetrahydro-2H-thiopyranyl)urea (22), and 3-(4-acetoxycyclohexyl)-1-(2-chloroethyl)-1-nitrosourea (23) are as active, and have better chemotherapeutic indices than does BCNU.



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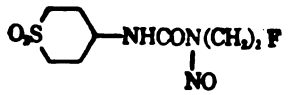

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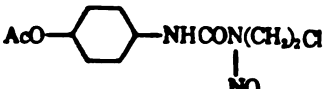

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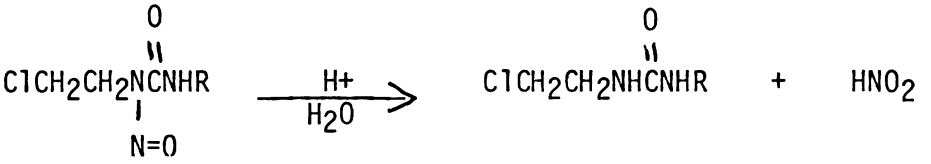
Chemical synthesis of alkylnitrosoureas was carried out by nitrosation of the corresponding ureas. A typical procedure is the addition of sodium nitrite (either neat or as an aqueous solution) in small portions to a cold, stirred solution of urea in hydrochloric acid or formic acid at 0-5°C. The symmetrical urea like bis(2-chloroethyl)urea gives BCNU only. An unsymmetrical 1,3-disubstituted urea generally yields a mixture of nitrosated products, i.e., N-1 and N-3 nitrosoureas. The position of nitrosation or the ratio of the isomers is dependent on several factors: 1) the nucleophilicity of the nitrogen which is influenced by the substituent[48]; 2) the presence of water in the reaction mixture[49]; 3) the steric hindrance of the N-alkyl substituent[50]. A certain degree of nucleophilicity is necessary for the ureas to be nitrosated. For example, diethyl N,N'-carbonyl diglycinate resists nitrosation under the condition which 1,3-bis(2-chloroethyl)urea is readily nitrosated. Some exceptions have been reported, however, in which the position of nitrosation was not related to the relative nucleophilicity[49]. The effect of water on the isomeric ratio was demonstrated in many compounds. For example, 1-(2-bromoethyl)-3-phenylurea gave approximately a 1:1 mixture of N-1 and N-3 nitrosation when reacted with sodium nitrite in 85% formic acid, whereas in 98%-100% formic acid only N-1 nitrosation was obtained. The steric hindrance of the cyclohexyl group results in exclusive formation of

1-(2-chloroethyl)-1-nitroso-3-cyclohexylurea in anhydrous nitrosation. In contrast, the steric effect for the cyclopentyl or cycloheptyl analogs gave a mixture of two isomers. In addition to the cyclic alkanes, noncyclic tertiary branching also demonstrates steric control of nitrosation[51].

1. Chemical Reactions

Chloroethylnitrosoureas react in basic, neutral and acidic aqueous solutions. The stability of these compounds is pH dependent. They are very unstable at pH above 8 and have a half-life less than 5 minutes. Stability increases at lower pH and reaches a maximum at pH 4 to 5, with a half-life of 400-500 minutes. In highly acidic solutions (pH < 2), they decompose very rapidly and may survive for only a few seconds[9]. This dependency of stability of chloroethylnitrosoureas on pH suggests that they undergo both acid and base catalyzed mechanisms of decomposition. Garrett and coworkers studied the solvolysis kinetics of various N-alkyl-N-nitrosoureas in solution at pH 6 to 7.8 phosphate buffer and found the rates were first order in substrate and hydroxide ion, and the log K vs pH profile was linear with a slope of +1.0[52]. Lasker et al.[53] also investigated the aqueous decomposition of BCNU in phosphate buffer at pH 6 to 8. The regression coefficient they obtained was also +1.0 indicating a specific base-catalyzed decomposition for chloroethylnitrosoureas in aqueous solution. There is no evidence of phosphate catalysis. The rate constant was the same at any particular pH and temperature with different ionic strength and concentrations of phosphate buffer.

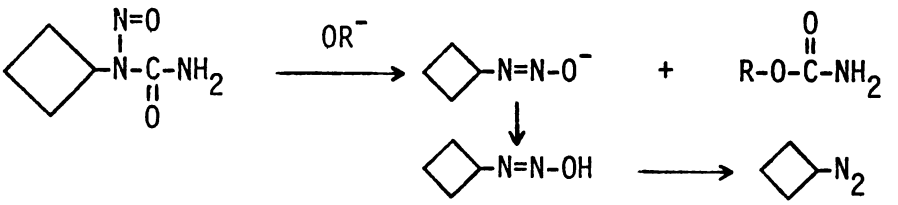
Nitrosoureas decompose by a different mechanism in very acidic solution (pH < 2)[54]. Nitrous acid is liberated rapidly by proton catalysis, which constitutes the basis for the colorimetric determination of nitrosoureas[55]. At pH 3-5, the rate of decomposition increases as the pH decreases[9,56]. The regression coefficient was nonzero and not equal to -1,



which indicated a general acid catalysis[56]. The rate of decomposition is also influenced by the presence of other acids.

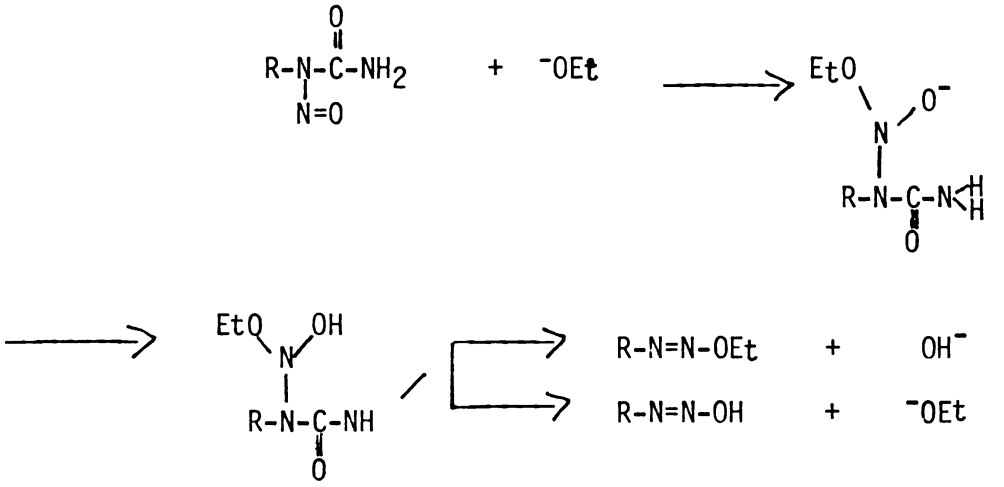
Most mechanistic studies have been conducted in basic media. Three different mechanisms have been proposed for the base-induced decomposition of nitrosoureas. The mechanistic studies were performed with alkylnitrosoureas rather than chloroethylnitrosoureas, but the mechanism of decomposition is considered to be the same. An early study of Applequist and McGreer[57] suggested a scheme of alkoxide-induced decomposition of N-nitroso-N-cyclobutylurea to diazocyclobutane in ether at -50°C (Scheme II).

Scheme II



This scheme implied that the initial step involved ethoxide ion attack on the carbonyl group. However, this mechanism was rejected by Jones and Muck[58,59]. They proposed ethoxide attack on the nitroso nitrogen based on the fact that they were unable to detect ethyl carbonate as a reaction product (Scheme III). It may be noticed that the structurally related compounds, N-nitroso-N-alkylurethanes and N-nitroso-N-alkylamides are thought to undergo competitive reaction mechanisms in which alkoxide ion may attack either the nitroso nitrogen or carbonyl carbon.

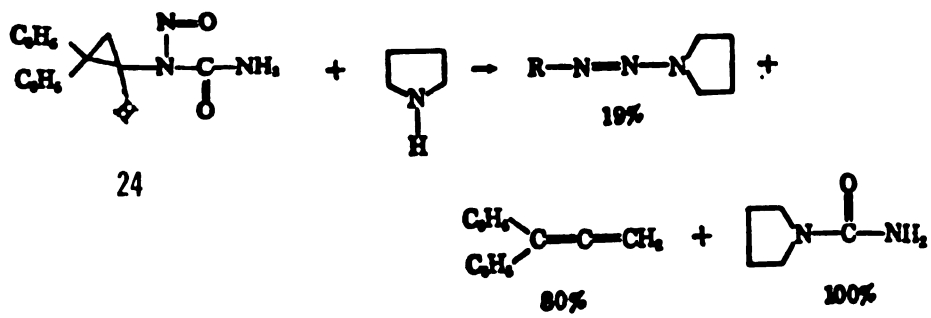
Scheme III



Montgomery et al. investigated the decomposition of BCNU in aqueous solution. Acetaldehyde, hydrochloric acid, nitrogen and derivatives of 2-chloroethylisocyanate, which included chloroethylamine, 1,3-bis(2-chloroethyl)urea, and 2-(2-chloroethylamino)-2-oxazoline were detected. They also found that the reaction products at pH 12 with 1 mole of base were primarily NaCl, acetaldehyde and 1,3-bis(2-chloroethyl)urea. However, 2-oxazolidone was obtained instead of urea when 2 moles of base were used[63]. Colvin and coworkers analyzed the volatile reaction products generated from BCNU (5×10^{-2}M) at pH 7.4 and 37°C in phosphate buffer[64]. The major products were 2-chloroethanol (< 32%) and acetaldehyde (< 16%). Vinyl chloride (< 2%) and 1,2-dichloroethane (< 1%) were observed as minor products. Approximately 50% of the radioactivity was present as nonvolatile material and was not identified. Reaction products of CCNU (0.6 mM) in 0.1 M phosphate buffer at pH 7.4 were determined by Reed et al.[65] and quantitated. Cyclohexylamine (32%) and 2-chloroethanol (18-25%) were found as the major products, along with acetaldehyde (5-10%), vinyl chloride (1-2%), and ethylene (1-2%). BCNU gave 15% 2-chloroethylamine under the same conditions. Montgomery and coworkers[66] reinvestigated the decomposition of BCNU and CCNU at higher concentration and compared the product distribution

in different solutions. They isolated 21% acetaldehyde and 44% 2-chloroethanol from 19.6 mM BCNU in phosphate buffer for 6 hours, and 48% acetaldehyde and 10% 2-chloroethanol from 11 mM CCNU in phosphate buffer for 21 hours. Reaction in distilled water gave lower production of 2-chloroethanol from BCNU and CCNU. There are other reports involving the quantitation of reaction products in aqueous solution from chloroethylnitrosoureas. In general, 2-chloroethanol, 2-chloroethylamine and acetaldehye are found as major products, while 1,3-bis(2-chloroethyl)urea, vinyl chloride, 1,2-dichloroethane, 2-oxazolidone, and 2-(2-chloroethylamino)-2-oxazoline are minor products. These products can account for less than 45% of the starting nitrosourea and several unidentified nonvolatile products are also formed. Further evidence that favored attack by the ethoxide at the nitroso nitrogen was reported by Jones, Muck and Tandy[60]. They observed up to 19% of triazene and urea from the reaction of 2,2-diphenycyclopropyl-N-nitrosourea (24) in the presence of pyrrolidine (Scheme IV). This observation suggested that an amine catalyzed decomposition of alkylnitrosoureas occurred by attack on the nitroso nitrogen.

Scheme IV



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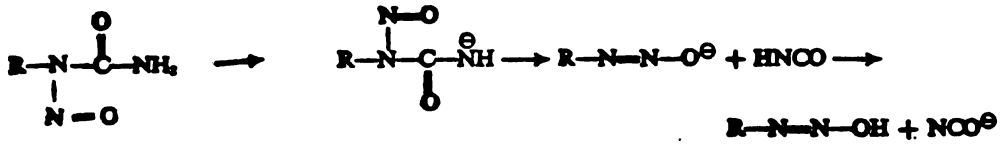
Hecht et al.[61] proposed a third mechanism involving initial proton abstraction at the urea nitrogen, and disagreed with the proposed mechanisms involving nucleophilic addition to the nitroso nitrogen or carbonyl group (Scheme V). When one considers the acidity of urea (pKa ≈ 16) and of

Scheme V



nitrosourea (pKa ≃ 8-9)[52,62], and the basicity of alkoxide ion, proton transfer appears to be a more attractive first step rather than nucleophilic displacement. According to this mechanism, the rate of decomposition is more dependent on basicity rather than nucleophilicity. Indeed, the half-life of N-nitroso-N-methylurea in the presence of phenoxide is shorter than that in thiophenoxide, which is a weaker base and stronger nucleophile than phenoxide. The authors did not give very direct, strong evidence for this proposal, however. Additional support for this proposal is that N',N'-disubstituted analogs which have no N-3 proton, have greater stability in basic solution. N-(2,2-diphenylcyclopropyl)-N',N'-dimethyl-N-nitrosourea does not liberate nitrogen when heated under reflux in heptane[59]. Colvin and coworkers[63] synthesized and studied the reaction of 1-chloroethyl-3,3-dimethyl-1-nitrosourea in aqueous solution. The very slow decomposition of this compound in aqueous solution suggested that an N-3 proton is necessary for the decomposition of alkylnitrosoureas. The mechanism of decomposition of chloroethylnitrosoureas is most probably the same as the alkylnitrosoureas discussed above, with removal of the N-3 proton as the first step. Several papers have dealt with the products formed from the reaction of chloroethylnitrosoureas in aqueous solution.

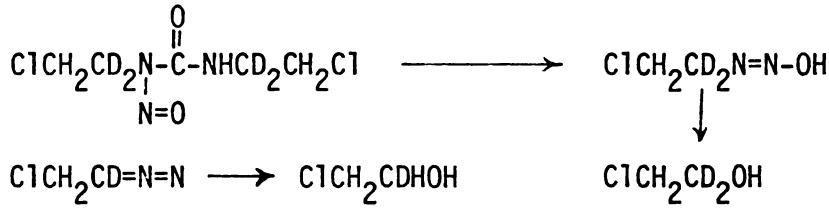
2. Alkylating Species

Chloroethylnitrosoureas are thought to be alkylating agents. They form two reactive intermediates which are alkylating species and carbamoylating species. It is quite clear that the alkylisocyanate formed from the N-3 side of the molecule has carbamoylating activity. However, the actual

structure of the alkylating species has long been the focus of intense investigations.

The first alkylnitroso compound found to have anticancer activity is N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 2). Skinner et al.[67] suggested that the biological effects of MNNG were due to its decomposition to diazomethane. It was therefore assumed that the active species generated from alkylnitrosoureas at physiological conditions were also diazoalkanes[52]. Garrett et al. suggested a scheme in which 1,3-disubstituted-1-nitrosoureas might decompose to yield an isocyanate and a precursor of a diazomethane[52]. Actually, N-nitroso-N-alkylamide[58], N-nitroso-N-alkylurea[59], N-nitroso-N-alkylcarbamate[68], and N-nitroso-N-alkylurethane[69] do give diazomethane in strong base-induced reactions. No evidence for the presence of diazomethane could be found in neutral aqueous decomposition of these compounds[63]. Studies with MNNG[70], MNU[71,72], and N-ethyl-N-nitrosourea[73] labeled with ^{14}C, ^{3}H, or ^{2}H on the methyl groups of the alkylnitrosation products contained the same ratio of isotopes as the parent compounds. These results excluded a diazoalkane as the reactive intermediate. This was further illustrated by Brundrett et al.[13] using deuterated BCNU; with α-d_4-BCNU the product, 2-chloroethanol, contained two deuteriums, which is inconsistent with the prediction of 2-chlorodiazoethane as an intermediate (Scheme VI).

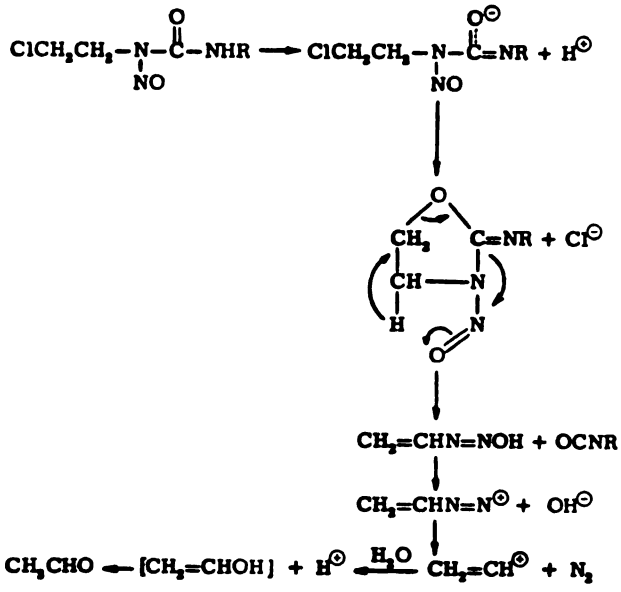
Scheme VI



Montgomery and coworkers[63] reported that the decomposition of BCNU and CCNU in water was anomalous and yielded primarily acetaldehyde rather than 2-chloroethanol. They suggested a mechanistic sequence via an oxazolidine intermediate, which then breaks down into ethyldiazohydroxide and isocya-

nate (Scheme VII). Since they found no acetaldehyde formed from the reaction of 2-chloroethylamine with nitrous acid, which proceeds via a chloroethylcarbonium ion, they proposed the oxazolidine intermediate instead of a sequence via the carbonium ion.

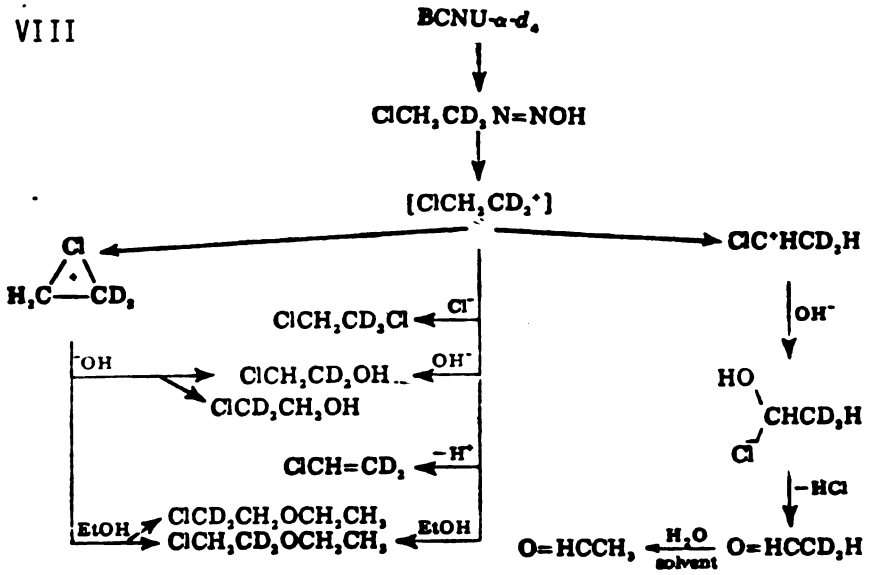
Scheme VII



Later Colvin and coworkers[64] identified 2-chloroethanol acetaldehyde, vinyl chloride, and 1,2-dichloroethane as the reaction products from ^{14}C-BCNU in neutral aqueous solution. The product distribution was similar to that from the reaction of chloroethylamine and nitrous acid in aqueous solution. They also isolated acetaldehyde from this diazotization. They suggested that the reactive intermediate was the chloroethylcarbonium ion. Reed and coworkers[65], who studied the degradation of CCNU and MeCCNU in buffer under physiological conditions, also suggested the 2-chloroethylcarbonium ion as the alkylating species. They proposed a mode of degradation that involved loss of the urea hydrogen as a proton, with formation of the corresponding isocyanate and 2-chloroethylazohydroxide. In 1975, Montgomery and coworkers reinvestigated the decomposition of six chloro-

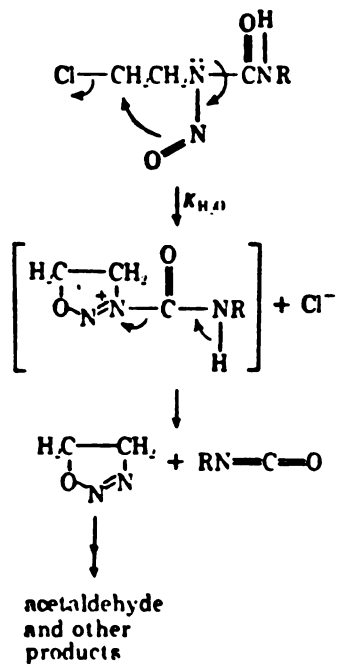
ethylnitrosoureas in distilled water and buffered aqueous solution. They observed that more chloroethanol and acetaldehyde are formed in buffered aqueous solution, and that the distribution varied with pH. In order to explain this result they proposed another reaction intermediate, the vinyl cation as a precursor of acetaldehyde, in addition to the chloroethylcarbonium ion[66]. However, they were still unable to account for the product distribution changes in different media. Brundrett and coworkers[13] reported a more exclusive result that favored the chloroethylazohydroxide intermediate. They conducted the decomposition reaction with deuterated BCNU. The deuterium distribution in the product acetaldehyde formed from tetradeuterated BCNU (α-d_4 or β-d_4) was inconsistent with the presence of the vinyl cation intermediate and they proposed that the 2-chloroethylcarbonium ion rearranged to 1-chloroethylcarbonium ion and the cyclic carbonium ion. The major product from BCNU, 2-chloroethanol (60%), is mostly unarranged (90%). They suggested that the reaction of chloroethylazohydroxide proceeds via a S_N2 mechanism. Only part of the azohydroxide goes through a 2-chloroethylcarbonium ion transition state which then rearranges to the more stable 1-chloroethylcarbonium ion or cyclic carbonium ion (Scheme VIII).

Scheme VIII



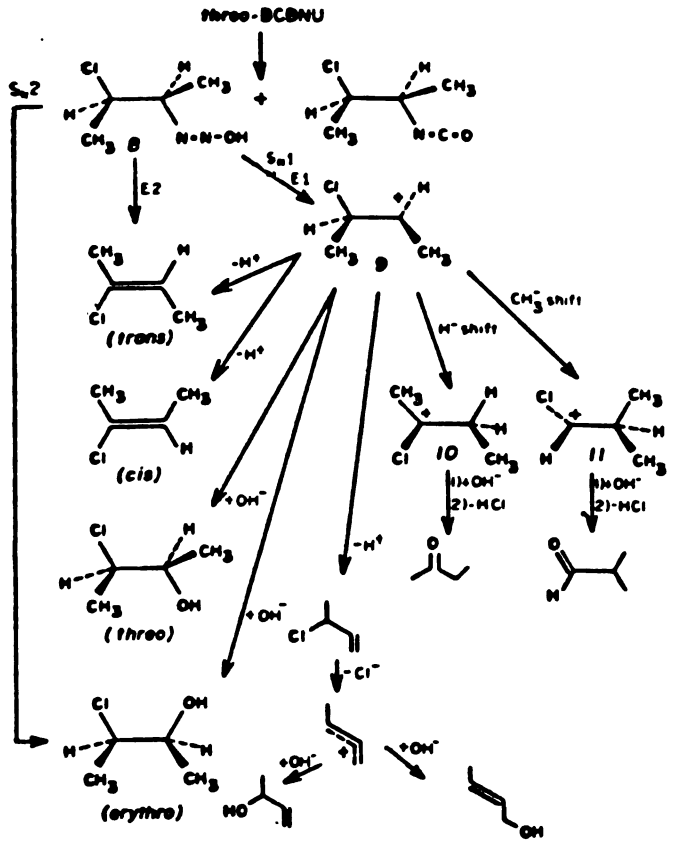
Based upon measurements of the rate of chloride ion formation, Chatterji[56] proposed another mechanism, which involved a oxadiazole intermediate as the precursor of acetaldehyde in neutral solution (Scheme IX). This is not consistent with the deuterium distribution reported by Brundrett et al. Chloride ion production is not a good parameter to measure, because there are several pathways that may lead to the formation of the chloride ion.

Scheme IX



The mechanism of decomposition of chloroethylnitrosoureas was further illustrated by Brundrett et al.[74] They synthesized 1,3-bis(threo-3-chloro-2-butyl)-1-nitrosourea (threo-BCBNU) and erythro-BCBNU and studied the decomposition in buffered solution. Erythro-BCBNU gave predominantly threo-3-chloro-2-butanol and cis-2-chloro-2-butene, while threo-BCBNU gave predominantly the erythro-butanol and transbutene (Scheme X). The stereochemistry of the products 3-chloro-2-butanols and 2-chloro-2-butene indicated that a significant fraction of these products were formed via reaction of 3-chloro-2-butylazohydroxide with S_N2 and E2 stereochemistry as well as S_N1 and E1 reactions involving the secondary 3-chloro-2-butyl

Scheme X



carbonium ion. There was 2/3 of the product, 3-chloro-2-butanol, in the inverted stereochemistry. The carbonium ion produced by BCBNU is secondary and hence a more energetically favorable species than the primary carbonium ion that BCNU would produce. Therefore, the decomposition of BCNU would be expected to involve S_N2- E2 reaction to a much greater extent than BCBNU. They suggested that the alkylating reaction of BCNU is probably 2-chloroethylazohydroxide.

In summary, chloroethylnitrosoureas decompose in aqueous solution to give 2-chloroethanol in neutral and alkaline conditions and acetaldehyde at more acidic conditions. The reactive intermediates are 2-chloroethylazohydroxide and an alkylisocyanate. Chloroethylazohydroxide can react with water throught a S_N2 mechanism to form 2-chloroethanol. Part of the azohydroxide reacts through the 2-chloroethylcarbonium ion which rearranges to a more stable 1-chloroethylcarbonium ion, from which acetaldehyde is

formed. The alkylisocyanate can react with water to form a carbamate, which is not stable enough to be isolated; rapid loss of carbon dioxide gives an alkylamine, a major reaction product. The alkylisocyanate can also react with the newly formed alkylamine to give urea. Thus the formation of urea is concentration dependent.

3. Alkylation and Carbamoylation

The antitumor activity of chloroethyl-1-nitrosoureas is thought to result from the alkylating and/or carbamoylating intermediates formed during its chemical degradation. Binding of chloroethylnitrosoureas with macromolecules have been studied with radioisotope labeled nitrosoureas. Incubation of leukemia L1210 cells with BCNU labeled with ^{14}C in the chloroethyl (alkylating) or in the carbonyl (carbamoylating) group indicated that covalent binding was associated with the crude protein fraction. When reacted with DNA, nucleohistone or histone, the largest quantity of radioactivity was associated with the histone. Incubation of carbonyl-^{14}C labeled BCNU with lysine gave a product that was chromatographically similar to N^6-(2-chloroethylcarbamoyl)lysine[14]. Cheng and coworkers examined the interaction of CCNU in three different systems, with L1210 leukemia cells in mice, in a suspension and with isolated nucleic acids and proteins[11]. In all three systems radioactivity from the cyclohexyl moiety was bound extensively to proteins. Radioactivity from the chloroethyl group was bound to both nucleic acids and proteins but to a much lesser extent than the cyclohexyl moiety. Macromolecules used for studies of binding with CCNU included polylysine, albumin, histone, poly C, poly G, poly A, poly U, cytochrome C, ribonuclease A, globulin, DNA and RNA. Polylysine and albumin were the most active in binding the cyclohexyl-labeled compounds and poly G , poly C and tRNA were the most active in

binding chloroethyl labeled CCNU. Connors and Hare studied the binding of BCNU to TLX 5 lymphoma and a cell line with acquired resistance to BCNU. Both cell lines showed essentially similar binding results[75]. They demonstrated that the nuclear proteins are particularly susceptible to binding by cyclohexyl-^{14}C labeled CCNU.

Carbamoylation of amino acids, peptides, and proteins by CCNU and cyclohexylisocyanate at approximately physiological conditions was reported to be at the α-amino groups of amino acids, the terminal amino groups of peptides and proteins, and the ε-amino groups of lysine moieties[76]. Carbamoylation of terminal α-amino groups occurred as readily, or more readily than, the carbamoylation of the ε-amino groups. Carbamoylation of amino groups of amino acids or peptides by BCNU gives (2-chloroethylcarbamoyl)amino products which may cyclize to form (2-oxazolin-2-yl)amino groups. This cyclization cannot happen following CCNU carbamoylation[76]. It was also found that the cyclohexyl group bound to the nuclear proteins of L1210 cells is labile under mildly alkaline conditions (pH 8 or 9), yet stable under mildly acidic conditions. A small amount of the cyclohexyl moiety was bound to histones in stable, nondialyzable form[77]. Further investigations of binding of CCNU to chromatin and nucleosomal fractions of Hela cells demonstrated that CCNU binds specifically to the extended euchromatin fraction of Hela cell genome[78]. CCNU alkylates a small fraction of the total genome in a nonrandom manner and preferentially occurs in the region associated with the nucleosome core.

The products that have been isolated from the reactions of haloethylnitrosoureas with macromolecules are N^6-(2-chloroethylcarbamoyl)-lysine[14], 3-hydroxyethel-CMP, 3-β-fluoroethylcytitidine, and 3-N^4-ethanocytidine[79]. N^6-(2-chloroethylcarbamoyl)lysine was isolated after incubation of BCNU with lysine[14]. When BCNU was reacted with polycytidylic acid, two deriva-

tives of CMP, 3-hydroxyethyl-CMP and 3,N^4-ethanol-CMP were identified in the hydrolysate of the polymer[80]. The reaction product of BCNU and poly G after chemical and enzymatic degradation was 7-(β-hydroxyethyl)GMP[81]. Incubation of BCNU with poly C and poly G in aqueous solution at 37°C and pH 7 produced approximately 0.33% and 0.07% substitution. Under the same conditions, there was relatively little reaction with poly A and poly U[81]. Reaction product of bis-fluoroethylnitrosourea and cytidine was 3-β-(fluoroethylcytidine. Fluoroethylcytidien undergoes intramolecular cyclization to form 3,N^4-ethanocytidine[79]. Although alkylation has been suggested as the major mode of action of chloroethylnitrosoureas, the binding of these compounds to proteins through carbamoylation is found to be much greater than the binding to nucleic acids through alkylation. Covalent binding results from carbamoylation and alkylation with proteins, nucleic acids or other macromolecules and will certainly cause some biological effects. But it is still not clear how to relate each of the chemical reactions to the biological effect. The significance of many of the findings concerning the reactions of chloroethylnitrosoureas with macromolecules is unknown. For example, what are the consequences of the difference between BCNU cyclization and CCNU after carbamoylation of amino groups; the specific binding to euchromatin fraction of Hela cell genome; the alkylation favorable to poly C and poly G or to poly A and poly U? Is the biological effect of carbamoylation significant at doses that produce cytotoxic alkylation?

In addition to the alkylating and carbamoylating activity, the distribution of chloroethylnitrosoureas in the body is an important factor affecting their chemical activity. Most of the studies in this area focus on the relationship between lipophilicity and antitumor activity. Hansch, Smith, Engle, and Wood reported a quantitative structure-activity relationship of 23 nitrosoureas, using a 75% ILS in ic inoculated L1210 bearing

mice as the biological endpoint. The optimum lipophilic character they found for nitrosoureas occurs at log P values near -0.6. The LD_{10}s for the nitrosoureas are also parabolically related to log P, with maximum toxicity occurring at a log P value of 0.4. This suggested that the nitrosoureas with more hydrophilicity would be more potent and less toxic[45]. Montgomery and Mayo investigated the antitumor activity of 14 nitrosoureas against Lewis lung carcinoma in mice[46]. They found that the ideal log P value was 0.83 (-0.2 to 1.34), and suggested that the region -0.1 to 0.0 was worthy of more intensive exploration in the search for active drugs. They were unable to parameterize the electric or steric effects of substituents on the NH group. Nitrosoureas with a phenyl group attached directly to the urea nitrogen are completely inactive. Compounds with carboxylic acid groups removed only one carbon from the NH moiety are less active than expected, but congeners with carboxylic groups well removed from the NH behave as predicted from structure-activity correlation. Montgomery attempted to generalize the structural requirements for high activity, and he found that most of these structures contain a saturated five- or six-membered ring. The importance of this structural feature is emphasized by the relatively low activity of the open-chain analogs of MeCCNU and the cyclohexenyl analogs of CCNU. Other groups present in highly active compounds include the 2-haloethyl groups and certain carboxylic acids[82].

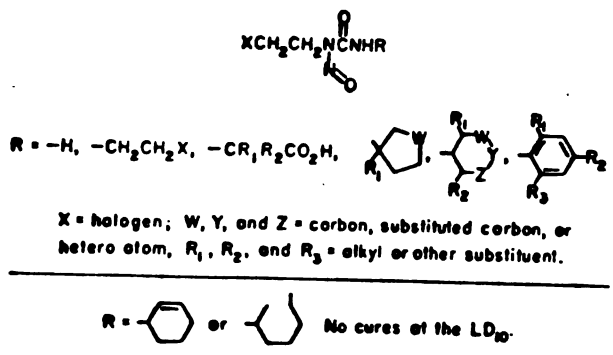


Figure 1. Nitrosoureas highly active against L1210 leukemia

About 80 nitrosoureas were evaluated using a 50% cure rate of ip implanted L1210 leukemia as the biological endpoint and LD_{10} as a measure of toxicity. Two parabolic curves were obtained that unfortunately, have the same apex, indicating that there is no separation of activity and toxicity based on partition coefficients (Figure 2,3). In general, compounds most active against the Lewis lung carcinoma were most active against other solid tumors, indicating that the structural characteristics necessary for activity against solid tumors are probably fairly general[83]. Although some exceptions were noted, compounds most active against the early form of the disease were most active against the established tumor.

Nonrefined structure activity relationships include parameters for alkylating and carbamoylating activity as well as solubility. The alkylating activity has been determined by the reaction of nitrosoureas with 4-(p-nitrobenzyl)pyridine. A highly conjugated alkylation reaction product was formed and the optical density was measured[84]. Carbamoylating activity was determined by the reaction of nitrosoureas with radioactive-^{14}C labeled lysine. Alkylation may also occur in this case but it is small in comparison to the extent of carbamoylation. Wheeler and coworkers suggested that carbamoylating activity, alkylating activity and solubility are all important in determining the degree of antitumor activity[85]. Their study indicated that carbamoylating activity played a dominant role in determining toxicity, as reflected in the LD_{10}, and in the therapeutic index. Alkylating activity was a greater factor in determining the single ip dose ED_{99} and the 50% 45-day survivors than in determining the LD_{10}. It is also important in determining the therapeutic index. The octanol/water partition coefficient is a major factor in determining toxicity and hence therapeutic indecies. However, the correlation coefficients obtained for the linear regression equations for the relationship between biological and chemical

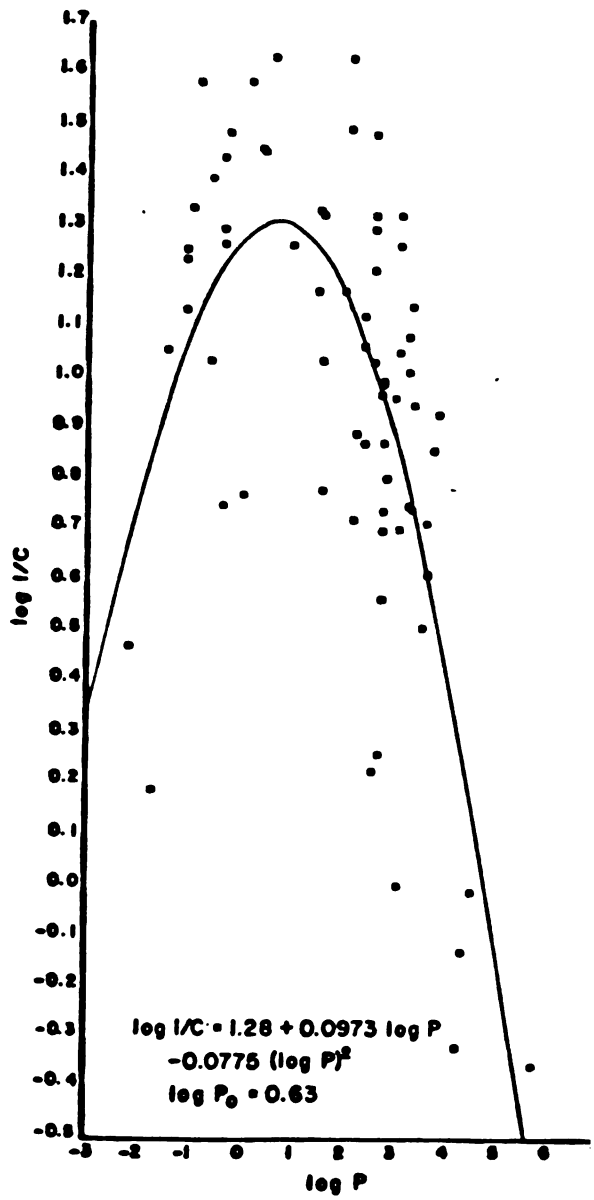


Figure 2. Selected nitrosoureas vs L1210 Lekemia (ip).

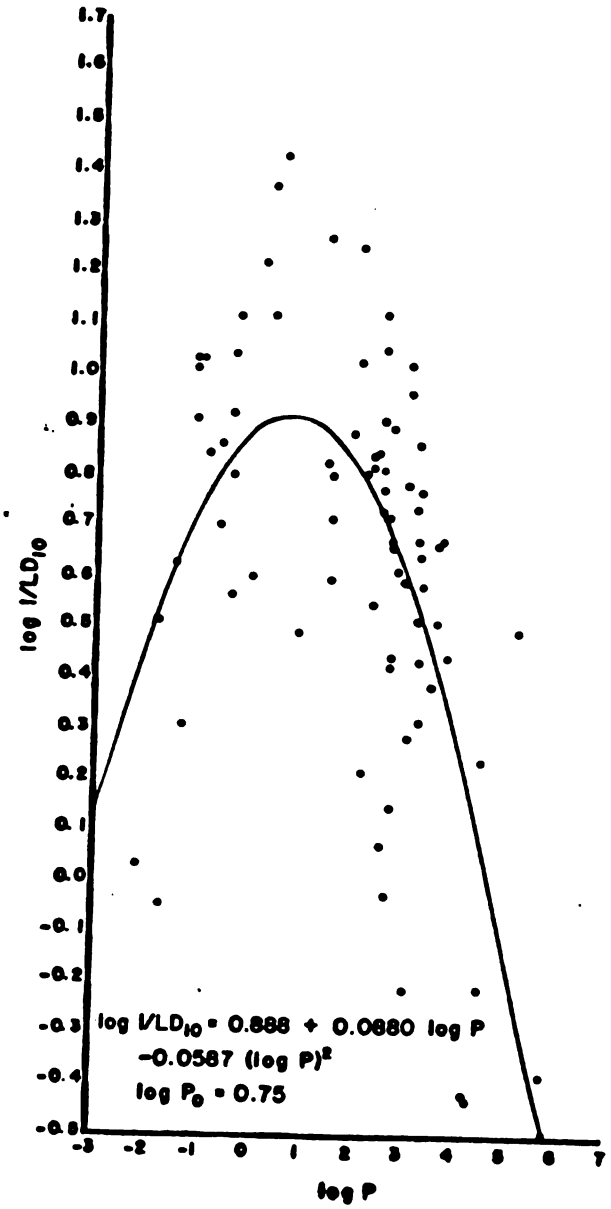
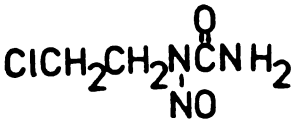


Figure 3. Toxicity of selected nitrosoureas.

and physicochemical parameters are relatively poor, so that no significant correlation was established. A linear regression analysis was also performed by Panasci and coworkers to study the correlation of in vitro carbamoylating activities, alkylating activities, and chemical half-lives with the biological activities of six chloroethylnitrosoureas in mice[86]. There was a highly significant inverse linear relationship between alkylating activity and the chemical half-life of the nitrosoureas and a significant linear relationship between the molar LD_{10} and alkylating activity or the chemical half-life. The greater the alkylating activity, the lower the LD_{10}. Carbamoylating activity did not demonstrate a significant linear correlation with the molar LD_{10}. Water solubility did not function as an independent variable for either of these biological activities. None of these three chemical parameters demonstrated a significant linear correlation with antitumor activity.

Colvin et al. compared the antitumor activity of some chloroethylnitrosoureas, such as 1-chloroethyl-1-nitrosourea (CNU, 24), 1-chloroethyl-3,3-dimethyl-1-nitrosourea (dMCNU, 25) and BCNU, and explained the antitumor activity on a chemical basis[10]. BCNU and CNU are active against



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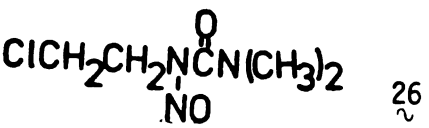

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L1210 leukemia cells while dMCNU is not active. Both BCNU and CNU generate chloroethanol as a decomposition product, probably through the chloroethylcarbonium ion (or diazonium ion). On the contrary, dMCNU is stable in aqueous solution. It was also pointed out that CNU did not generate an organic isocyanate moiety, did not inhibit RNA synthesis and processing but was an antitumor agent. It was, therefore, suggested that organic isocyanate generation is not required for antitumor activity and that the antitumor activity of chloroethylnitrosoureas is due to the formation of an

alkylating species. Lastly, dMCNU was shown to be active against L1210 leukemia in vivo, probably because of N-demethylation to a more labile form[87].

4. Biodisposition of Chloroethylnitrosoureas

Early research on the biodisposition of chloroethylnitrosoureas followed the in vivo fate of labeled compounds. Wheeler and coworkers studied the distribution of radioactivity from ^{14}C-labeled BCNU in tissues of mice and hamsters after intraperitoneal administration of the agent[88]. Radioactivity was found in all tissues examined including brain, liver, kidney, lung, spleen, heart and blood. Comparable quantities were found in various tissues of mice after ip injection of chloroethyl-^{14}C-BCNU and carbonyl-^{14}C-BCNU to BDF_1 mice. Urinary excretion accounts for the major portion of the isotope after intravenous injection of BCNU[89]. The radioactivity recovered was 72% in 24 hours from mice, 67% in 24 hours from monkeys, 29% in 6 hours from dogs and 43% in 24 hours from patients. Approximately 10% of the initial radioactivity was excreted as CO_2 in two patients following oral and intravenous administration. The disposition of CCNU is similar to that of BCNU. In mice, 10 to 20% of the carbonyl and 4 to 6% of ethylene carbon was recovered as expired CO_2 one day after parenteral or oral dosage of CCNU. The parent drug was excreted primarily by the kidney, with excretion being essentially complete during the first 24 hours in rodents and monkeys; however, the excretion was more protracted in dogs[90]. The urinary excretion of radioactivity in rodents after ip or oral administration was 75% while in dogs after iv administration it was 37 to 64%. The radioactivity levels in plasma and cerebrospinal fluid were roughly parallel. The relative intensity of radioactivity varied with different animals and positions of the label on the ethylene, carbonyl or

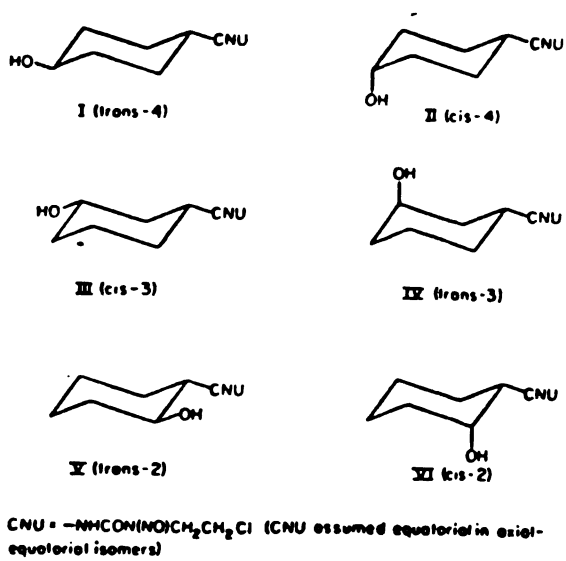
cyclohexyl group[90,91]. Since no effort was made to isolate any unreacted drug, the radioactivity detected in these studies was probably from the degradation products as well as the nitrosoureas.

BCNU rapidly degrades in mouse and dog plasma with two exponential phases after parenteral dosage. The half-life of the initial phase is about 5 minutes while the second phase extends over one hour[90]. Sponzo and coworkers studied the physiological disposition of radioactively labeled CCNU and MeCCNU in patients[92]. CCNU was labeled at the chloroethyl, carbonyl or cyclohexyl moiety, while MeCCNU was labeled at the carbonyl or cyclohexyl group. Significant plasma levels of radioactivity were detected in as early as 10 minutes. Approximately 60% of the radioactivity administered was excreted in the urine within 48 hours and more than 50% was excreted in the first 12 hours. Radioactivity was found in the CSF of patients given both drugs. The authors were unable to detect any intact drugs in plasma, CSF, or urine samples after one hour. Levin and coworkers studied the pharmacokinetics of BCNU in 20 patients[47]. Plasma levels of BCNU were measured using a technique of selective ion monitoring chemical ionization mass spectrometry[93]. The disappearance of BCNU in patients has a half-life of 15.6 minutes. Analysis of the pharmacokinetics of BCNU in 20 patients using a two-comparment open model demonstrated a volume of distribution of 3.25 liter/Kg, a clearance of 56 ml/Kg and a transfer constant from the central compartment to the outside (k_{10}) of 0.0324 minutes^{-1}. It was suggested that the pharmacokinetics of BCNU in patients may be affected by the percent of body fat and the lipid content of the serum[47].

The metabolic reactions of chloroethylnitrosoureas has been investigated in vivo and in vitro. Most studies of metabolism of chloroethylnitrosoureas have focused on CCNU and MeCCNU in the past five years; much less is known about the metabolic reactions of BCNU and other chloroethyl-

nitrosoureas. May, Boose, and Reed first showed that CCNU was a substrate for rat liver microsomes and that the metabolic reaction required O_2 and NADPH. The enzyme activity was inactivated by heating and partially inhibited when the reaction was carried out under 80% CO and 20% O_2[94]. Hilton and Walker also observed 50% inhibition by CO[2]. The enzyme system was inducible by pretreatment of phenobarbital. The phenobarbital pretreated microsomes gave a type I difference spectrum in the presence of CCNU, with a K_S value of 4×10^{-5}M. The type I binding spectrum obtained from cyclohexane with a K_S of 7.4×10^{-4}M has very similar characteristics to that of CCNU[94]. If one recalls that cyclohexane is a substrate for cytochrome P-450 dependent enzymes[95,96], then all these properties indicate that CCNU is also a substrate for cytochrome P-450 dependent enzymes. Hill and coworkers showed that CCNU is not a substrate for glutathion-S-transferase present in the soluble fraction of mouse liver[97].

All of the metabolites of CCNU formed from in vitro microsomal enzyme systems are products of monohydroxylation on the cyclohexyl ring (Figure 4). At present, five metabolites have been identified. May and coworkers found



CNU = $-NHCON(NO)CH_2CH_2Cl$ (CNU assumed equatorial in axial-equatorial isomers)

Figure 4. Possible hydroxylated metabolites from CCNU.

cis-hydroxy CCNU as a metabolite from cyclohexyl-^{14}C-CCNU in rat liver microsomes. The metabolite was isolated by selective solvent extraction and purified by liquid chromatography. Nuclear magnetic resonance (NMR) spectroscopy was used also for structure determination since the metabolite was compared with standard cis-4-hydroxy CCNU and trans-4-hydroxy CCNU[94]. Chemical ionization mass spectra had a molecular ion of the metabolite sixteen mass units higher than that of CCNU. EIMS gave evidence that dehydration occurred during fragmentation and contained fragments that were consistent with an unsaturated ring. These data all supported the hydroxylation of the cyclohexyl moiety. The presence of the nitroso group was shown by the method of Loo[55]. May, Boose and Reed further isolated and identified four metabolites, cis- and trans-3-hydroxy CCNU and cis-trans-4-hydroxy CCNU[98]. High pressure liquid chromatography was used in the separation of these metabolites. The metabolites were identified both by mass spectra and NMR and then compared to the synthetic standards. The position of ring hydroxylation and the identity of each geometric isomer were established with NMR by use of a shift reagent in conjunction with spin decoupling techniques. Hilton and Walker identified the metabolites by generation of N-(2,4-dinitrophenyl)derivatives. When treated in hot alkali, CCNU and its hydroxylated metabolites release their cyclohexyl moiety as cyclohexylamine and aminocyclohexanol respectively. The DNP-derivatives of these amines have been separated by high-pressure liquid chromatography and identified by co-chromatography with authentic DNP-aminocyclohexanols. The metabolites identified were trans-2-hydroxy, cis- and trans-3-hydroxy, and cis- and trans-4-hydroxy CCNU[100,101]. Montgomery and coworkers developed a HPLC system using a reverse phase column in which trans-4-, cis-4-, trans-3-, trans-2 and cis-2-hydroxy CCNU were eluted in thirty minutes[102].

Quantitative results reported by different laboratories have been inconsistent. May and his coworkers reported that the hydroxylated metabolites from normal rat liver microsomes were 40% trans-3-, 30% cis-3-, 21% cis-4-, and 9% trans-4-hydroxy CCNU. Hilton and Walker found 53% cis-4-hydroxy CCNU formed. The yields of the other metabolites in 40 minutes were 30% for trans-3-hydroxy, 13.8% for trans-2-hydroxy, 2.6% for trans-4-hydroxy and only a trace of cis-3-hydroxy CCNU found. A total of 77% of CCNU was metabolized.

As mentioned before, the microsomal enzymes that are responsible for the metabolism of CCNU are inducible by treatment with phenobarbital. The rate of metabolism of CCNU by liver microsomes from rats pretreated with phenobarbital is increased. CCNU has V_{max} of 67.5 nmoles/min/mg protein and a K_m of 0.24 mM while the normal rat liver microsomes give a V_{max} of 42.5 nmoles/min/mg protein and a K_m of 0.4 mM[101]. May et al. reported a six-fold increase in rate of metabolism following phenobarbital treatment. 3-Methylcholanthrene, which induces some forms of cytochrome P-450, reduces the total CCNU hydroxylation[103]. Phenobarbital pretreatment changes the distribution of CCNU hydroxylation; the cis-4-hydroxy-CCNU yield was increased 9-19 fold, while a 5-fold increase was reported for trans-4-hydroxy CCNU, a two-fold increase for trans-3-hydroxy CCNU, and a three-fold increase for cis-3-hydroxy CCNU[98,103]. Phenobarbital pretreatment leads primarily to the formation of cis-4-hydroxy CCNU (77.4%) with some trans-3-hydroxy CCNU (11.3%) and small amounts of other metabolites.

Ring hydroxylated CCNU metabolites are also formed in vivo in rat and man. The metabolites isolated from rat plasma in 20 minutes after administration of CCNU (5 mg/Kg) were 54% for cis-4-hydroxy, 22.6% for trans-3-hydroxy, 11.5% for trans-4-hydroxy, 8% for trans-2-hydroxy and 3.9% for cis-3-hydroxy CCNU. Ninety-six percent of CCNU was metabolized

in 20 minutes[101]. In addition to ring hydroxylation, in vivo metabolism gave thioacetic acid as a major urinary metabolite of CCNU when rats were administrated chloroethyl-^{14}C-CCNU[103]. Only cis-4-hydroxy and trans-4-hydroxy CCNU were isolated from humans[100]. Similar effects of phenobarbital pretreatment were found with in vivo and in vitro metabolism of CCNU.

Cis-4-hydroxy CCNU was the major metabolite isolated from rat plasma, 61.5% in 2 minutes. Other metabolites were trans-4-hydroxy, 21.4%, trans-3-hydroxy, 9.3%, trans-2-hydroxy, 7.8% and cis-3-hydroxy CCNU, trace. It is interesting to note that the yield of trans-4-hydroxy CCNU in phenobarbital induced in vivo is much higher than that of the in vitro systems[101].

Farmer and coworkers synthesized deuterated analogues of CCNU and studied their metabolic reaction and antitumor activity. Deuterium was introduced into the cyclohexyl ring at positions susceptible to metabolic hydroxylation, namely CCNU-d_4 (2,2',6,6'-d_4), CCNU-d_6(3,3',4,4',5,5'-d_6) and CCNU-d_{10}(2,2',3,3',4,4',5,5',6,6'-d_{10}). Selective deuteration induced a metabolic switch that directed metabolism away from the sites of isotopic substitution. There is only a small isotope effect for the overall hydroxylation process. The total yield of the hydroxy derivatives from CCNU-d_{10} was 74% of that from CCNU but the relative proportions of each hydroxy derivatives were very similar. CCNU-d_4 yielded much less trans-2-hydroxy-CCNU whereas the yield of this isomer from CCNU-d_6 was correspondingly greater[104].

The metabolism of BCNU has not been well studied. BCNU is a substrate for a microsomal enzyme of mouse liver; the metabolic reaction of BCNU was studied by Hill et al. [105] NADPH is required for the metabolic reaction, and cannot be substituted by NAD, NADP or NADH. Magnesium ions are not required and did not stimulate the reaction. When the reaction was performed under an atmosphere of nitrogen, product formation was reduced

by 55%. Under an atmosphere of carbon monoxide the reaction was moderately inhibited. Another indication that BCNU was a substrate for cytochrome P-450 dependent microsomal enzymes was obtained from the competitive inhibition of the oxidation of nicotine to 5-hydroxynicotine and of cyclophosphamide to aldophosphamide. The K_m for BCNU with liver microsomes is 1.7 mM. The metabolite of BCNU after incubation with microsomal enzymes was separated by thin-layer chromatography and compared with the authentic compound, N,N'-bis(2-chloroethyl)urea (BCU) for identification. The structure was further characterized to be BCU by mass spectrometry. Quantitative calculation of metabolite formation based on radioactivity indicated that 20% of BCNU was converted to BCU in 6 minutes in a mouse liver microsomal preparation.

BCNU was also shown to be metabolized to a polar product by glutathione-S-transferase, an enzyme that is present in the soluble portion of mouse liver homogenate[99]. It has a K_m of 0.6 $\pm$ 0.1 mM and a V_{max} of 0.8 nmole/min/mg liver under conditions of optimum pH 7.9 and SHH concentration. The metabolite was purified by thin-layer chromatography. Analysis by field desorption mass spectrometry gave peaks of m/e 532 and 534 which suggested a structure of $K_2GS\text{-}CH_2CH_2NHCH_2CH_2Cl$. No further identification has been made.

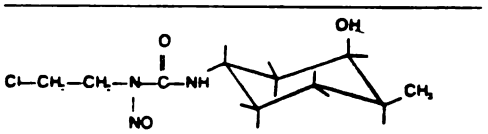
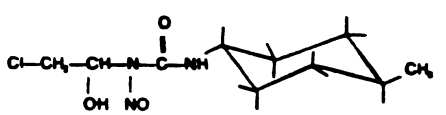
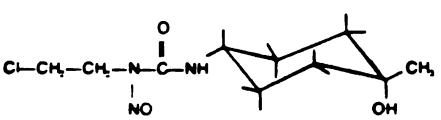
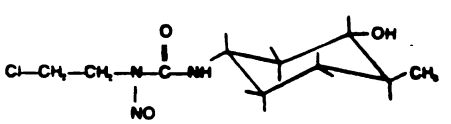
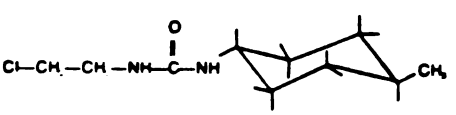
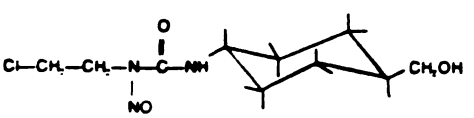
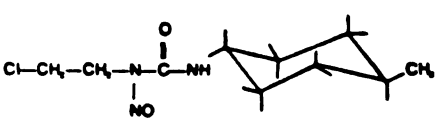
Phenobarbital pretreatment has been shown to dramatically affect the pharmacokinetics and antitumor activity of BCNU. Rats pretreated with phenobarbital showed a 1.5 to 2.1 fold increase in clearance of BCNU compared with the control group[106]. It did not produce more toxic BCNU degradation products, but led to a 100% reduction of BCNU antitumor activity.

The metabolism of MeCCNU is similar to CCNU in that ring hydroxylation occurs. Additional metabolism has also been observed, however. Hill and coworkers studied the microsomal metabolism of nitrosoureas and showed that

MeCCNU is a substrate for microsomal enzymes[105]. The products of the metabolic reaction are ring hydroxylated derivatives that were partially characterized by mass spectrometry. The rate of disappearance of MeCCNU is 1.48 nmoles/min/mg protein, which is much less than that of CCNU. The K_m value is 1.0 mM and V_{max} is 42.5 nmoles/min/mg protein. The MeCCNU metabolites from incubation with a rat liver microsomal preparation have been purified by HPLC and quantitated by Reed and May[107]. Among the metabolites (Table I), 34% was found to be hydroxylation of methyl group on the cyclohexyl ring, 20% was hydroxylated on the cyclohexyl ring in the cis-3-, trans-3-, and cis-4- positions. In addition to these products, MeCCNU was reported to be denitrosated to MeCCU (24%), and 5% was hydroxylation on the α carbon of the chloroethylmoiety. These metabolites were further characterized by NMR and mass spectrometry[108].

Table I **Structure of methyl CCNU metabolites**

Properties of CCNU

Structure	Structure
	
	
	Not identified
 Methyl-CCNU	

Denitrosation is more important in this analog since it accounts for 24% of the products. In CCNU metabolism, more than 90% occurs as hydroxy-

lation on the cyclohexyl moiety. However, the percentage of the total metabolism for MeCCNU compared to CCNU was very low, only 3-5%, and formation of these metabolites was not very significant.

Microsomes from rats pretreated with phenobarbital showed about a 3-fold increase in the hydroxylation rate while phenobarbital-treated mice microsomes were induced 8-fold. However, in both species, the induced hydroxylation rate was about 4 nmoles/min/mg protein. When microsomes from phenobarbital-induced rats were used a mixture of 80% CO:20% O_2 decreased the rate of formation of all metabolites to 14% of that in 80% N_2:20% O_2[108].

The metabolism of chloroethylnitrosoureas is dependent on the nature of the N-3 substituents. The only other position subjected to microsomal enzyme metabolism is the nitroso group. Both BCNU and MeCCNU are reported to be denitrosated to give the corresponding ureas. Methylene groups of the CCNU cyclohexyl ring are vulnerable to oxidation so that CCNU is metabolized primarily on the C-4 position with over 90% of the metabolic reaction occurring on the cyclohexyl moiety. When the 4-position is substituted with a methyl group, as in MeCCNU, this site of metabolism is blocked. Thus the rate of metabolism is decreased and oxidation of the methyl group adjacent to the aliphatic ring and ring hydroxylation occur along with denitrosation. BCNU has no favorable hydroxylation site. Metabolism is very slow and only denitrosation products are isolated. Denitrosation reactions have been shown to be one of the metabolic pathways for other nitroso compounds. N-Butylurea and N-(3-hydroxybutyl)urea were isolated from the urine of mice given radioactively labeled N-nitroso-N-butylurea[109]. In this case, only 5% of the dose was due to denitrosation and 60% of the radioactivity was excreted from expired air. Kawachi et al. reported when N-methyl-N'-nitro-N-nitrosoguanidine is given to rats, it is excreted in the urine as the denitrosated N-methyl-N'-nitroguanidine[110]. Tanada and

Sano reported that N-methyl-N'-nitroguanidine and nitroguanidine were the major urinary metabolites from rats given radioactive MNNG through a stomach tube[111].

The hydroxylated CCNU metabolites have altered physico-chemical properties but retain the chloroethylnitrosourea function. The metabolic reactions of CCNU lead to the hydroxylation of this compound which result in changes in physico-chemical properties. Increases in polarity will effect physiological disposition protein binding, excretion, and probably the antitumor activity. Johnston and coworkers[112] synthesized cis- and trans-4-hydroxy CCNU and investigated their antitumor activity. At LD_{10}, both metabolites cured essentially all animals of implanted ip or ic leukemia L1210. At non-toxic levels the metabolites are at least as active as CCNU but on a weight basis were more effective and more toxic. They suggested that the antitumor activity of CCNU is due primarily to its metabolites. Wheeler and coworkers[113] compared the properties of the six isomeric hydroxy CCNU's, all of which have been identified as metabolites of CCNU. There are significant differences in the physiochemical, chemical and biological properties of these metabolites and the properties of some of them are significantly different from those of CCNU. These properties are summerized in the following table (Table II).

All of the isomeric metabolites are effective against intracerebrally implanted L1210 leukemia and are more toxic than CCNU on a molar basis but have a slightly better therapeutic index. On a molar basis the trans-2-isomer has more than a two-fold greater lethal toxicity than CCNU, while the cis-2-, cis-3-, and cis-4-isomers are about as toxic as CCNU and the trans-3- and trans-4-isomers have intermediate toxicity. The metabolites that have high alkylating activities are the most toxic on a molar basis. Cis- and trans-2-hydroxy CCNU have the lowest carbamolylating activity which can

TableII Properties of metabolites of CCNU.

Compound	Log P*	$T_{\frac{1}{2}}$+	Per cent of value for CCNU						
			Alkylating activity ‡	Carbamoylating activity#	LD_{10}@	ED_{50}& i.p.	ED_{50}& i.c.	ED_{50}/LD_{10} i.p.	ED_{50}/LD_{10} i.c.
CCNU	2.83	100	100	100	100	100	100	0.77	0.73
Chlorozotocin	-1.02	46	489	4	38	30	-	0.62	-
cis-2-OH CCNU	1.06	76	177	1	98	56	56	0.44	0.42
cis-2-OAc CCNU	2.56	83	218	50	82	58	-	0.55	-
trans-2-OH CCNU	1.30	66	329	6	41	16	29	0.29	0.52
trans-2-OAc CCNU	2.56	92	213	100	60	30	-	0.39	-
trans,trans-2,6-diOH	-	70	577	0.3	33	10	33	0.24	0.72
cis-3-OH CCNU	1.25	91	133	21	98	47	72	0.37	0.54
trans-3-OH CCNU	1.28	84	112	61	74	41	53	0.42	0.53
cis-4-OH CCNU	1.11	93	136	104	90	54	51	0.47	0.41
trans-4-OH CCNU	1.00	87	140	100	82	36	48	0.33	0.43

* Logarithm of the distribution coefficient in the octanol-water system. as determined experimentally. calculated or estimated with the aid of high-pressure liquid chromatogrophy.
‡ Half-life in phosphate buffer. pH 7.4, 37°C. The half-life of CCNU under these conditions is 53 min.
‡ Alkylating activity measured by the absorbance of the solution containing the product obtained upon reaction with NBP. Equimolar quantities were used in the tests.
# Carbamoylating activity measured by the quantity of radioactivity present as reaction products after incubation of the compound with ^{14}C-L-lysine. Equimolar quantities were used in the tests.
@ LD_{10}'s for BDF_1 mice were calculated on a molar basis, and those molar doses were compared with the molar LD_{10} for CCNU.
& Dosage (single dose i.p. day 1 only) giving 50 per cent long term (45 days or more) survivors when 10^5 leukemia L1210 cells were implanted i.p. or 10^4 leukemia L1210 cells were implanted i.c. Dosages were expressed on a molar basis for comparisons.

be explained by intramolecular carbamoylation, but have similar LD_{10}, ED_{50} and therapeutic indices. The biological and biochemical properties of the 2-hydroxy metabolites of CCNU were further studied by Heal and co-workers[114]. In terms of molar doses resulting in the death of 10% normal mice, the cis- and trans-2-isomers were 2- and 3-fold more toxic than was CCNU in normal mice. In comparing the antitumor activity produced by a maximum nonlethal dose for each compound, the trans isomer had activity identical to that of CCNU (ILS 412%), and the cis isomer had considerably less activity (ILS 152%). The low carbamoylating activity of the 2-hydroxy metabolites was shown to have no association with the reduced myelotoxicity, although chlorozotocin, which has low carbamoylating activity, causes less bone marrow toxicity. The bone marrow toxicity was assayed by a murine model of the human bone marrow colony formation. The decrease in surviving colonies was not significantly different from that recorded with the parent compound.

Chapter III. ANALYTICAL CHEMISTRY

A lack of appropriate analytical methodology has limited study of the chemical and metabolic reactions of chloroethylnitrosoureas. These methods must have sufficient sensitivity to detect the parent chloroethylnitrosoureas, as well as metabolic and chemical degradation products at microgram and submicrogram/ml concentrations. Analytical techniques are limited by the chemical properties of the nitrosourea function. BCNU and CCNU are thermally unstable and decompose with gas evolution at about 100°C. In aqueous solution, they are reasonably stable between pH 3.5 and 5.5 ($t_{\frac{1}{2}}$ = 5 hr), but decompose more rapidly at pH 7.4 ($t_{\frac{1}{2}}$ = 50 min). As a consequence, these compounds are not amenable to gas chromatography but can be analyzed with high performance liquid chromatography. However, because of the low molar absorptivity of the nitrosourea chromophore (λ_{max} = 232, ε = 6000), chemical instability and sample-handling losses, this approach lacks the sensitivity required for human plasma analysis when variable wavelength detectors are used. A sensitivity limit of approximately 5 µg/ml serum is possible with this method. A colormetric method has been used for the analysis of nitrosoureas by the quantitative liberation of nitrous acid under strongly acidic conditions. The nitrous acid thus formed is determined by the Bratton-Marshall method[115] using sulfonilamide as a reagent[55]. This technique provides near-adequate sensitivity but lacks the specificity. A chemical ionization mass spectrometric method for the analysis of BCNU in biological samples was developed by Weinkam et al.[93] that has sufficient sensitivity for patient pharmacokinetic studies. A stable deuterium-labeled BCNU internal standard is used in this method. Selective ion monitoring is used to determine the relative amounts of the deuterated and non-deuterated BCNU protonated molecular ions after direct insertion of the sample extract

into the source.

This analytical technique was used for the analysis of BCNU. In this work, quantitative direct reaction mixture analysis methods using probe insertion chemical ionization mass spectrometry (CIMS) were developed for water soluble, nonvolatile chemical decomposition products. Gas chromatography selective ion monitoring was used for the assay of more volatile decomposition products. Reaction products from incubations in serum were analyzed by gas chromatography-mass spectrometry and metabolites formed in vitro and in vivo were analyzed by high performance liquid chromatography.

Analysis of BCNU by Chemical Ionization Mass Spectrometry

The spectrometric system used is a Finnigan 3200 Mass Spectrometer equipped with a dual electron impact/chemical-ionization source and operated in the chemical ionization mode using isobutane as a reagent gas. The spectrometer is interfaced with a Data General Corporation Nova 830 commercial computer that has a 64K core memory and a Diablo 44 10-megabyte dual disk system. Data output was provided by a Model 4010 terminal and hardcopy device. It has the capacity to monitor up to 6 ions simultaneously in the selected ion monitoring mode.

An octadeuterio analog of BCNU labeled on the four methylene positions (BCNU-d_8) is used as an internal standard. A standard solution of BCNU-d_8 in hexane, stored at -60°C, was calibrated with reference to known amounts of BCNU. For each experiment, a 0.2 to 1 ml sample of aqueous buffer, serum or incubation mixture is added to a vial containing known amount (an amount approximately equal to the sample) of internal standard from a stock solution. The sample is extracted with 1 to 3 ml ether three times. At times, the extraction of biological samples required centrifugation for 2 minutes at 2000 rpm in a Damon/IECPR-6000 centrifuge to achieve better

separation. The ether extracts were combined and stored at -60°C until analysis. The combined ether extracts were evaporated under nitrogen at room temperature before analysis. The residues were redissolved in a small amount (about 20 μl) of absolute ethanol or methylene chloride and an aliquot was placed on the ceramic tip of the direct-insertion probe. After evaporation of the solvent the sample was inserted into the mass spectrometer. Mass spectrometry parameters were established to monitor the protonated molecular ions, MH^+ 214 and 216 (BCNU-$^{35}Cl_2$ and $^{35}Cl^{37}Cl$) and 222 and 224 (BCNU-d_8-$^{35}Cl_2$ and $^{35}Cl^{37}Cl$). Dwell time on each channel was 75 msec with a total data acquisition time of 40 seconds. The probe was inserted to within 3 cm of the source block (110°C) where it was warmed by induction. After sufficient ion current had been generated, as indicated by the oscilloscope signal level, the probe was withdrawn. No isotope effect could be detected during the process of evaporation. Data display programs plot the total ion current accumulated during the dwell time, which is normalized for differences in dwell times between channels. Scale factor and baseline can be adjusted for each channel. Peak height ratios and peak areas also can be determined by using cursor-selected baseline points. A typical selected-ion monitoring scan for BCNU is shown in figure 5. Also shown are the peak height measurement lines used to determine the d_0/d_8 peak height ratio.

The protonated molecular ions of BCNU occur as MH^+ 214, 216 and 218, with a ratio close to 9.3/6.2/1.0, which is characteristic of a molecule containing two chlorine atoms in the ratio of their natural abundance. Figure 6 shows the mass spectra of plasma extract background and plasma containing 3 μg/ml BCNU. It is noted that the background does not interfere with the assay. A similar pattern is observed for BCNU-d_8 at MH^+ 222, 224 and 226. When the ratios of ions 214/216 and 222/224 are computed,

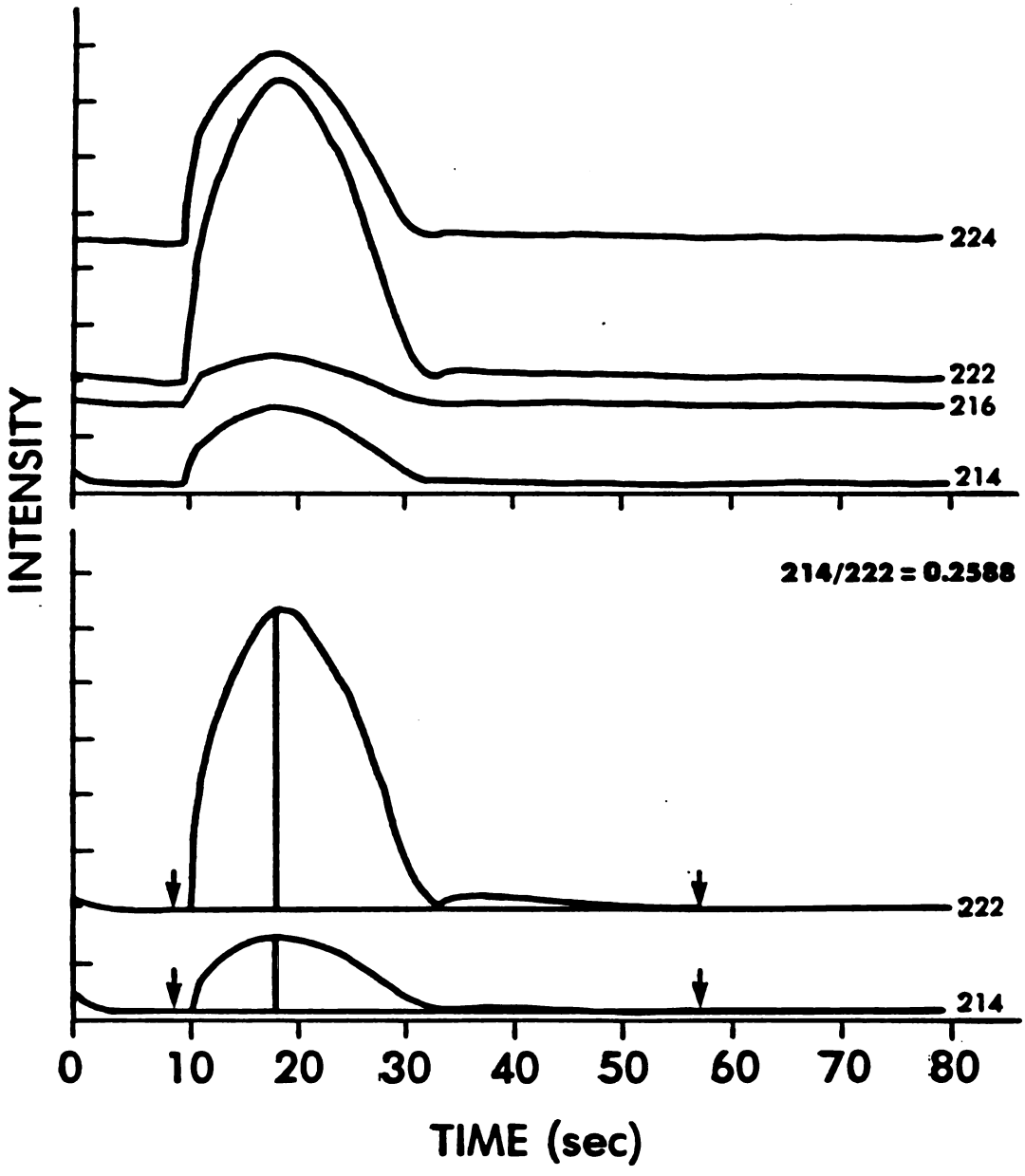


Figure 5. Selected-ion monitoring scan of BCNU showing the evaporation curves of the four monitored ions (upper) and the peak ratio determination of a pair of curves. Cursor-selected baseline points are indicated by small arrows. The base line and tangent to the peak maxima are drawn by computer; the computed peak-height ratio is 0.2588.

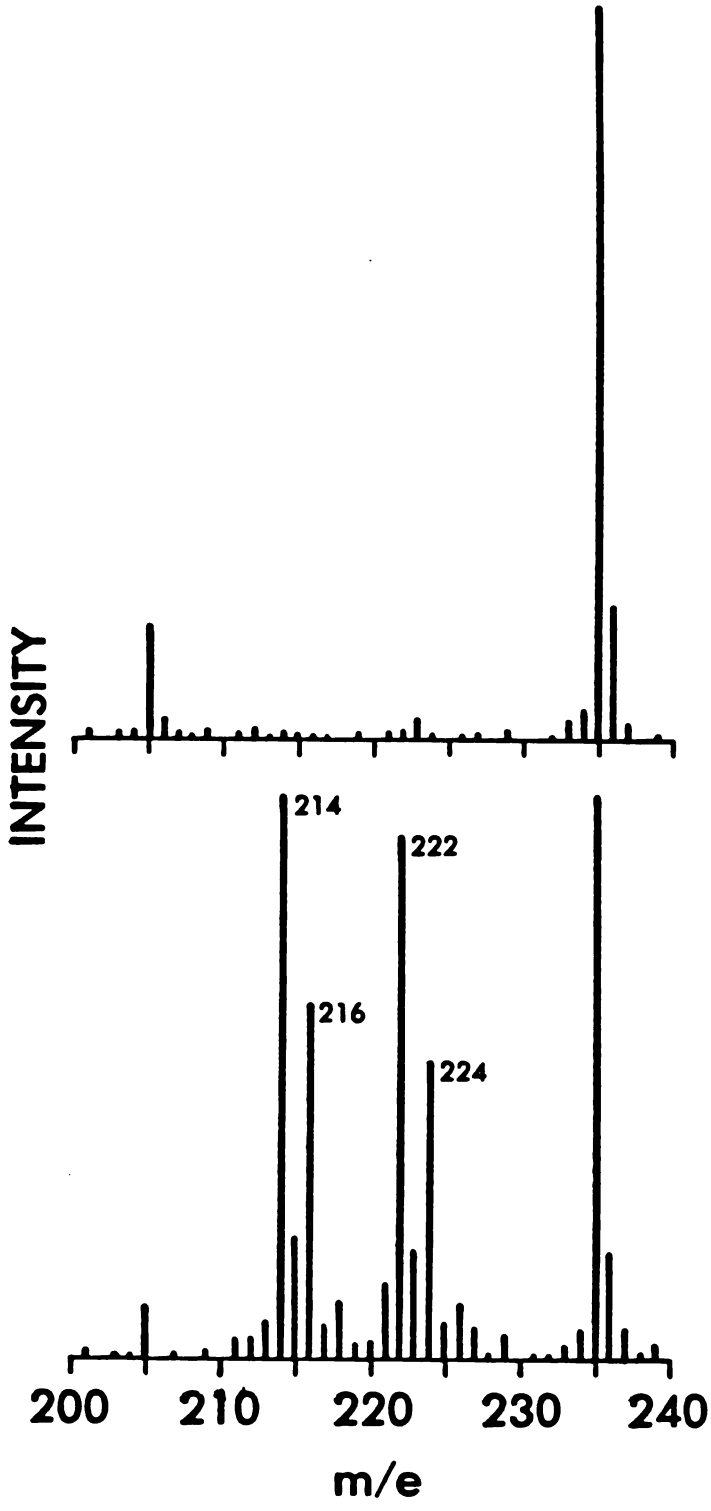


Figure 6. An isobutane CIMS scan of an ether extract of 0.5 ml plasma (upper) and plama containing BCNU and BCNU-d_8.

they should approximate 1.50, which is the ratio of the natural abundance of $^{35}Cl_2/^{35}Cl^{37}Cl$ ratio. The drug/standard ratio is determined from both 214/222 and 216/224 ratios, which should be identical. If any of these fail, it is an indication that some interfering ion current is being measured along with BCNU ions and that the integrity of the data point is questionable. The sensitivity limit for the mass spectrometer used in these analyses was about 10^{-13} mole, and the lower limit for the peak ratio measurement of pure BCNU and BCNU-d_8 mixture was 10^{-11} mole. This loss is caused by evaporation before sample insertion and thermal decomposition of BCNU in the ion source.

Direct Reaction Mixture Analysis by Probe Insertion Chemical Ionization Mass Spectrometry for Chemical Degradation Products

A new approach to quantitative analysis of a mixture of reaction products was developed. The BCNU chemical decomposition products in the reaction mixture was analyzed without prior separation by direct probe insertion chemical ionization mass spectrometry. Unreacted BCNU has to be removed because fragments interfere with the products in the mass spectra. Most of the volatile products will be extracted by ether and are measured separately. Since most of the volatile products have been quantitated by other researchers, this study was directed towards the assay of previously unidentified water soluble products.

Procedure

Mass spectra were obtained on a Finnigan Model 3200 Mass Spectrometer equipped with a chemical ionization source. Isobutane was used as a reagent gas (0.5 torr). Ion chamber temperature was maintained at 150°C. Samples were introduced on and evaporated from a direct insertion probe, a smooth quartz rod that was heated by conduction from the ion source block.

A Nova 830 computer was used to control selected ion monitoring and to perform data analysis.

A 5 to 10 ml aliquot was removed from the reaction mixture of BCNU in phosphate buffer and extracted with hexane to remove unreacted BCNU. A known amount of CCU as internal standard was added to the aliquot, and the mixture was lyophilized. The residue was dissolved in a small amount of methanol and placed on the direct insertion probe. At an ion chamber temperature of 150°C all compounds were exhaustively evaporated within 5 minutes (figure 7). Thermal degradation of the chloroethylureas and oxazolines was avoided and CI induced fragmentation was minimized under these conditions. Selected ion monitoring profiles of the molecular ion of each mixture component and a known amount of internal standard, CCU, were integrated over the 3-5 minute period of exhaustive sample evaporation. Reaction products were quantitated by reference to standard curves generated from measurement of the total ion current produced from known amounts of synthetic materials. The total ion current includes ion current from the molecular ion and fragments and their isotope ions. A correction factor for total ion current, which is based on individual CI spectra, is shown on Table III. The isobutane CI spectra relative ion intensities of the individual compounds are shown in Table IV. Table V shows the CI spectra using methane as reagent gas. A standard curve for each mixture component can be generated by integrating the evaporation profile of a binary mixture of standard and synthetic product (Table VI). Regression analysis shows that these curves are linear with high correlation coefficients. The average standard deviation of points from the curve is 0.09. Standard curves contained product-to-standard ratios between 0.5 and 15.0.

Simultaneous introduction of several compounds into a chemical ionization source may lead to interactions between protonated molecular ions

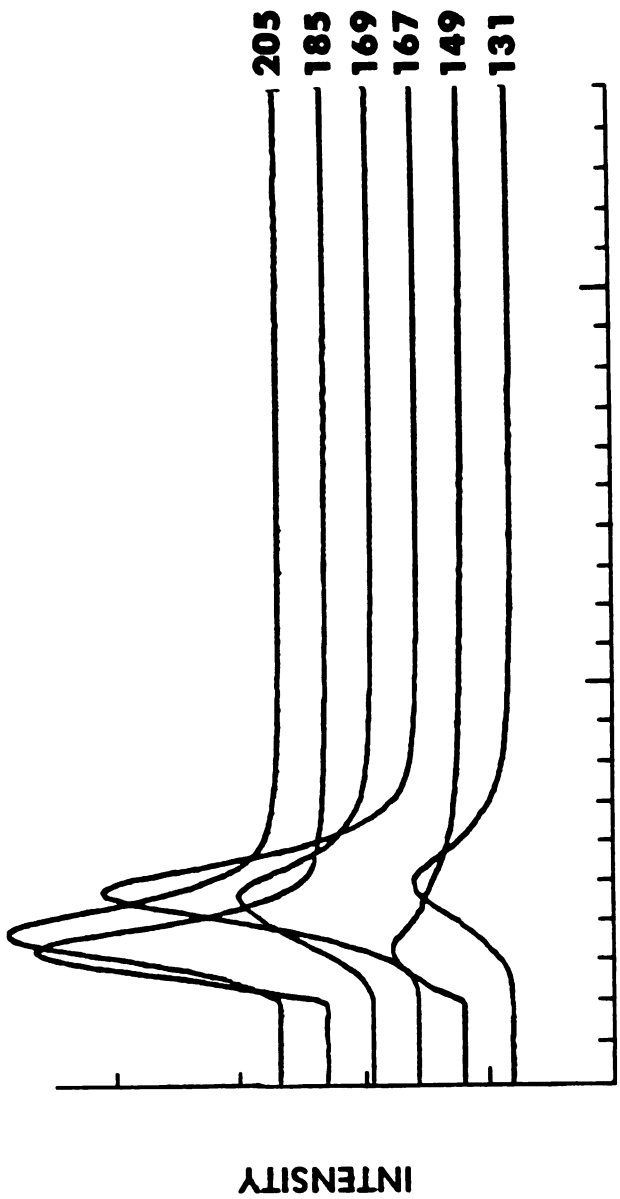


Figure 7. Selected ion monitoring evaporation profile obtained during the simultaneous introduction of five BCNU reaction mixture components into the CI source.

Table III Corrections for Total Ion Current

compound	ion monitored	No. of carbon	No. of chlorine	correction factor*
CCU	205	8	1	(205) x 1.4
	169	8	0	((169)-(167) x 0.33)[a] x 1.1
BCU	185	4	2	(185) x 1.8
	149	4	1	(185) x 0.3[b] x 1.4
HCU	167	4	1	(167) x 1.4
	131	4	0	(131) x 1.1
CAO	149	4	1	((149-(185) x 0.3)[c] x 1.4
oxazolidone	88	3	0	(88) x 1
chloroethyl-amine	80	2	1	(80) x 1.4

* correction factor for each ion includes ^{13}C and ^{37}Cl

a If HCU is also present in the reaction mixture, its ^{37}Cl ion has m/e of 169 too, that can be calculaed from molecular ion.

b The percentage of BCU fragmentation is constant under certain condition and is measured every time.

c Fragment of BCU account for ion 149 too.

Table IV Chemical Ionization Mass Spectra of Synthetic BCNU Reaction Products (Isobutane, 150°C)

compound	m/e (relative intensities[a], ion)
1-(2-chloroethyl)-3-cyclohexylurea (CCU)	205-208 (100,MH), 169-170 (6, MH-HCl)
1,3-bis(2-chloroethyl)urea (BCU)	185-190 (100, MH), 149-152 (6, MH-HCl)
1-(2-chloroethyl)-3-(2-hydroxyethyl)urea (CHU)	167-170 (100, MH), 149-152 (1, MH-H_2O), 131-132 (8, MH-HCl), 80 (3), 62 (15, $HOCH_2CH_2NH_3$)
1,3 bis(2-hydroxyethyl)urea (BHU)	149-150 (100, MH), 131-132 (2, MH-H_2O), 62 (12, $HOCH_2CH_2NH_3$)
2-oxazolidone	88-89 (100, MH)
chloroethylamine	80-83 (100, MH), 44 (2, MH-HCl)
2-(2-chloroethyl)-3-oxazoline (CAO)	149-152 (100, MH), 113-114 (6, MH-HCl), 86 (2)
2-(2-hydroxyethyl)-1,3-oxazoline (HAO)	131-132 (100, MH), 113-114 (3, MH-H_2O), 86 (10)

[a] Relative intensities include ^{13}C and ^{37}Cl isotope ions intensities.

Table V Chemical Ionization Mass Spectra of Synthetic BCNU Reaction Products (Methane, 150°C)

compound	m/e (relative intensities[a], ion)
CCU	205-208 (100, MH), 169-170 (52, MH-HCl), 123-125 (19), 98 (18), 87 (36), 80-82 (36)
BCU	185-190 (100, MH), 149-152 (78, MH-HCl), 113 (26, MH-2HCl), 80-82 (77)
CHU	167-170 (11, MH), 149-150 (5, MH-H_2O), 131 (26, MH-HCl), 80-82 (10), 62 (100, $HOCH_2CH_2NH_3$)
BHU	149-150 (10, MH), 131 (7, MH-H_2O), 118 (4), 102 (4), 90 (8), 88 (7), 62 (100, $HOCH_2CH_2NH_3$)
CAO	149-152 (76, MH), 113 (100, MH-HCl), 86 (29)
2-oxazolidone	88-89 (100, MH), 70 (3)

Table VI Standard Curves Based on Protonated Molecular Ion Current for Each Compound Relative to CCU, MH 205, vs. the Amount of Each Compound Relative to CCU

compound	slope	y-intercept	correlation coefficient	n
BCU	0.964	-0.37	0.977	10
CHU	0.499	-0.10	0.996	6
BHU	0.570	-0.18	0.972	10
CAO	0.116	0.11	0.991	9
2-oxazolidone	0.447	-0.21	0.986	7
chloroethylamine	1.22	-0.50	0.989	7

and neutral molecules of the same or different structure. Hydrogen bonded complexes may form ($MH^+ + M' \longrightarrow (MHM')^+$), and exothermal proton exchange ($MH^+ + M' \longrightarrow M + M'H^+$), may occur if neutral molecule partial pressure in the ionization chamber is high enough to allow collisions with MH^+ ions. Both interactions may be avoided by limiting the amount and rate at which sample or mixture components are introduced into the ion source. Since the BCNU mixture contains more than one product, it was necessary to determine if the set of standard curves obtained from binary mixtures can be applied to a mixture containing three or more components. A mixture of known amounts of three components and CCU was introduced as quaternary mixture to the mass spectrometer following the same procedure. Peak height ratios of total ion currents for each component were measured and compared with that of binary mixtures. Figure 8 shows representative peak ratios obtained from mixtures containing three or more components. The standard deviation of these points from curves obtained from binary mixtures is 0.17 (Table VI). The linearity and slopes of linear regression fits of these ratios are in agreement with binary mixture standard curves. In some cases the ion intensity ratios were corrected for the presence of minor fragment ions having the same mass (Table III).

Discussion

These results show the usefulness of direct probe insertion mass spectrometry as a method for quantitative analysis of reaction mixtures provided that each of the mixture component is available for use in generating a standard curve. The slopes of the standard curves vary over a ten-fold range, which reflects the relative ionization efficiency of each compound (Table VII). Mass discrimination at the electron multiplier and mass filter are minor factors. The probability of proton transfer from the

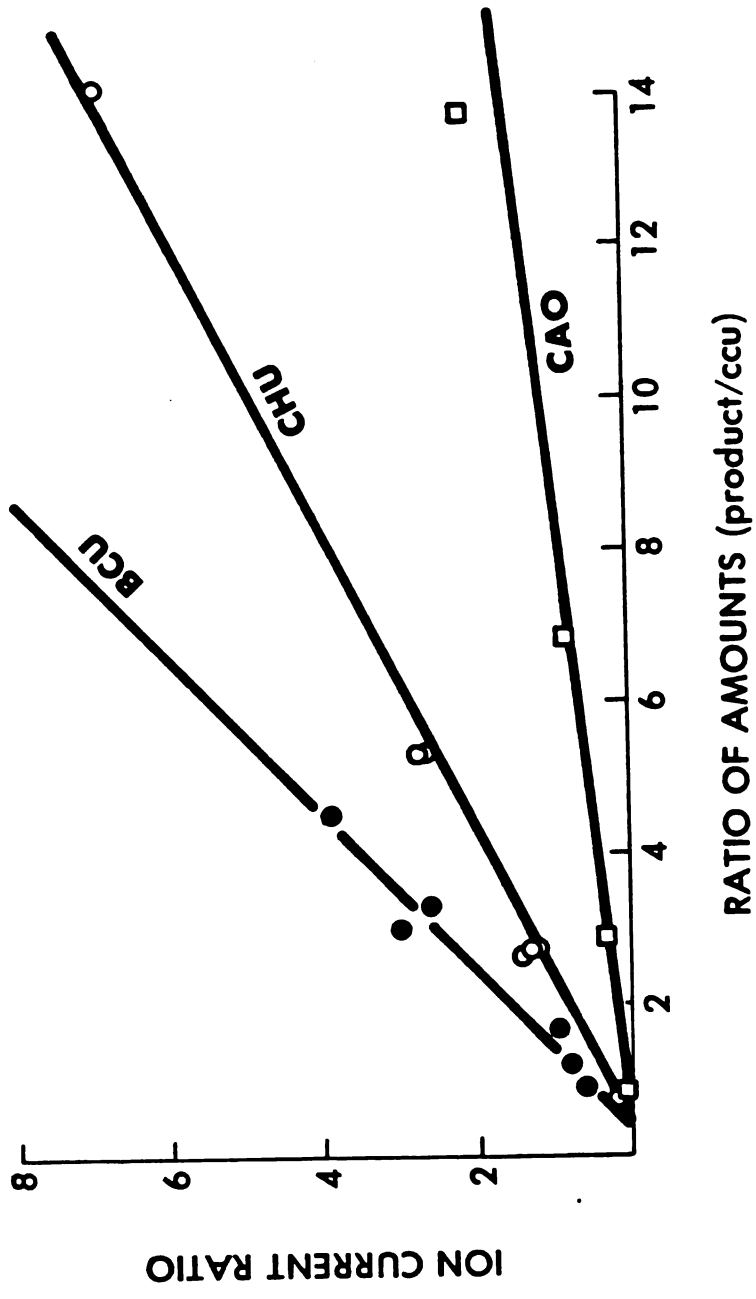


Figure 8. Standard curves for BCU, CHU and CAO obtained from binary mixtures, and data points from the same compounds introduced as quaternary mixtures.

Table VII Ionization Efficiency Relative to 1-(2-Chloroethy)-3-cyclohexylurea

compound	MH^+	ionization efficiency	std. dev., %	n
CCU	205	1.00	-	
BCU	185	1.14	0.18	6
CHU	167	0.51	0.06	6
EHU	149	0.39	0.16	10
CAO	149	0.10	0.09	5
2-oxazolidone	88	0.25	0.03	7
chlorethylamine	80	0.91	0.19	6

reagent ion $C_4H_9^+$ to each component is apparently dependent on molecular structure. Each of these compounds should have a higher proton affinity than isobutylene, the conjugate base of the reagent gas ion and should, therefore, be protonated efficiently. Chloroethylamine and CAO are both protonated in neutral aqueous solutions, but the respective ionization efficiencies are 0.91 and 0.10. The ionization efficiency of CAO is increased slightly from 0.1 to 0.21 using methane as the reagent gas (Figure 9). The ionization efficiencies of four closely related ureas are also different. These results suggest that a significant quantitative error may be encountered if the ionization efficiencies of apparently similar compounds are assumed to be equal.

Protonation efficiencies of the synthetic materials may be determined by monitoring the fragment ions in addition to the protonated molecular ion of each component and by including isotope peaks in the total ion current. Association ions $(M-C_4H_9)^+$, and $M-H^+$ ions were less than 1% of the total ion current. The mass range of ions monitored in these experiments was m/e 80 to 225. It should be noted that the transmission efficiency of a quadrupole mass filter may change for ions of different mass. Care must be taken to maintain the instrument in a consistent operating condition so that the slopes of the standard curves and apparent relative ionization efficiencies are not altered. Slopes of standard curves for known amounts of each mixture component can be compared to determine the relative protonation efficiencies of these compounds. These slopes showed product-to-standard ratios between 0.5 and 15.0 (Table VI) indicating that there was a wide range of protonation efficiency for these structures and that a standard curve must be obtained for each component that is to be quantified.

Chemical and physical properties limit the range of application of this quantitative approach to relatively nonvolatile compounds and compounds that

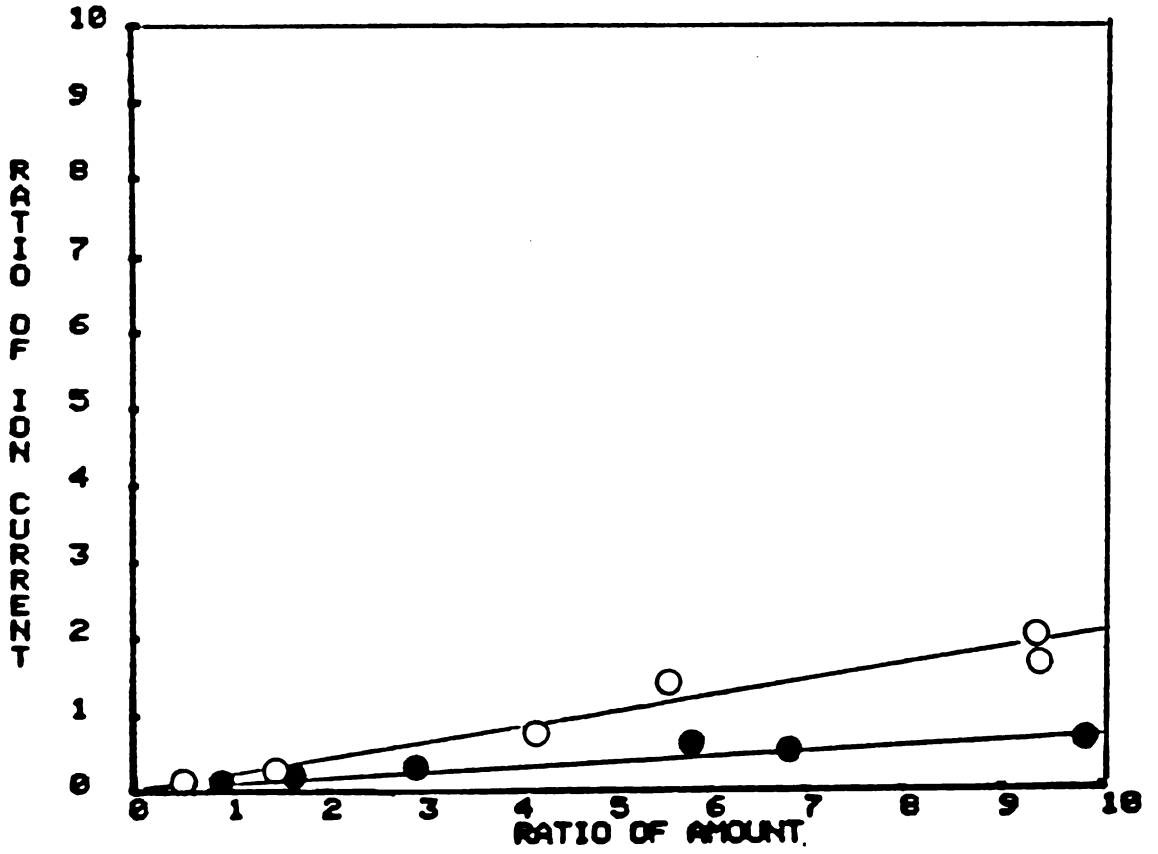


Figure 9. Total ion current of 2-(2-chloroethylamino)-2-oxazoline following ionization with methane (○) and isobutane (●).

can be volatilized reproducibly from a direct insertion probe. Chloroethylamine must be introduced as a salt to avoid losses due to evaporation during the probe insertion process. Sample loss due to evaporation may be indicated by an instantaneous rise in the SIM profile and by a negative y-intercept in a standard curve. Incomplete evaporation of a compound due to decomposition leading to nonvolatile products is more difficult to detect. If it leads to insufficient sensitivity or irreproducible evaporation characteristics, this approach may not be applicable. 2-(2-Chloroethylamino)-2-oxazoline hydrochloride decomposes on heating to give BCU. The possibility that this compound may be decomposing on the probe to give nonvolatile products was considered; however, the same standard curve was obtained when this compound was introduced as the more stable and volatile free base. An aqueous mixture containing known amounts of five components was prepared and analyzed using the same procedure needed for the analysis of a BCNU chemical reaction mixture (Table VIII). There was no significant interfering ion current present. The method of quantification was able to provide an accuracy ($\pm$ 15-20%) that was suitable for the analysis of small amounts of product produced in this complex reaction mixture.

A major advantage inherent in the use of this method of analysis is the speed with which the assay can be developed and data acquired. This approach may not be suitable to the analysis of unknown reaction products unless the factors that determine the ionization efficiency of CI reagent gases are understood.

Analysis of 2-Chloroethanol by Gas Chromatography

Gas chromatography was performed on a Varian 2100 chromatograph equipped with flame ionization detector. Quantification of 2-chloroethanol was performed using a KOH-2% Carbowax 20M on 100/120 Gas-Chrom Q column. The assay was conducted at column temperature of 70^{o}C, injector and

Table VIII Analysis of a Known Aqueous Mixture of BCNU Reaction Products

component	mixture		observed	
	nM	(% total)	nM	(% total)
BCU	2.79	(25.5)	2.84 ± 0.44	(25.4)
HCU	2.35	(21.5)	2.35 ± 0.27	(21.1)
BHU	3.01	(27.6)	2.87 ± 0.44	(25.8)
2-oxazolidone	2.29	(21.0)	2.26 ± 0.27	(20.3)
CAO[a]	0.48	(4.4)	0.82 ± 0.11	(7.4)
total	10.92		11.14	

[a] BCU cyclizes to give CAO in aqueous solution.

detector temperature of 120°C. Helium was used as carrier gas. Cyclohexanol was used as internal standard and the peak height ratios were measured. A standard curve was obtained by plotting the 2-chloroethanol/cyclohexanol peak height ratio against the ratio of amounts of 2-chloroethanol and internal standard. Standard curves cover a range of 0.3 to 9.6 (Figure 10). 2-Chloroethylamine can be quantified under the same conditions and with cyclohexanol as the internal standard. The standard curve is shown in Figure 10. Table IX shows the mixture of 2-chloroethanol, 2-chloroethylamine and cyclohexanol used to generate their standard curves. The gas chromatograph of these compounds is shown in Figure 11.

Procedure

An aliquot of 1 to 2 ml of reaction mixture was added to a known amount of cyclohexanol and extracted with ether. The ether extract was directly injected into the gas chromatography.

Direct insertion mass spectrometry is not useful for the analysis of 2-chloroethanol because it is too volatile. BCNU, which is also extracted by ether, does not interfere with 2-chloroethanol under these experimental conditions. Injection of BCNU to the column at the temperature used gave no peaks. Quantification of 2-chloroethylamine can be done by the same procedure, if the reaction mixture is basified before ether extraction.

Analysis of BCNU Reaction Products by Selected Ion Monitoring Gas-Liquid Chromatography-Mass Spectrometry

BCNU reaction products formed in the presence of bovine serum albumin or human serum were analyzed by a GC-MS system. These reaction mixtures contained high concentrations of interfering material that prevented the use of direct insertion mass spectrometry methods. The mass spectrometer used in this system is a Finnigan 3200, which is connected with a gas liquid

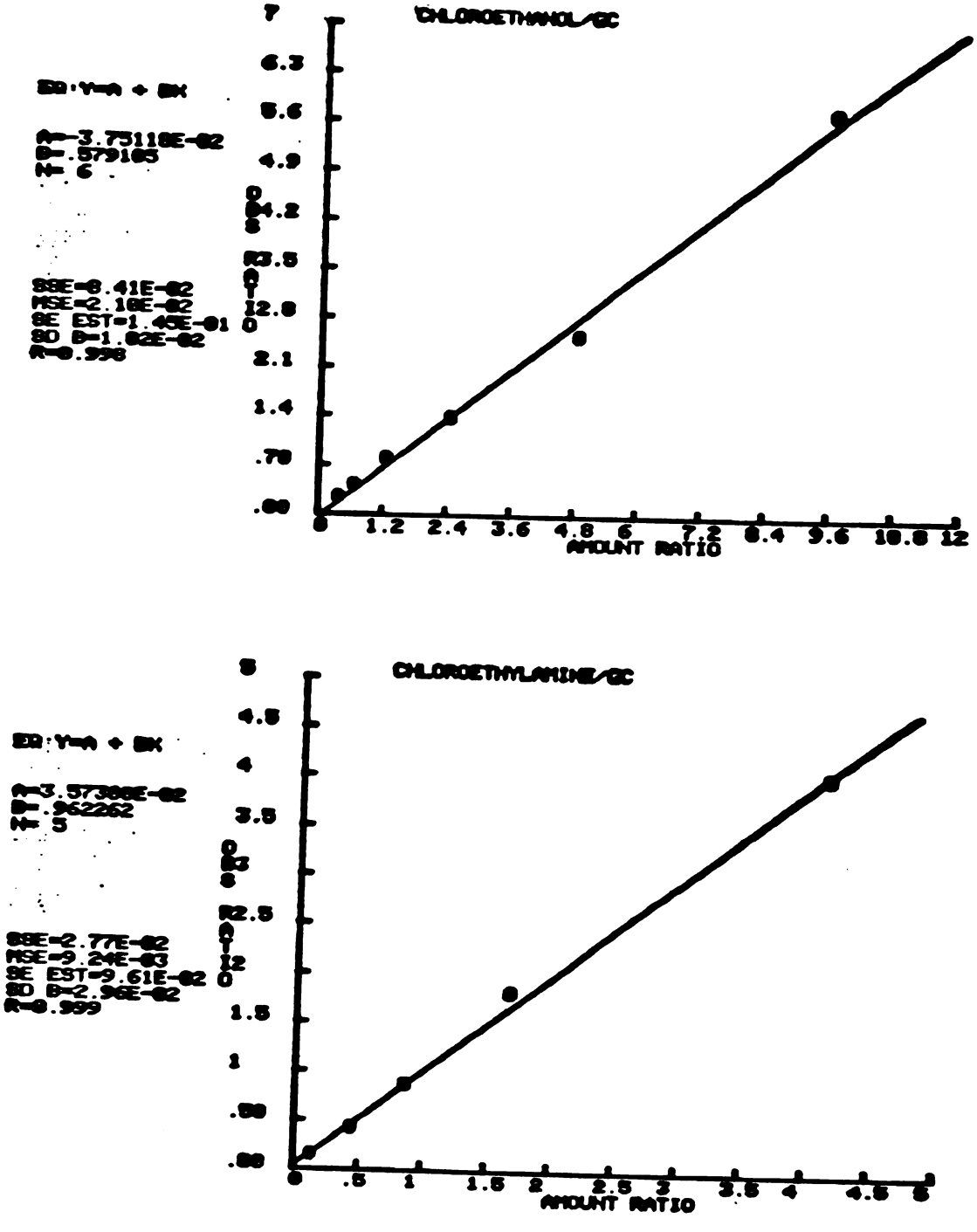


Figure 10. Standard curves for chloroethanol (upper) and chloroethylamine by gas liquid chromatography using cyclohexanol as the internal standard.

Table IX Mixture of 2-Chloroethanol and 2-Chloroethylamine and Their Internal Standard Cyclohexanol Used to Generate Their Standard Curves

2-chloroethanol	calc. amount ratio	obs. pk ht ratio
	0.3	0.18
	0.6	0.36
	1.2	0.73
	2.4	1.31
	4.8	2.5
	9.6	5.64

2-chloroethylamine	calc. amount ratio	obs. pk ht ratio
	0.1	0.10
	0.41	0.38
	0.83	0.82
	1.65	1.77
	4.13	3.96

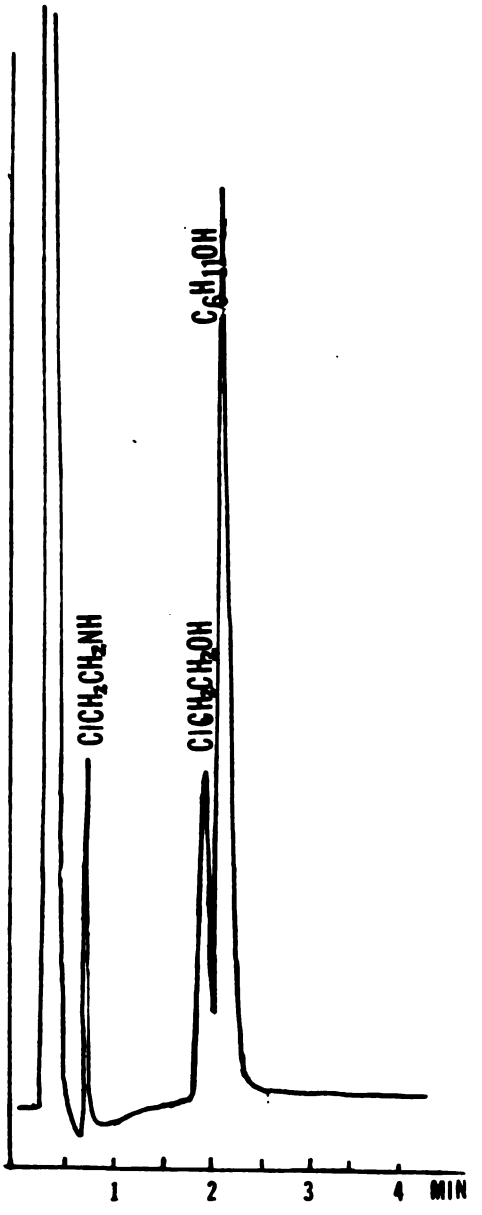


Figure 11. Gas liquid chromatograph of 2-chloroethanol and 2-chloroethylamine and their internal standard cyclohexanol. Column: 2% KOH-2% Carbowax 20M on 100/120 Gas Chrom Q. Temperature: 90°C.

chromatography Finnigan 9500. Data were displayed on a Linear Model 385 recorder as total ion current and also interfaced with a computer system Nova 830. Figure 12 shows the configuration of the GC-MS system. Chromatography was performed using a 2% KOH-2% Carbowax 20M on 100/120 Gas-Chrom Q column with methane carrier and reagent gas for mass spectrometry.

Selected ion monitoring chemical ionization technique was used for the analysis of water soluble products. Benzyl alcohol was used as internal standard. Four ions with m/e 80, 88, 91, 113 corresponding to chloroethylamine, 2-oxazolidone, benzyl alcohol, and BCU (Table X), were monitored and the total data acquisition time was 15 minutes. The column temperature was initially set at 70^{o}C and jumped to 200^{o}C after the first peak, chloroethylamine, was eluted. Temperature of the separater and transfer line were kept at 200^{o}C. Gas pressure was 0.6 torr. Cyclohexanol was used as internal standard for the analysis of the ether soluble product, chloroethanol. These ions, the first from chloroethanol with a m/e of 63 and the latter two from cyclohexanol with m/e of 81 and 83 were monitored. The total acquisition time was 6 minutes. A typical spectrum of chloroethanol and cyclohexanol is shown in Figure 13. Some of the ions monitored were not molecular ions, but dehydrated or dehydrochlorinated products. Table X shows the structures and m/e of the monitored ions.

Peak areas of each ion were integrated and the ratio of each ion to the ion of standard compound was calculated. Standard curves were obtained for each compound by plotting the ratio of the peak area against calculated amounts (Figure 14).

Procedure

Chloroethanol was analyzed by adding a known amount of cyclohexanol to a 100 µl sample aliquot and 60 µl of ether. After thorough vortexing

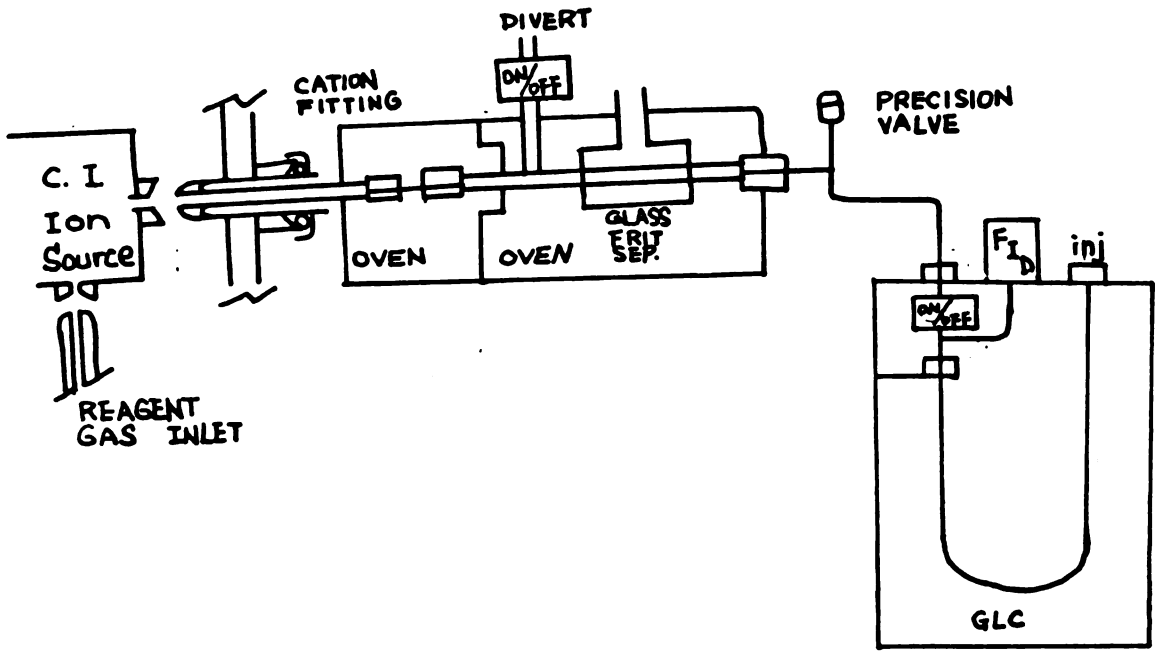
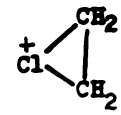
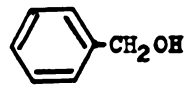
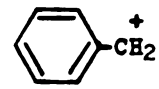
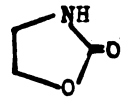
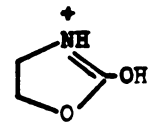
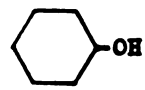
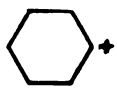
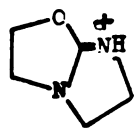
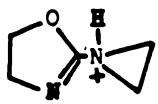


Figure 12. Configuration of the GC-MS system.

Table I Ions Monitored for the Selected Ion Monitoring Gas Chromatography-Mass Spectrometry

compound	monitored ion	emp. formula	m/e
$H_2NCH_2CH_2Cl$	$H_3\overset{+}{N}CH_2CH_2Cl$	C_2H_7ClN	80
$HOCH_2CH_2Cl$		C_2H_4Cl	63
		C_7H_7	91
		$C_3H_6NO_2$	88
		C_6H_{11}	83
BCU		$C_5H_9N_2O$	113
			

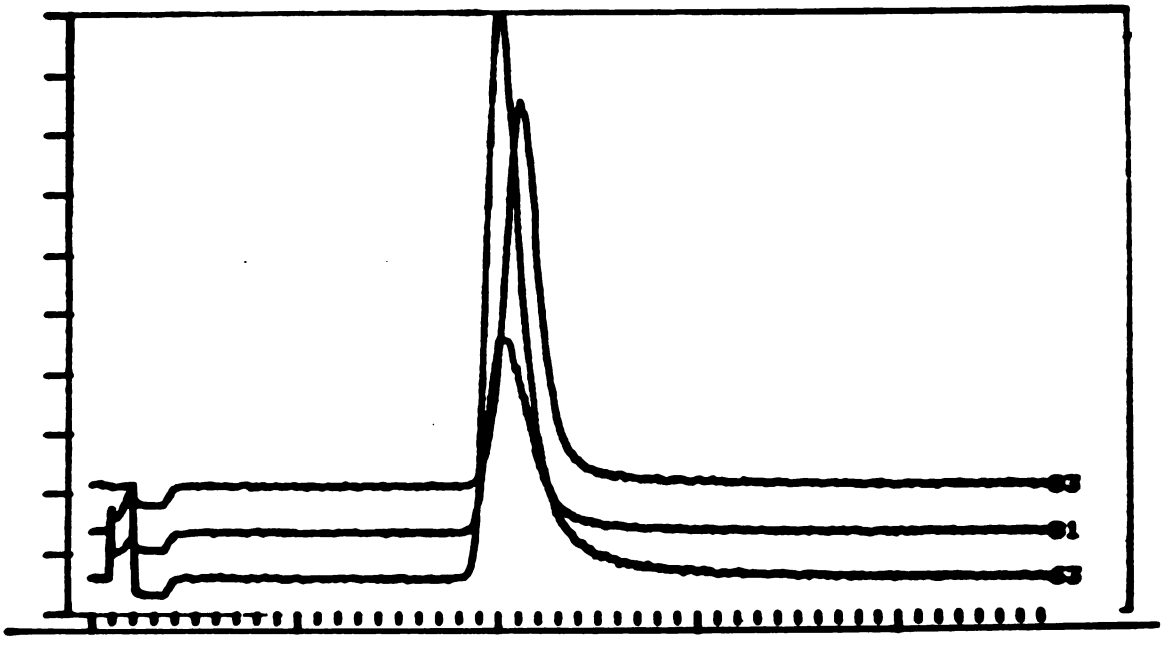


Figure 13. Evaporation curves of chloroethanol and cyclohexanol from GC-MS.

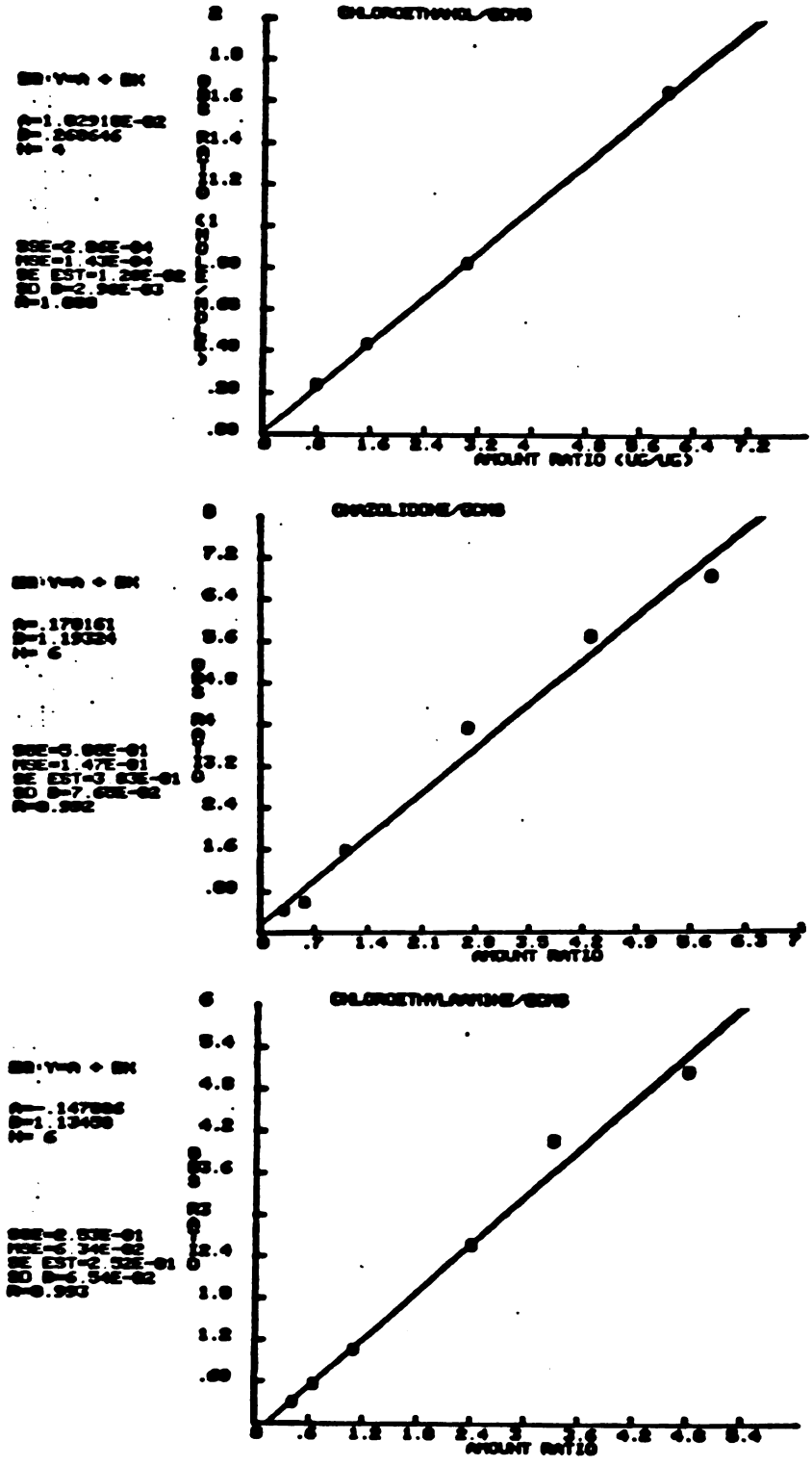


Figure 14. Standard curves for chloroethanol, chloroethylamine and oxazolidone obtained from GC-MS system.

and phase separation, 1 µl of the ether layer was injected into the GC-MS. The peak area of ions 63 to 83 was measured and the ratio determined.

Water soluble products, such as chloroethylamine, 2-oxazolidone and BCU, were analyzed from 250 µl aliquots following extraction with hexane to remove unreacted BCNU. Approximately 3 ml of methanol was added to the aqueous solution and filtered through a glasswool filter to remove the precipitated protein. The methanol solution was concentrated under nitrogen gas and a known amount of benzyl alcohol was added as an internal standard. The ratios of peak areas for each ion of interest to the ion of standard compound were determined.

Discussion

The GC-MS quantitative system has the advantage that each compound was separated before entering the ion chamber of the mass spectrometer. Retention times on the glc column and the fragmentation pattern of mass spectra provided qualitative information. Quantitative analysis can be achieved at the same time by measuring the peak area ratios. Compounds which are poorly resolved on the glc column can be quantitated by monitoring different fragment ions. The sensitivity limit of the SIM-GC-MS is around 100 picograms of material injected on the column.

Efforts have been made to measure the reaction products by a single injection. At the column temperatures used, unreacted BCNU in the reaction mixture interfered with the measurements of reaction products, namely ions with a m/e ratio at 88 and 113 (Figure 15). Therefore, it is necessary to remove any unreacted BCNU from the reaction mixture before analysis. Figure 16 shows SIM-GC-MS evaporation profiles from standard compounds (a) and the reaction mixture (b) at an initial column temperature of 70°C which was later raised to 200°C. When the temperature of the column was

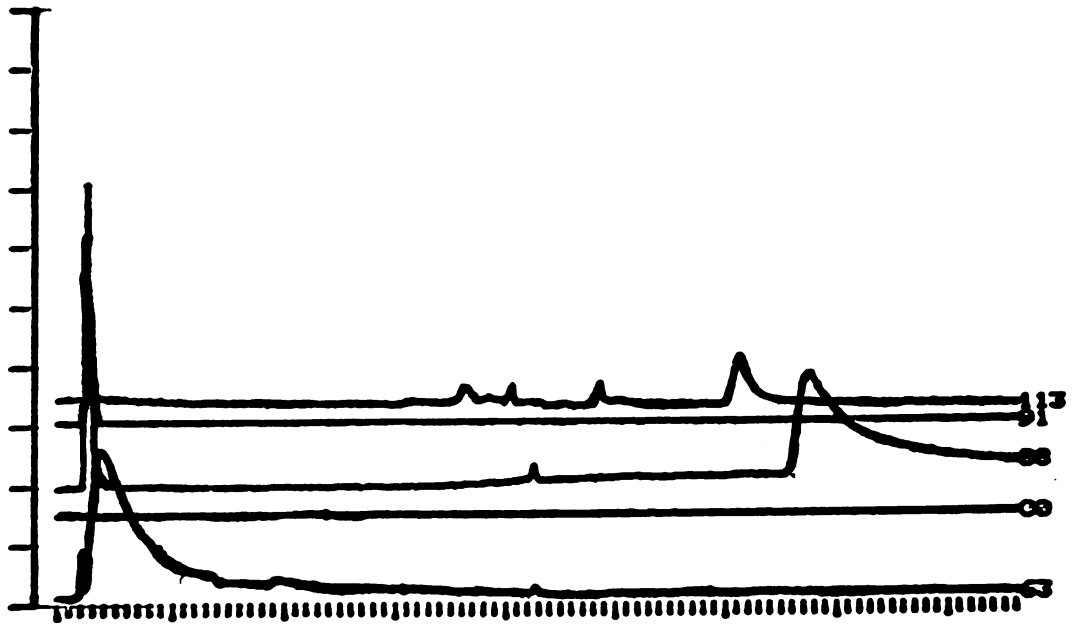


Figure 15. SIM-GC-MS evaporation profiles of BCNU with injector at 90°C, shows interfering with ions 113 and 88.

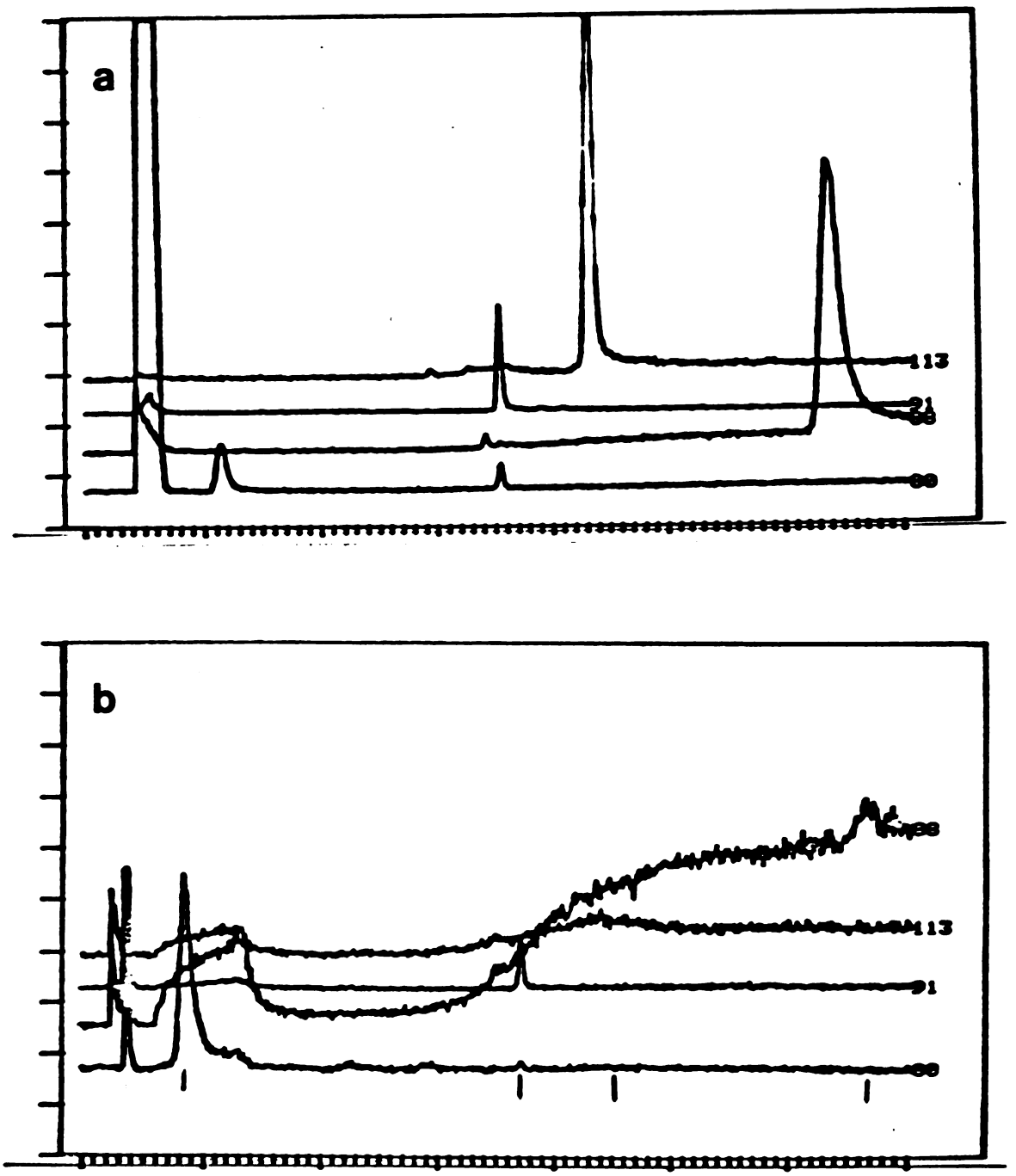


Figure 16. SIM-GC-MS evaporation profiles from (a) a mixture of standard compounds of chloroethylamine, 2-oxazolidone, BCU and benzyl alcohol, (b) a BCNU reaction mixture.

Chapter IV. CHEMICAL REACTIONS OF BCNU, CCNU IN PHOSPHATE BUFFER

A. Introduction

BCNU and other chloroethylnitrosoureas are not biologically reactive in their parent form, but generate alkylating intermediates through chemical reactions in aqueous media. The active alkylating agent is thought to be 2-chloroethylazohydroxide or diazonium ion. The reaction of BCNU in aqueous solution at physiological temperature and pH determines the fraction of parent drug subject to activation and degradation processes. The rate of BCNU decomposition is highly dependent on pH. BCNU is relatively stable at pH 4-5, where its half life exceeds 500 min, but it degrades rapidly under basic conditions, with a half life of 5 minutes at pH 8.0. BCNU disappearance at pH 7.4 is first order with a half life of 50 min. The rate of disappearance is independent of buffer and salt effects, but shows specific general base catalysis.

Colvin and coworkers have analyzed the volatile reaction products generated from the 1-(2-chloroethyl) moiety of BCNU (5×10^{-2}M) at pH 7.4 and 37°C in phosphate buffer containing 0.1 M NaCl[64]. 2-Chloroethanol (< 32%) and acetaldehyde (< 16%) were identified as major products. Vinyl chloride (< 2%) and 1,2-dichloroethane (< 1%) were observed as minor products. Approximately 50% of the radioactivity was present as nonvolatile material and was not identified. Montgomery and coworkers have confirmed these results; they isolated 2-chloroethanol (40%) and acetaldehyde (20%) after 2 hours at 50°C. 2-Chloroethylamine and 1,3-bis(2-chloroethyl)urea were also identified as products[63].

The reaction of CCNU in phosphate buffer, 6×10^{-4}M, is similar to BCNU. 2-Chloroethanol (18-25%), acetaldehyde (5-10%) and cyclohexylamine (32%) are formed after 3 hours at 37°C. CCNU has a half-life of 48 min

under these conditions. The products are similar to those observed for other alkyl nitrosoureas.

The yields of these products from 2-chloroethylnitrosourea reactions are lower than those reported from other alkyl nitrosoureas under the same reaction conditions[116]. Only a fraction (< 45%) of BCNU and CCNU reaction products have been identified, and unidentified nonvolatile products are known to be formed. Additional chloroethylnitrosourea reactions that were determined through the analysis of nonvolatile reaction products are discussed in the following section.

B. Experimental Methods

1. Synthesis of Standard Compounds - Possible Decomposition Products of BCNU

1,3-Bis(2-chloroethyl)urea (BCU), 1-(2-chloroethyl)-3-cyclohexylurea (CCU), 1,3-bis(2-hydroxyethyl)urea (BHU), 1-(2-chloroethyl)-3-(2-hydroxyethyl)urea (CHU), 2-(chloroethylamino)-2-oxazoline (CAO), and 2-cyclohexyl-2-oxazoline were synthesized for identification of the reaction products and as standards in quantitative analysis. The syntheses of these compounds are based on previously reported methods for similar compounds. The purpose of each synthetic procedure is to obtain the desired compound in pure form and in sufficient quantities for the studies and therefore optimization of reaction conditions was not always pursued. Better synthetic procedures might be devised in many cases.

Proton magnetic resonance (PMR) spectra were obtained on Varian A-60 and XL-100 spectrometers. Mass spectra were performed using a Finnigan 3200 mass spectrometer operated in the chemical ionization mode with isobutane reagent gas. HPLC utilized an Altex pump with a Waters C_{18}-uBondapak column. Column effluent was monitored at 205 nm using a

Hitachi 100-10 spectrophotometer. PMR data are reported as ppm downfield from TMS = 0. CIMS data are reported as the nominal mass m/e. Relative intensity values include ^{13}C and ^{37}Cl isotope peaks.

1,3-Bis(2-chloroethyl)urea, BCU. 2-Chloroethylisocyanate (1.97 g, 18.7 mmol) was added to a filtered solution of 2-chloroethylamine hydrochloride (2.12 g, 18.2 mmol) and potassium hydroxide (1.24 g) in 5 ml of isopropanol. The solution was stirred for two hours at room temperature and solvent evaporated under reduced pressure. The solid residue was washed with water and crystallized from methanol-ether (1:1,v/v) to give 3.4 g, 18.3 mmol, of BCU, mp 126.5-127.5 (Lit [117] 126.5-128°C). PMR (DMSO-d_6): 3.3 (CH_2N), 3.5 (CH_2Cl), 6.1-6.1 (NH). CIMS (isobutane, 150°C), m/e (rel. int.), 185 (100), 149 (6).

1-(2-Chloroethyl)-3-cyclohexylurea, CCU, was prepared from cyclohexylisocyanate and 2-chloroethylamine hydrochloride using the above procedure, mp 120-121°C (Lit [118] 130-132°C). PMR (DMSO-d_6): 3.3(CH_2N), 3.6 (CH_2Cl), 6.6 (NH)(NCH), 0.9-1.9 (C_6H_{10}). CIMS (isobutane, 150°C), m/e (rel. int.), 205 (100), 169 (6).

1,3-Bis(2-hydroxyethyl)urea, BHU, was prepared from 2-aminoethanol and 2-oxazolidone according to Brundrett et al.[13], mp 86-88°C (Lit [119] 86°C). PMR (DMSO-d_6): 3.1 (CH_2O), 3.4 (CH_2N), 4.7 (OH), 6.1 (NH). CIMS (isobutane, 150°C),m/e (rel. int.) 149 (100), 132 (2), 80 (3), 62 (15).

1-(2-Chloroethyl)-3-(2-hydroxyethyl)urea, CHU, was prepared from 2-aminoethanol and 2-chloroethylisocyante using the above procedure, mp 83-85°C. PMR (DMSO-d_6) : 3.1 (CH_2O), 3.3-3.5 (CH_2N), 3.5 (CH_2Cl), 6.3 (NH) (Figure 17). CIMS (isobutane, 150°C), m/e (rel. int.), 167 (100), 149 (1), 131 (8), 62 (15) (Figure 18).

2-(Chloroethylamino)-2-oxazoline, CAO, was prepared in 50% yield from BCU following the method of Kreling and McKay[120], mp 103-104°C and 118-

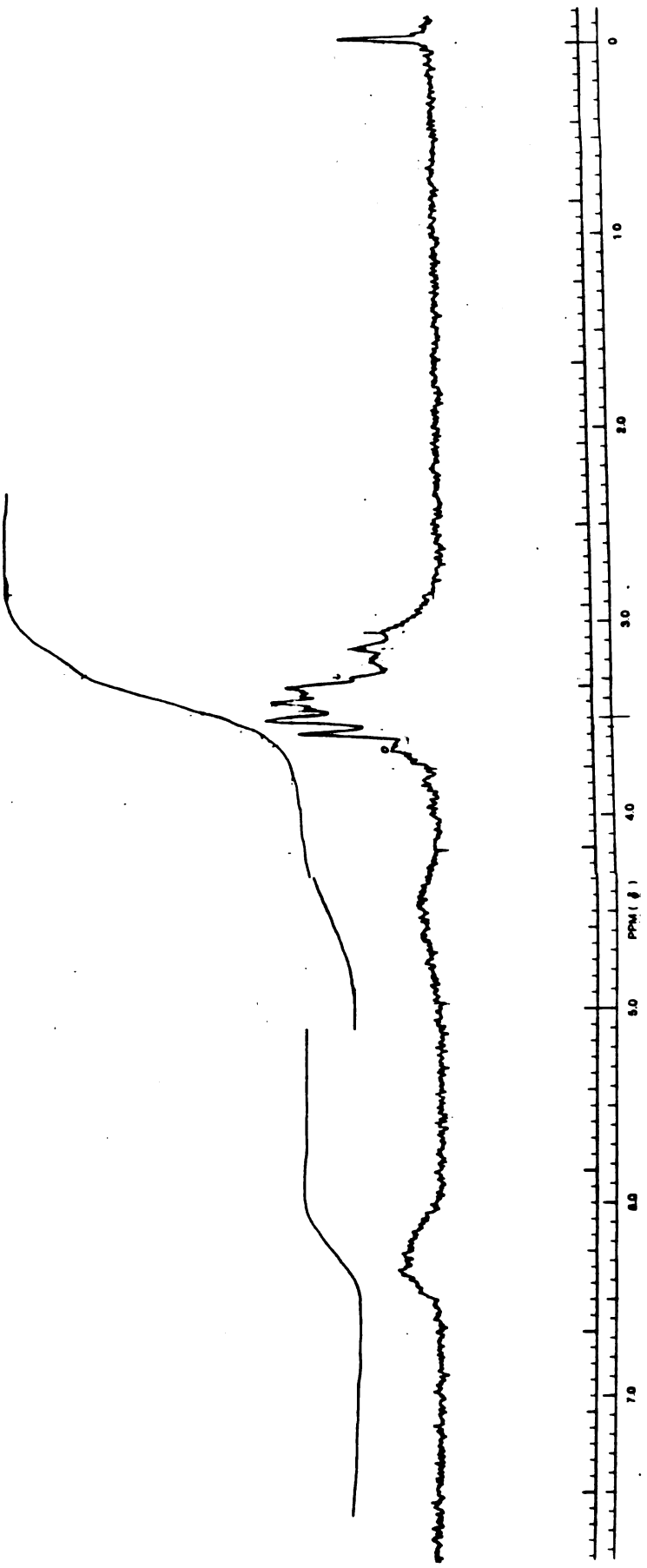


Figure 17. NMR spectrum of CEU, (1-(2-chloroethy)-3-(2-hydroxy-ethyl)urea).

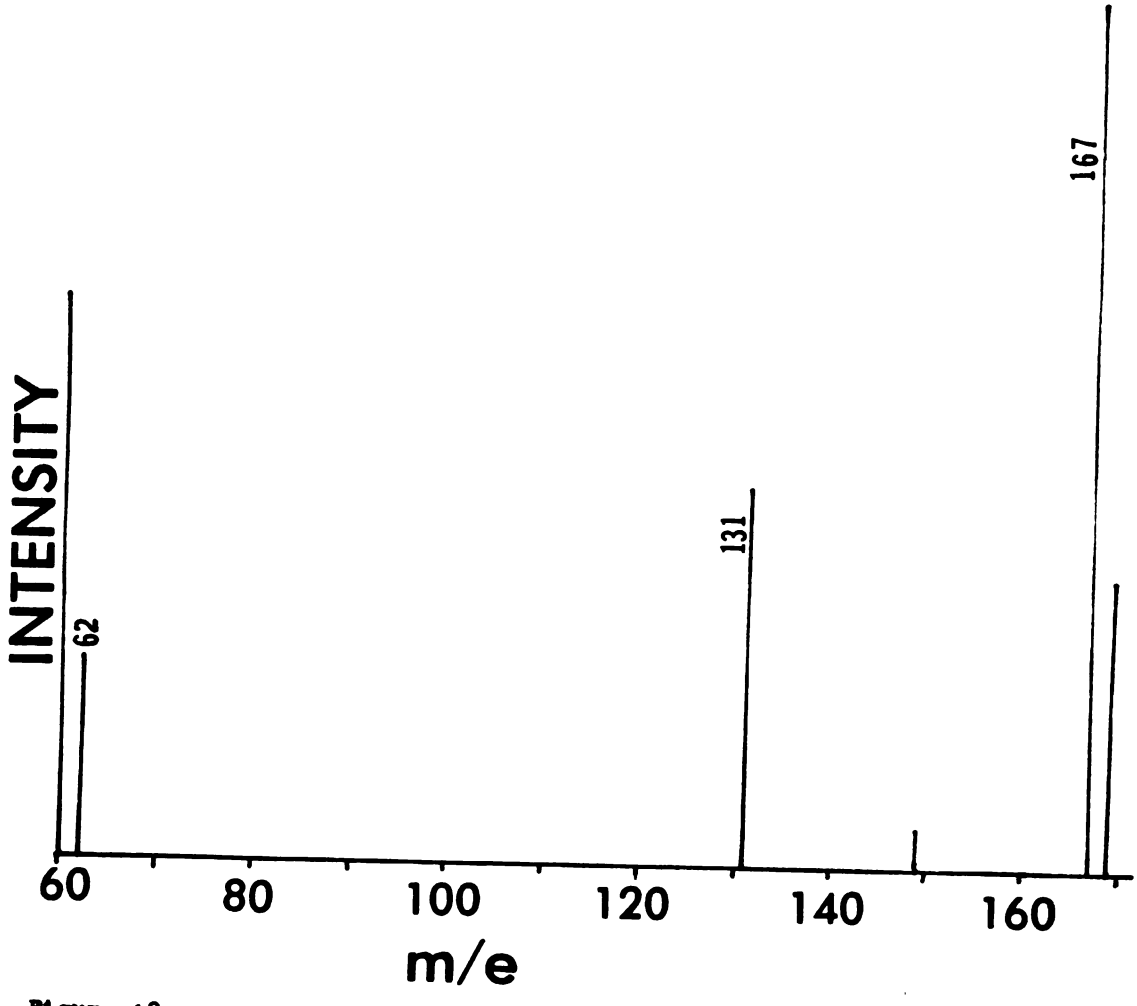


Figure 18. Mass spectrum of CHU, (1-(2-chloroethyl)-3-(2-hydroxyethyl)urea).

120°C (Lit [120] 103-104°C and 121-123°C). PMR (DMSO-d_6) 4.85 (CH_2N), 3.9 (CH_2O), 3.6 ($NHCH_2CH_2Cl$), 6.6 (NH). CIMS spectrum of the hydrochloride salt was the same as observed for BCU, suggesting that ring opening occurred. The free base was prepared by stirring CAO·HCl with AG1-10x (hydroxide form) ion exchange resin in methanol for 5 hours. The resin was filtered, washed with methanol, and the combined methanol fractions were evaporated to give the free base in 18% yield, mp 82-84°C. PMR ($CDCl_3$): 4.45 (CH_2N), 3.9 (CH_2O), 3.6 ($NHCH_2CH_2Cl$), 6.5 (NH).

2-Cyclohexyl-2-oxazoline was prepared from CCU (0.48 g, 21 mmol) by refluxing in 5 ml water for 90 min. The hot reaction mixture was filtered and the pH adjusted to 9.0 using 2.5% NH_4OH. This solution was extracted with ethyl acetate and evaporated to give 0.18 g (38% yield), mp 127-128°C (Lit [118] 131°C).

2. Reaction of BCNU (CCNU) in Phosphate Buffer

BCNU (CCNU) in 0.2 ml of ethanol was dissolved in 10 ml of 0.07 M, pH 7.4, phosphate buffer to give 1.0×10^{-2} to 1.0×10^{-4} M solutions. Reactions were carried out at 37°C in septum capped tubes. At the end of the reaction period, a 2.0 ml aliquot was removed and analyzed for chloroethanol using gas chromatography. The remaining reaction mixture was extracted with 2 x 8 ml of ether containing a known amount of BCNU-d_8. The ether solution was analyzed for unreacted BCNU using the direct insertion CIMS method previously described. A known amount of CCU was added to the aqueous solution and the mixture was lyophilized. The residue was analyzed for nonvolatile products. 2-Chloroethylamine and CAO, which are protonated at pH 7.4, and 2-oxazolidone, BCU, CHU, and BHU were analyzed using direct insertion total ion summation methods and HPLC.

Chloroethanol was analyzed by GC on a 2% KOH-2% carbowax on 100/120

Gas-chrom Q column at 70°C. An aliquot of the above reaction mixture was mixed with a known amount of cyclohexanol or an internal standard and extracted with ether. The ether solution may be injected onto the column. Unreacted BCNU does not interfere with this assay at the column temperature used.

Ureas were analyzed with HPLC on a C_{18}-uBondapak reverse-phase column with 40% acetonitrile in water as solvent. The ureas, CCU and 1,3-dicyclohexylurea, were analyzed using BCU as a standard. Figure 19 shows the procedures of analysis of BCNU reaction products.

3. Formation of Nitrite Ion

The reaction mixtures from decomposition of BCNU, or CCNU in phosphate buffer were also analyzed for the presence of nitrite ion by the Bratton-Marshall method. At the end of the reaction, the reaction mixture was extracted with ether to remove unreacted parent compound and a 2 ml aliquot was taken from the aqueous portion. Sulfanilamide was added to the aqueous solution and mixed. After 5 minutes the Bratton-Marshall reagent (N-(1-naphthyl)ethylenediamine dihydrochloride) was added, mixed and allowed to stand at room temperature for at least 15 minutes before measuring the absorbance. Samples were read against the reagent blank at 540 nm using a Coleman Jr. Spectrophotometer.

C. Results

1. Identification of the BCNU Chemical Decomposition Products

Chemical decomposition products from the reaction of BCNU in phosphate buffer at 37°C were identified by GC, thin-layer chromatography, mass spectrometry and HPLC. The reaction mixture was extracted with ether and the ether extract was subjected to glc. The identity of chloroethanol was determined by comparison of the retention time of the product and standard

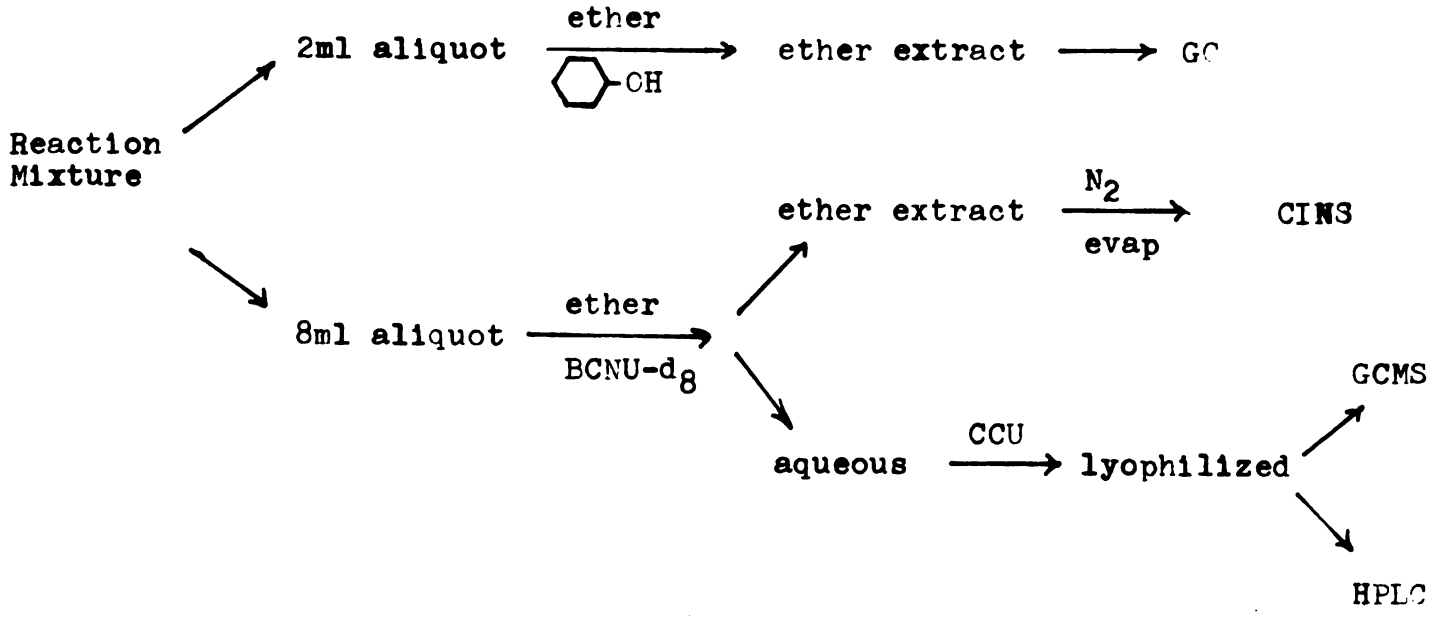


Figure 19. Assay procedures for the analysis of BCNU reaction products.

compound. Chloroethylamine was identified by the same method except that the reaction mixture was basified before ether extraction.

The isolation and identification of nonvolatile products from BCNU chemical reaction was made by thin-layer chromatography and mass spectrometry. Repetitive CI scans were made of lyophilysed reaction mixtures and extracts as these materials were being evaporated into the ion source. The limited protonated molecular ion fragmentation that occurs under CI conditions permits the preliminary identification of mixture components that are introduced simultaneously. Figure 20 shows an isobutane CI spectrum in which the protonated molecular ion (MH^+) of six nonvolatile BCNU reaction products are apparent. Tentative identification of these compounds may be made on the basis of ion masses and chlorine isotope ratios evident in the spectrum. The structures of these reaction products were confirmed by independent synthesis and comparison of the TLC rf values and GC retention times of reaction products and synthetic compounds (Table XI).

After BCNU was reacted in phosphate buffer at pH 7.4, 37^{o}C for 2 hours, the reaction mixture was washed with hexane and an aliquot of 1 ml of the aqueous phase was lyophilized. The residue was dissolved in a small volume of methanol and separated on a silica gel plate. The solvent system used was ethanol : dichloromethane = 1 : 4. The rf values of the products were compared with the corresponding synthetic standards. Another 5 ml aliquot was washed with hexane, lyophilized and separated by thin layer chromatography. The silica gel was divided into sections according to the rf of the standard compounds, extracted with methanol, and analyzed by chemical ionization mass spectrometry. The structures of these reaction products were confirmed by comparing mass spectra with those of standard compounds. Further evidence for 2-(2-chloroethyl)amino-2-oxazoline, which gave a tiny

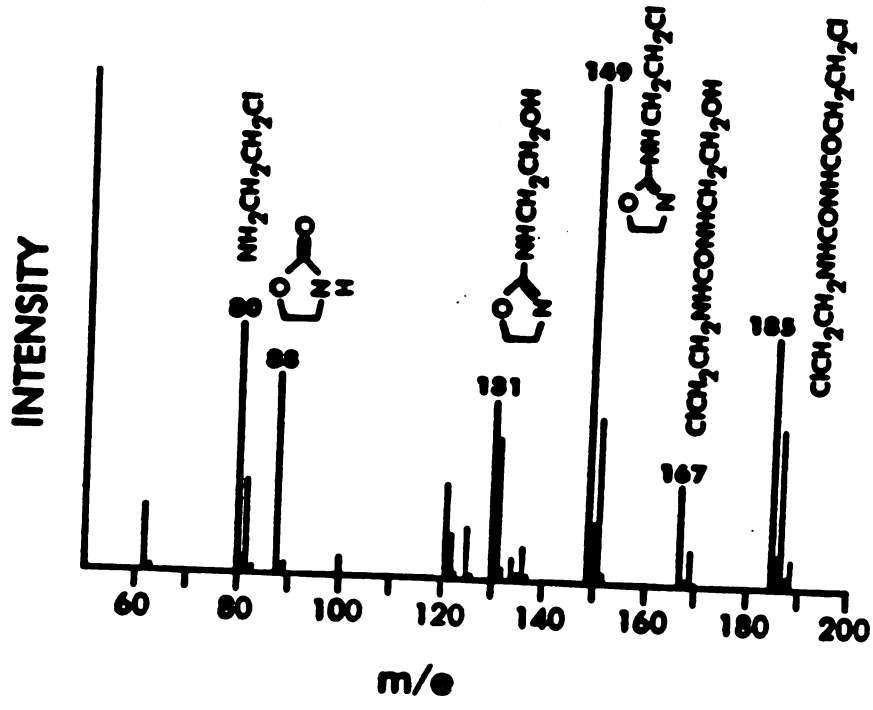


Figure 20. Isobutane CIMS of nonvolatile products formed during the reaction of BCNU in pH 7.4 phosphate buffer at 37 C. Unreacted BCNU was extracted with cyclohexane prior to lyophilization.

Table XI **TLC/MS quantitative analysis of BCNU reaction products formed in phosphate buffer, pH 7.4, 37°C.**

Compound	rf standard	rf product	MH^+ standard	MH^+ product
BCU	0.96	0.96	185, 149	185, 149
2-oxazolidone	0.83	0.83	88	88
CHU	0.71	-	167, 131	167, 131
CAO	0.24	-	149	149

peak on mass spectral analysis, was obtained from HPLC.

2. Quantitation of BCNU Chemical Decomposition Products

The direct insertion probe CIMS technique may be used for the quantitative analysis of ureas, oxazoline, 2-oxazolidone, and 2-chloroethylamine. Table XII shows the reaction mixture components measured after 2 hours reaction at 37°C in a pH 7.4 phosphate buffer. 2-Chloroethanol, acetaldehyde, and vinylchloride are derived from 2-chloroethylazohydroxide. The measured amount of 2-chloroethanol (< 31%) is in good agreement with the amount found by Colvin et al. under identical conditions[64]. Amounts of acetaldehyde and vinylchloride were not measured but were calculated using the published results[64,66] from the relative amount of 2-chloroethanol and these compounds. 2-Chloroethylamine and 2-oxazolidone are derived from 2-chloroethylisocyanate. The combination of these yields suggested that at least 50-60% of the BCNU-water reaction involves cleavage to alkylating species and isocycnates as shown in Scheme XII.

3. Kinetics and Reaction Pathways

Aqueous decomposition reactions were conducted using a mixture of BCNU and BCNU-d_8 labeled on the ethylene positions. The relative amounts of deuterium present in the products is indicative of the reaction pathway leading to their formation. Isotope ratios were computed from repeated CIMS scans of reaction mixture aliquots following extraction of unreacted BCNU. Table XIII shows the H/D ratios of starting BCNU/BCNU-d_8 and products formed from incubation of a 3×10^{-3} M BCNU solution. The isotope ratios of BCNU, 2-oxazolidone, 2-chloroethylamine, BCU, and HAO did not change during the reaction.

Figure 21 shows an isobutane CI spectrum of the 3×10^{-3} M 1.51 : 1 BCNU/BCNU-d_8 reaction mixture. The abundance of CAO-d_4 ion, m/e = 153,

Table XII Products from the reaction of BCNU (9.5×10^{-4} M) in pH 7.4 phosphate buffer at 37 °C after 2 hr.

Component	% Recovered[a]
2-Chloroethanol	25
Acetaldehyde	13[b]
Vinylchloride	3[b]
2-Chloroethylamine	11
2-Oxazolidone	26
BCU	4
HCU	2
BHU	1
CAO	12
HAO	2[c]
BCNU unreacted	19
Total recovered	80

[a] Percent of theoretical yield, assuming 1 mole of BCNU gives 1 mole of azohydroxide and isocyanate derived products.

[b] Calculated from 2-chloroethanol/acetaldehyde or vinylchloride ratios of refs. 13 and 14.

[c] Value obtained from a different experiment under similar conditions.

Scheme XII

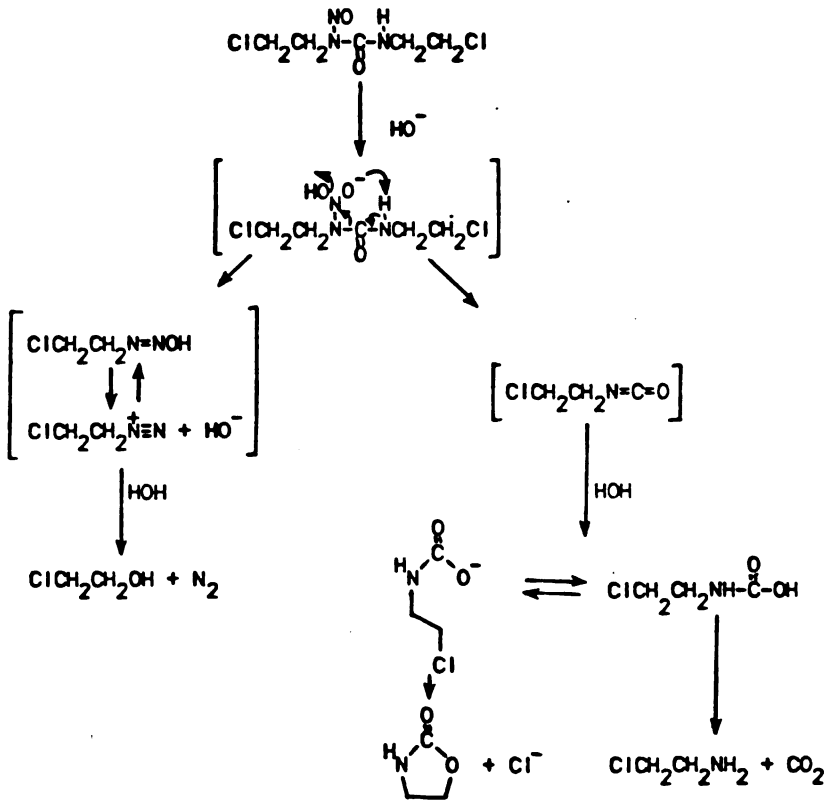

ClCH2CH2N(NO)-C(=O)-NHCH2CH2Cl
HO⁻
ClCH2CH2N=NOH
ClCH2CH2N≡N + HO⁻
HOH
ClCH2CH2OH + N2
ClCH2CH2N=C=O
HOH
ClCH2CH2NH-C(=O)-OH
Cl⁻
+ Cl⁻
ClCH2CH2NH2 + CO2

Table XIII BCNU-d_0/BCNU-d_8 isotope ratios from the reaction of 3×10^{-3} M total BCNU in pH 7.4 phosphate buffer at 37°C

Compound		H/D	(SD)
BCNU		1.51	(0.06)
2-Oxazolidone		1.62	(0.10)
2-Chloroethylamine		1.41	(0.10)

		d_0	d_4	d_8
BCU				
Recombination (calc.)		1.0	1.3	0.42
Denitrosation (calc.)		1.0	0.0	0.66
Observed		1.0	1.2	0.42
CAO				
From BCNU (calc.)		1.0	0.0	0.66
Observed	10 min	1.0	0.37	0.56
	20 min	1.0	0.69	0.43
	30 min	1.0	0.73	0.37
	80 min	1.0	0.89	0.35
HAO				
Observed		1.0	1.2	0.46

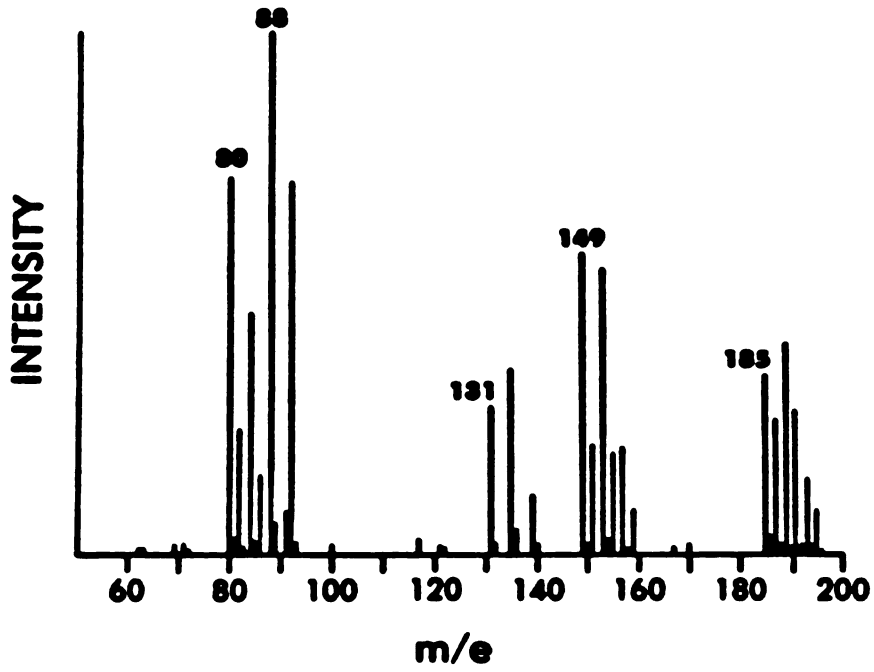


Figure 21. Mass spectra of products from a 3 x 10^{-3}M 1.5/1 mixture of BCNU/BCNU-d_8, showing 2-chloroethanol, m/e 80; 2-oxazolidone,88; HAO,131; CAO, 149; and BCU, 185.

is greatly reduced in the spectrum of the 4 x 10^{-4} M 1.02 : 1 BCNU/BCNU-d_8 reaction mixture (Figure 22). These results show that CAO may be formed from BCNU directly and from BCU cyclization. The amount that forms from BCNU is less at lower initial BCNU concentrations, as the amount of BCU formed under these conditions is reduced (Table XIV).

When 0.5 mM of BCU was incubated in phosphate buffer at pH 7.4, 37^{o}C for 100 minutes, CAO was formed in the reaction mixture. Figure 23 (a) shows the evaporation profile of the reaction mixture. By scaling up the m/e 149 curve, it is clear that the ion of m/e 149 is more volatile and evaporates first. The later part of the curve is superimposible with that of 185. Figure 23 (b) shows the evaporation curve of BCU. In this case, the m/e 149 ion is the fragment of the molecular ion 185. Both curves are superimposible with each other. The contribution from CAO to m/e 149 ion current calculated from the difference between peak ratio and area ratio of the reaction mixture profile was roughly 18%.

Chloroethylisocyanate in high concentrations of 0.1 ml in 0.5 ml of phosphate buffer gave a white precipitate at room temperature. CIMS of the precipitate indicated a structure of BCU. CIMS of the aqueous solution obtained after filtering the precipitate showed a mixture of BCU (minor), 2-oxazolidone and chloroethylamine. These are not fragments of unreacted chloroethylisocyanate. When chloroethylisocyanate was dissolved in dichloromethane and analyzed by CIMS, only trace amounts of m/e 88, 105, 107, 149, 151 were seen in the CI spectrum. Most of the chloroethylisocyanate evaporated during sample insertion at 150^{o}C due to the volatility of this compound.

The formation of nitrite ion during the reaction of chloroethylnitrosoureas with phosphate buffer was not favored. Only 1.5% yield from BCNU and 0.8% from CCNU was obtained after 2 hours at 37^{o}C. Little or no

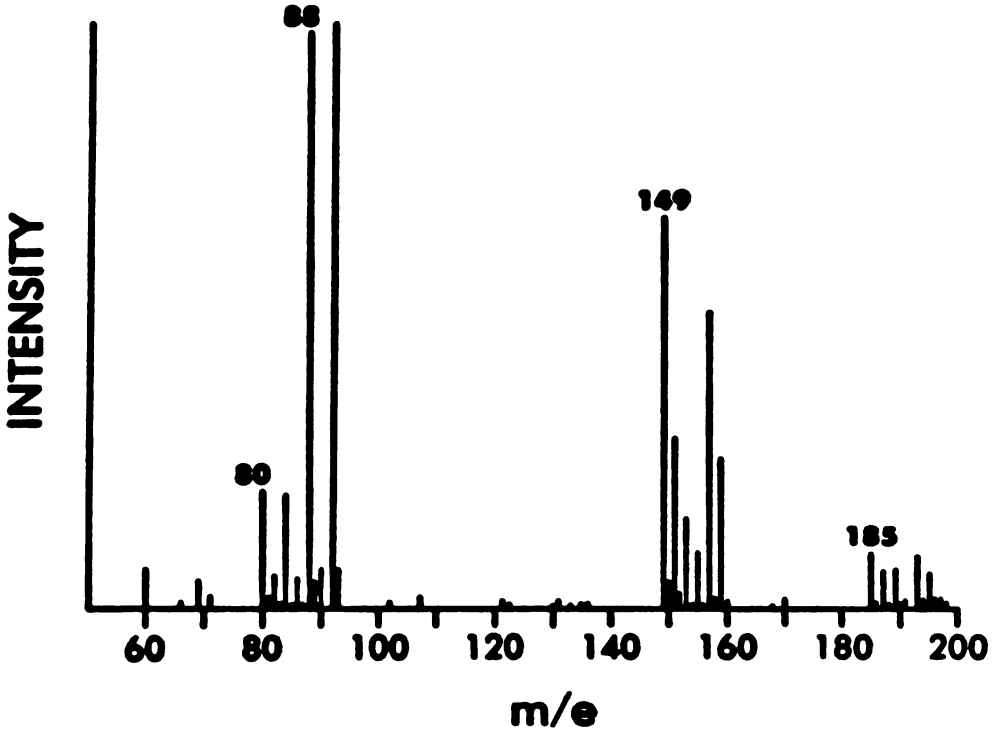


Figure 22. Mass spectra of products from a 4 x 10^{-4} M 1/1 mixture of BCNU/BCNU-d_8.

Table XIV Dependence of initial BCNU concentration on the origin of CAO.

Initial Concentration[a]	BCNU		CAO			% CAO from BCNU
	d_o	d_8	d_o	d_4	d_8	
3×10^{-3} M	1.0	0.66	1.0	0.73	0.37	44
8×10^{-4} M	1.0	0.84	1.0	0.22	0.78	87
4×10^{-4} M	1.0	0.98	1.0	0.22	0.77	89

[a] Reaction of BCNU and BCNU-d_8 at 37 °C for 30 min in pH 7.4 phosphate buffer.

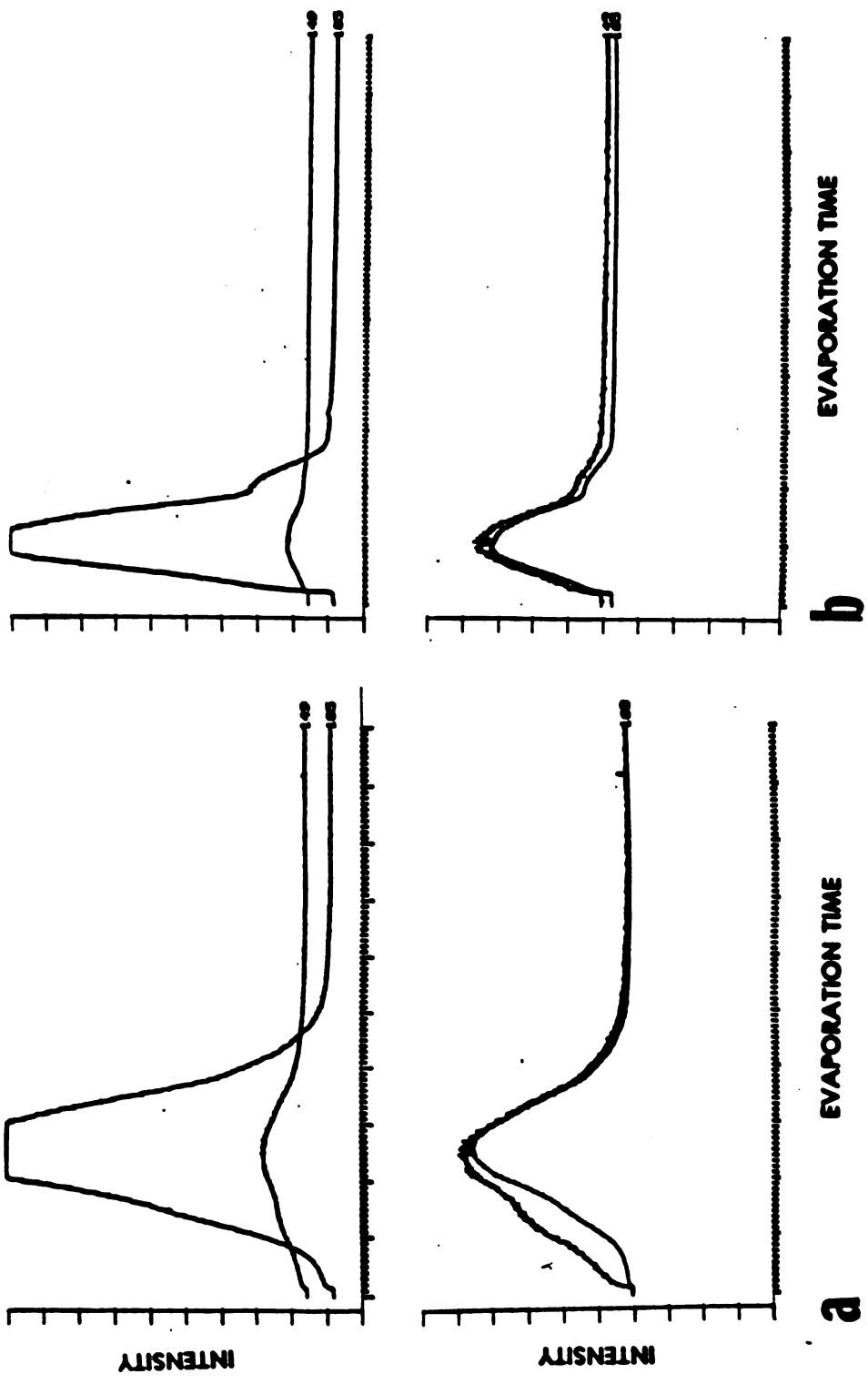


Figure 23. Evaporation profiles obtained from BCU reaction mixture in phosphate buffer (a), and pure BCU (b).

nitrite ion is present when the nitrosoureas are mixed with phosphate buffer and extracted immediately. Nitrite ion is stable under the reaction condition in phosphate buffer.

4. Chemical Reaction of CCNU in Phosphate Buffer

The chemical reaction products of CCNU on pH 7.4 phosphate buffer at 37°C were identified by thin-layer chromatography, mass spectrometry and HPLC. The reaction mixture was acidified to pH 4 and extracted with ether. The aqueous solution was then basified to pH 9 by NH_4OH and extracted with ethyl acetate. The ether solution and ethyl acetate extracts were subjected to mass spectrometric analysis.

Dicyclohexylurea (DCU), 1-(2-chloroethyl)-3-cyclohexylurea (CCU), cyclohexylamino-2-oxazoline and cyclohexylamine were identified as the reaction products. Mass spectra were taken for the preliminary identification. The residue from the lyophilized reaction mixture after 5 hours incubation of CCNU with phosphate buffer gave a mass spectrum with a major ion at m/e 100, and smaller peaks at 225, 205, 169. The mass spectrum was compared with that of pure CCNU, which had peaks at m/e 205 and 169 but not 225 (Figure 24). If CCNU was mixed with buffer for 5 minutes under the same condition and extracted with hexane, the hexane gave no peak at m/e 225. These results indicated that DCU (m/e 225) was a reaction product from the reaction of CCNU in phosphate buffer. CCU (m/e 205) and DCU were further identified by HPLC. CCNU generates a fragment of m/e 169 in CIMS, which is the same as cyclohexylamino-2-oxazoline, therefore, CCNU must be separated from the reaction mixture in order to obtain a spectrum of cyclohexylamino-2-oxazoline without interference.

The reaction mixture was acidified to pH 4 and extracted with ether to remove unreacted BCNU, cyclohexylamino-2-oxazoline was protonated at pH 4.

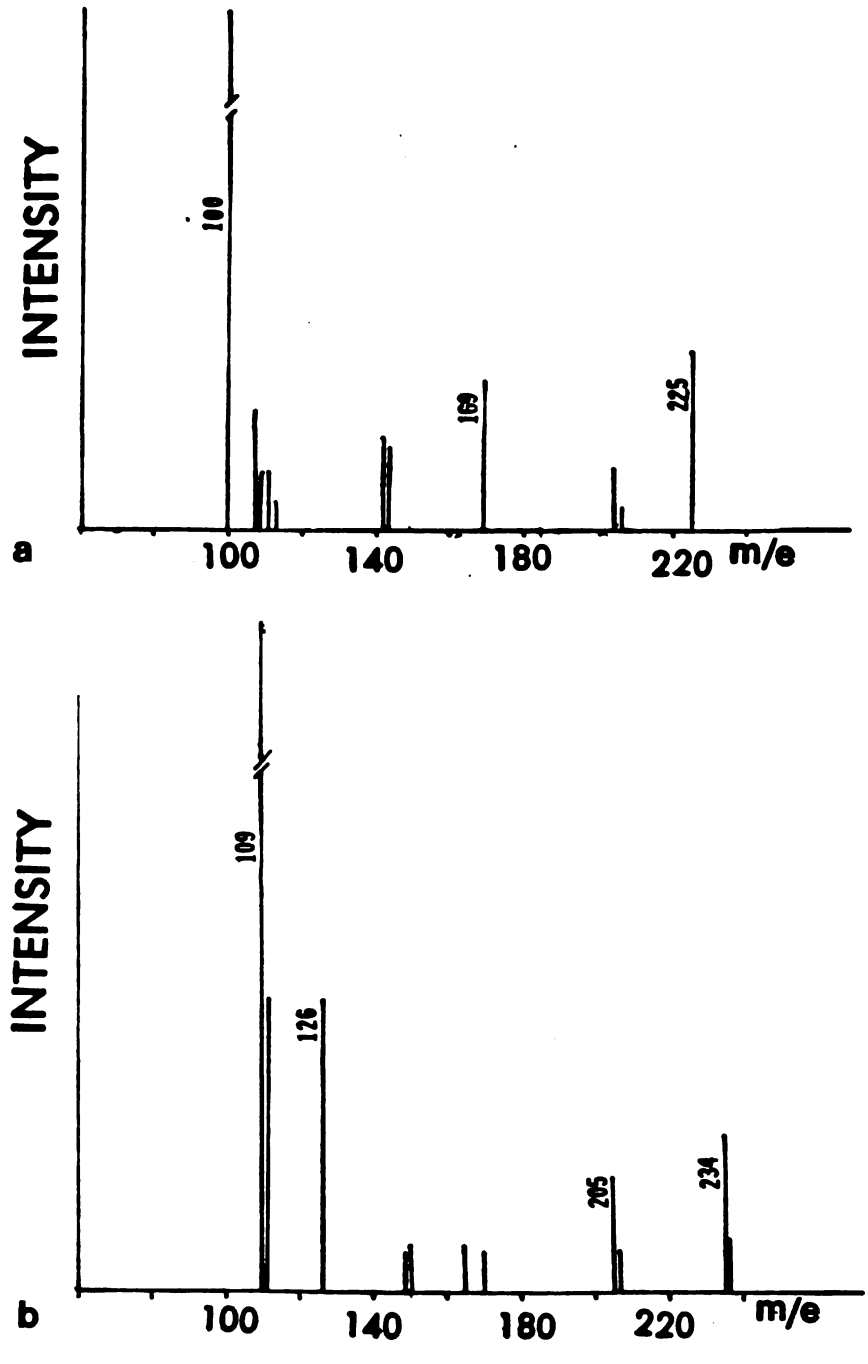


Figure 24. Mass spectra obtained from (a) lyophilized reaction mixture after 5 hours incubation of CCNU with phosphate buffer, pH 7.4, 37°C (b) pure CCNU.

The aqueous solution was then basified to pH 9 and extracted with ethyl acetate. Cyclohexylamino-2-oxazoline was extracted into the ethyl acetate layer and the CI spectrum of the ethyl acetate solution gave peaks at 169 and 100 corresponding to cyclohexylamino-2-oxazoline and cyclohexylamine (Figure 25). The structure was confirmed by TLC isolation and mass spectral analysis. The TLC rf value and mass spectrum were identical to that of the synthetic sample.

The ureas were quantified by HPLC using BCU as the internal standard. A C_{18}-uBondapak reverse phase column with a solvent system of 40% acetonitrile in water was used. Figure 26 shows the HPLC chromatograph of BCU, CCU, DCU. Standard curves were obtained for CCU and DCU by plotting the observed peak height ratios versus the amount ratios (Figure 27). The amount of oxazoline was estimated from mass spectral analysis. Table XV combines these results with those of Reed et al.[65] to show the distribution of CCNU reaction products formed in phosphate buffer with the concentration of CCNU at 1 mM.

D. Discussion

BCNU reacts in aqueous solution through cleavage to give azohydroxide and isocyanate, or cyclization to an oxazoline. The first reaction is shown in Scheme XI. The initial step in this reaction probably involves general base catalysis with the dissociation of the urea N-H bond as the rate determining step.

2-Chloroethylazohydroxide or the diazonium ion reacts with water by an S_N2 mechanism to give 2-chloroethanol and smaller amounts of acetaldehyde and vinyl chloride as described by Colvin et al.[64] 2-Chloroethylisocyanate reacts by addition of water to give a carbamic acid. This unstable intermediate may decarboxylate to 2-chloroethylamine and carbon dioxide, or it

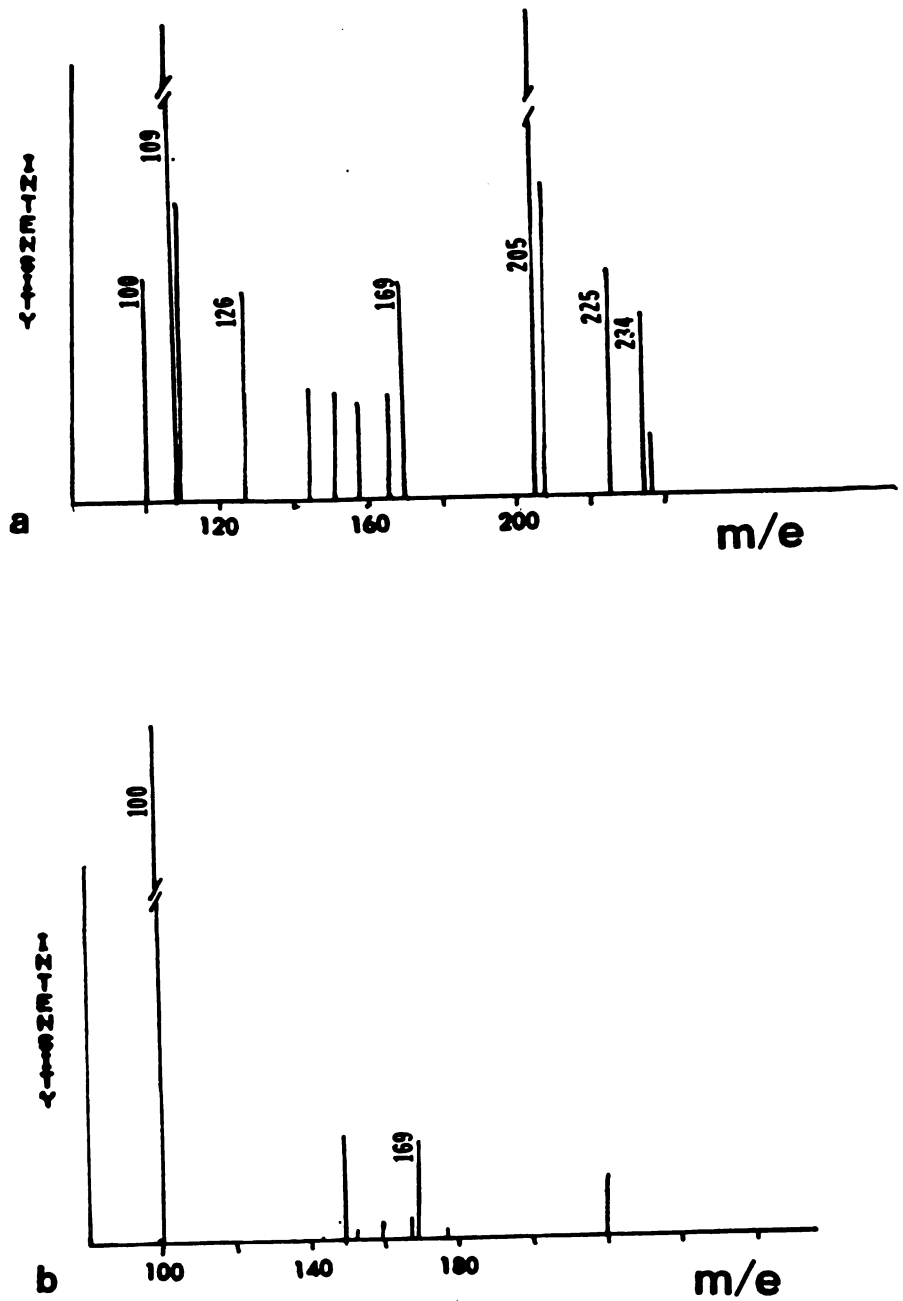


Figure 25. Mass spectra obtained from (a) ether extract of CCNU reaction mixture at pH 4, and (b) ethyl acetate extract of the aqueous portion at pH 9.

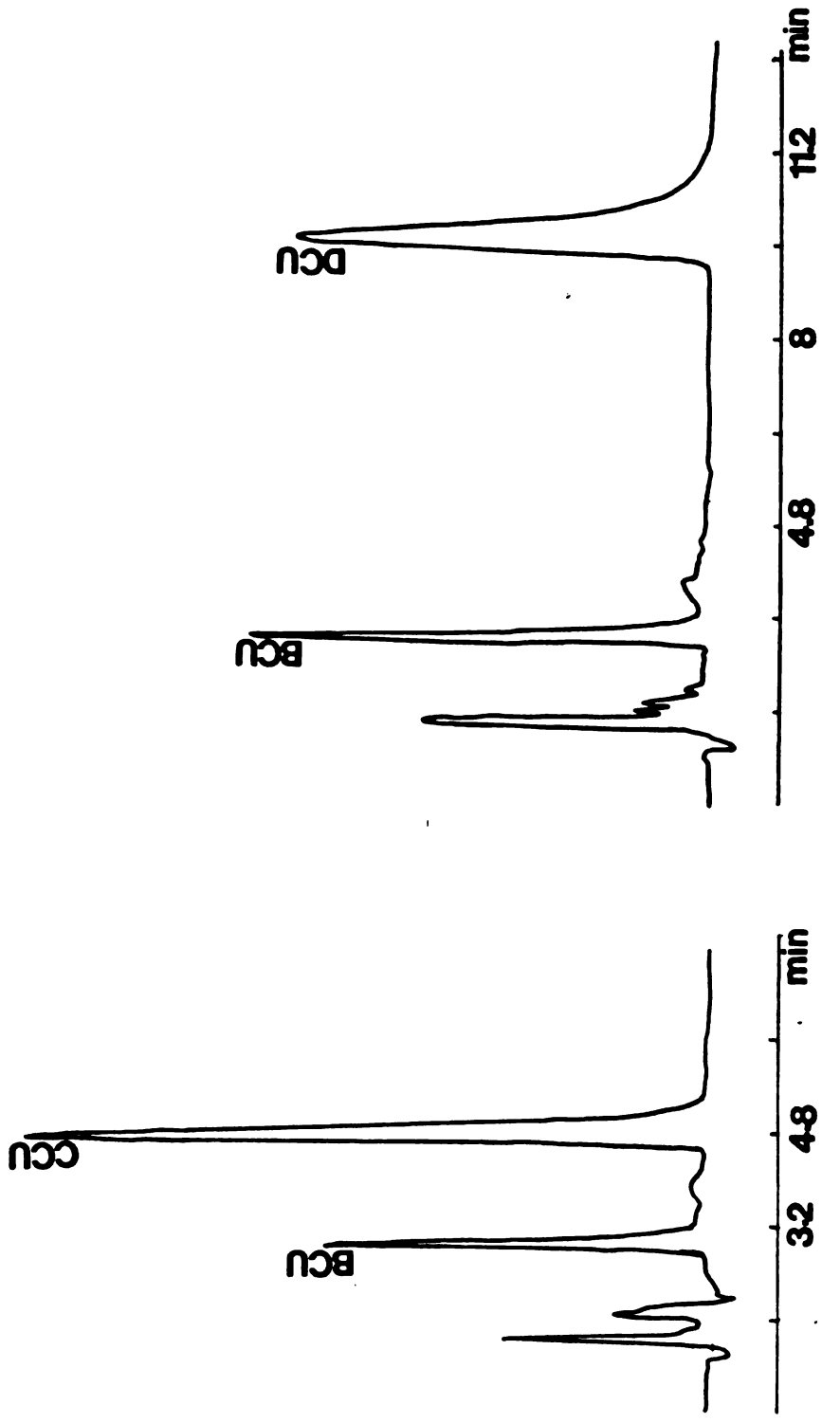


Figure 26. HPLC chromatograph for CCU, DCU and their internal standard BCU.

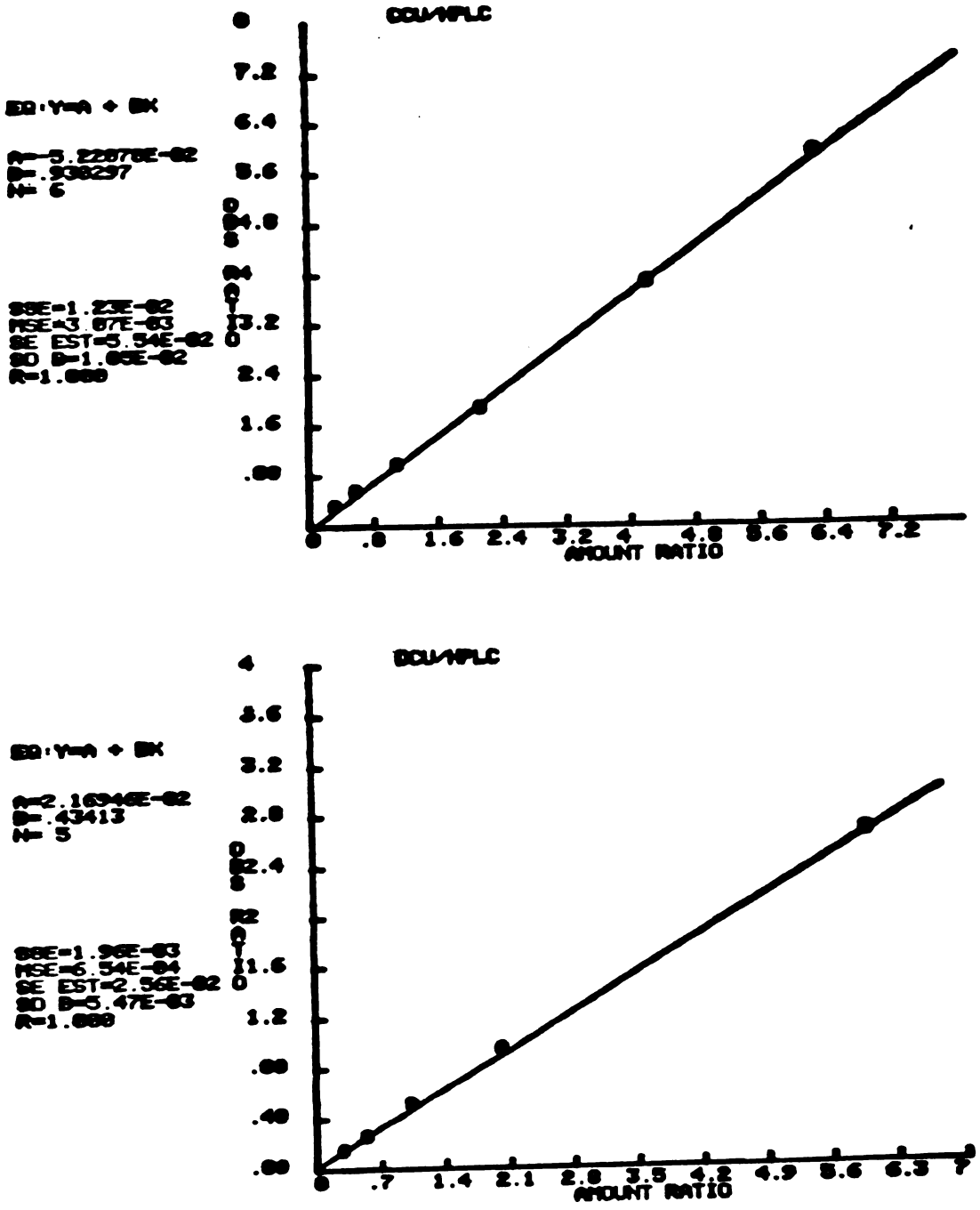


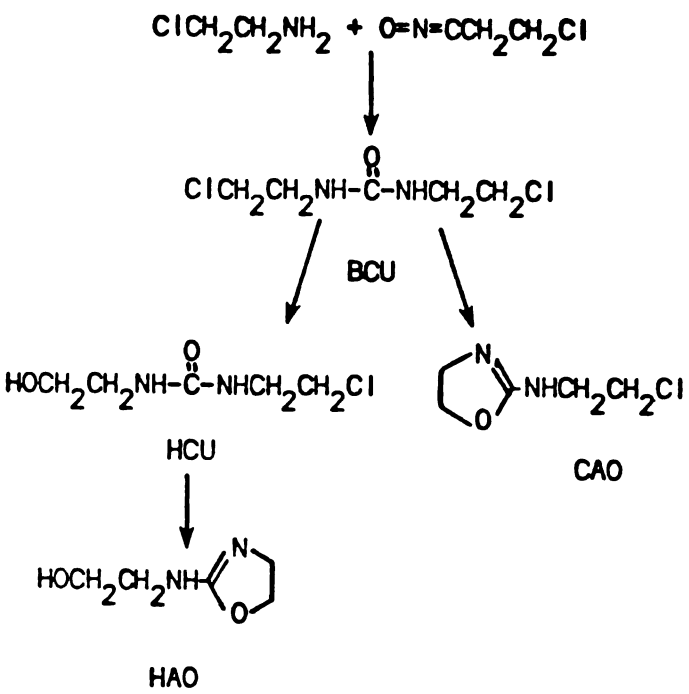
Figure 27. Standard curves for quantitative analysis of CCU and DCU by HPLC using BCU as the internal standard.

Table XV Products from the reaction of CCNU (1×10^{-3} M) in pH 7.4 phosphate buffer at 37 °C after 2 hr.

Component	Percent Recovered
2-Chloroethanol	18-25 [a]
Acetaldehyde	5-10 [a]
Cyclohexylamine	32 [a]
DCU	1
CCU	3-5
2-Cyclohexylamino-2-oxazoline	3-5
CCNU unreacted	12-17

[a] Calculated from the results of Reed, et al.

Scheme XI



may ionize. The carbamate can cyclize by displacement of chloride ion to give 2-oxazolidone. This reaction sequence is supported by the isotope data of Table XIII. 2-Oxazolidone formed from a BCNU, BCNU-d_8 mixture is depleted in deuterium, while 2-chloroethylamine is deuterium enriched. This suggested that 2-oxazolidone and 2-chloroethylamine are formed from a common intermediate, N-(2-chloroethyl)carbamic acid. The slower decarboxylation reaction occurs from a carbamic acid pool enriched in deuterium to the extent that the α and β-secondary deuterium isotope effects prevent the more rapid 2-oxazolidone cyclization reaction. If deuterium does not alter the decarboxylation rate, the cummulative effect on 2-oxazolidone formation from the carbamic acid is $K_{H/D}$ = 1.16, Scheme XII.

The kinetic isotope effects were derived from the following equations:

where I_H, I_D are carbamic acid-d_0 and -d_4, A_H, A_D are chloroethylamine-d_0 and -d_4, and O_H, O_D are 2-oxazolidone-d_0 and -d_4.

$$\frac{A_H}{O_H} = \frac{k_{H(a)}}{k_{H(o)}} \qquad \frac{A_D}{O_D} = \frac{k_{H(a)}}{k_{D(o)}}$$

$$\frac{\frac{k_{H(o)}}{k_{D(o)}}}{\frac{k_{H(a)}}{k_{D(a)}}} = \frac{k_{H(o)}}{k_{H(a)}} - \frac{k_{D(a)}}{k_{D(o)}} = \frac{O_H}{A_H} \cdot \frac{A_D}{O_D} = \frac{O_H}{O_D} \cdot \frac{A_D}{A_H} = 1.16$$

If the ratio of $k_{H(a)}/k_{D(a)}$ is greater than one, the ratio A_H/A_D must be greater than one from the equation 2. But the value observed from the experiment was less than one, therefore the ratio $k_{H(a)}/k_{D(a)}$ is equal to or less than one. Assuming there is no reverse isotope effect for the formation of chloroethylamine, the ratio is equal to one and thus $k_{H(o)}/k_{D(o)}$ is equal to 1.16.

Isocyanate generates carbamic acid as an intermediate, from which chloroethylamine and 2-oxazolidone are formed. Secondary isotope effect favors the formation of the amine from deuterated isocyanate. This can be explained by the higher transition state energy of the reaction from isocyanate-d_4 to oxazolidone-d_4. The reaction rate for d_4-oxazolidone is slower and the rate of the amine formation is relatively increased. Therefore, when a mixture of BCNU-d_0 and -d_8 were reacted with the buffer, a ratio of d_0 to d_4-chloroethylamine is less than one and d_0 to d_4-oxazolidone is greater than one. Further work may be done to determine if there is isotope effect on the reaction from isocyanate to amine.

The cleavage reaction is the major BCNU pathway under these conditions. Products derived from the initial intermediates account for 51-61% of reacted BCNU (67-80% products recovered). All of the major products of this reaction, 2-chloroethanol, acetaldehyde, 2-oxazolidone and 2-chloroethylamine, are toxic substances. 2-Oxazolidone displays delayed

toxicity[121].

BCU can be generated from BCNU through a denitrosation mechanism. In addition, 2-chloroethylisocyanate can react with 2-chloroethylamine to give BCU (Scheme XI). Reaction of a BCNU, BCNU-d_8 mixture will give BCU with the same isotope abundance as starting BCNU if it is formed by denitrosation, or as a d_0,d_4,d_8 mixture if formed from the 2-chloroethylisocyanate-amine combination. The BCU isotope abundances are equal to the amounts calculated for combination indicating that this is the only route of formation (Table XIII).

Urea formation is in competition with the isocyanate-water reaction of Scheme XI so that the amount of urea formed is related in a complex manner to the initial concentrations of BCNU. The yield of BCU is less than 10% if the initial BCNU concentration is below 10^{-3}M at physiological pH and temperature. BCU undergoes a cycloelimination of chloride ion to give CAO. Solvolysis of BCU gives HCU by a competitive reaction. HCU will also cyclize to give HAO.

This scheme is also supported by the isotope abundance data of Table XIII. HAO has the same isotopic composition as BCU, indicating that it is formed from this compound rather than by hydrolysis of CAO. The secondary reactions of BCU are slow relative to the rate of BCNU decomposition. The reaction products are important only when high concentrations (>10mM) of BCNU are reacted for several hours. The toxicity of CAO is not known, but other substituted 2-amino-oxazolines are highly toxic[121].

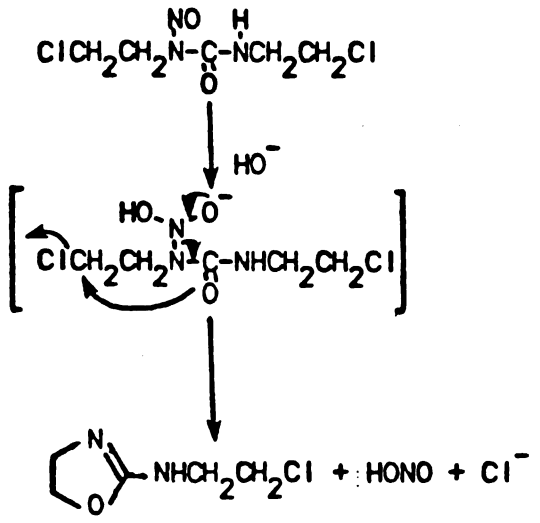
CAO may be formed in relatively high yield (20% of recovered products) from reaction of 10^{-3}M BCNU (Table XII). Reactions of more dilute BCNU, BCNU-d_8 mixtures (Table XIV) gave CAO-CAO-d_8 with little CAO-d_4, indicating that CAO may be formed directly from BCNU. CAO is formed by cyclization of BCU as discussed above. The amount of CAO formed directly

from BCNU is 3-6% in two hours at pH 7.4, 37°C. Scheme XIII shows a possible mechanism for this reaction. Nitrite ion, another product of this reaction, was found to be present in 1.5% yield after two hours.

Recent reports indicate that the N-nitroso group of dialkyl nitrosamines may act as a nucleophile to displace β-substituents (Scheme XIV). Analogous reactions of chloroethylnitrosoureas would yield 1-hydroxyl-1-nitrosoureas or possibly, ethylene glycol, on hydrolysis. No evidence was found for the presence of either of these products using mass spectrometric and GCMS selected ion monitoring methods of analysis. The electron withdrawing effect of the N-acyl group may decrease the nucleophilicity of the nitroso group and raise the energy of the positively charged intermediate formed from chloride ion displacement. This and poor leaving group properties of the chloride ion may combine to inhibit this reaction.

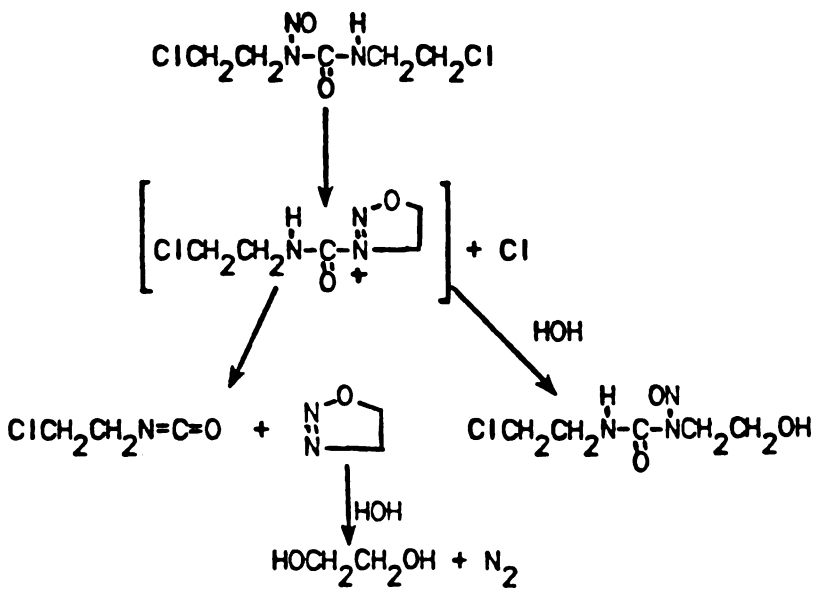
CCNU reactions in aqueous buffer occur primarily through the initial cleavage to chloroethylazohydroxide and cyclohexylisocyanate, Scheme XV. These intermediates decompose to chloroethanol, acetaldehyde and cyclohexylamine as previously described. Cyclohexylisocyanate reacts with water to give a carbamic acid, which can only decarboxylate to cyclohexylamine. 2-Oxazolidone cannot be formed from CCNU. Cyclohexylamine can react with cyclohexylisocyanate to give DCU if the initial CCNU concentration is above 10^{-3}M, in a similar manner as the BCU formation. A surprisingly high amount of CCU (11-25% recovered products), and its cyclization product, 2-cyclohexylamino-2-oxazoline, were formed during this reaction. This is not analogous to BCNU reactions as no BCU is formed directly from BCNU. Less than 0.8% nitrite is generated during the CCNU reaction; simple denitrosation does not appear to be the mechanism of formation.

Scheme XIII

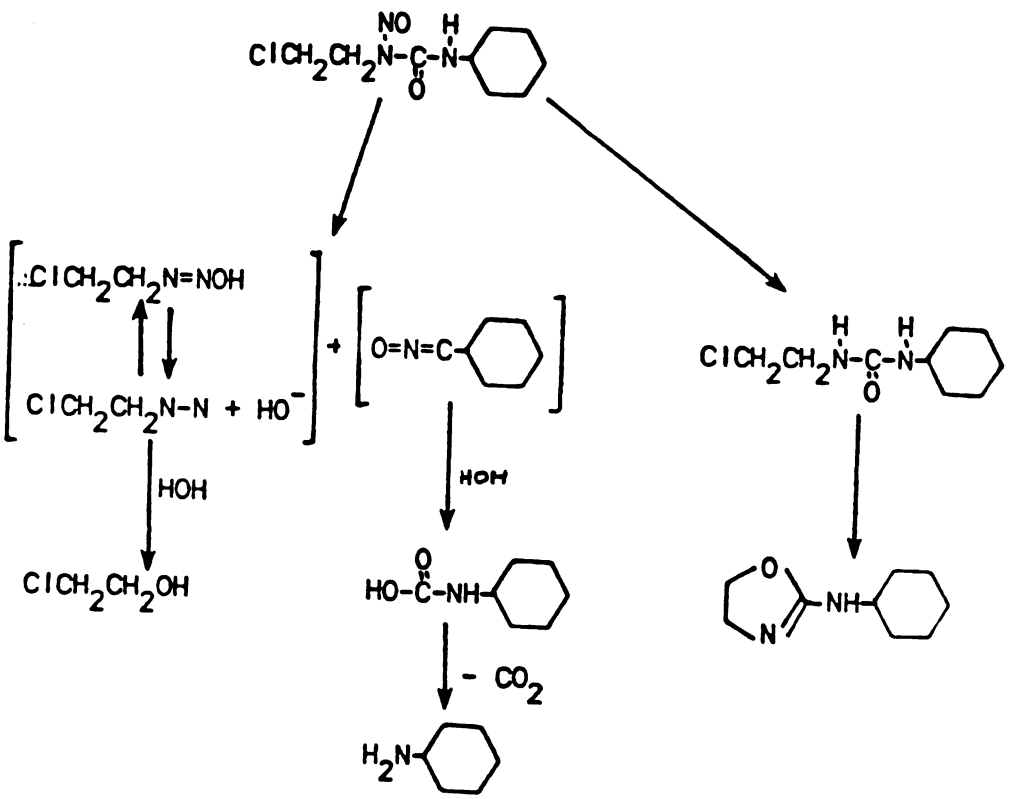


CAO

Scheme XIV



Scheme XV


ClCH2CH2N-C-N
NO H
O
:ClCH2CH2N=NOH
ClCH2CH2N-N + HO⁻
HOH
ClCH2CH2OH
O=N=C
HOH
HO-C-NH
O
- CO2
H2N
ClCH2CH2N-C-N
H H
O
O
N
NH

Chapter V. PROTEIN MEDIATED CHEMICAL REACTIONS OF CHLOROETHYLNITROSOUREAS

A. Introduction

As discussed in the previous section, chloroethylnitrosoureas are not active as the intact molecule but decompose rapidly in aqueous solution to the active intermediates. Cleavage to alkylating intermediates constitutes more than 80% of the reaction pathway for BCNU and CCNU in neutral aqueous solution. Aqueous decomposition reactions may be required to convert chloroethylnitrosoureas to active intermediates within tumor cells but may constitute only a minor fraction of in vivo biodistribution.

The rate of chloroethylnitrosourea decomposition in serum is much more rapid than the rate observed in aqueous buffer at pH 7.4, 37°C. When incubated in human serum, the half life of BCNU is reduced from 50 to 15 min, CCNU from 60 to 30 min, and MeCCNU from 57 to 29 min[122]. Serum protein components apparently catalyzed a BCNU decomposition reaction. The half life of BCNU in serum ultrafiltrate, from which compounds having a molecular weight greater than 25,000 Daltons have been excluded, is 42 min, which is approximately the same as the aqueous buffer half life[47]. Normal serum lipoprotein concentrations reduce the rate of aqueous and reaction by partitioning the more lipophilic chloroethylnitrosoureas into the lipoprotein core region[123].

Serum catalyzed reactions are important because they control, at least in part, the conversion of chloroethylnitrosoureas to active and toxic species. In the case of BCNU, the in vitro serum decomposition rate, $k = -0.048$ to -0.063 min^{-1}, is close to the patient plasma clearance rate, $k_{10} = -0.019$ to -0.045 min^{-1}, calculated assuming a two compartment open model[47]. Since patient pharmacokinetic parameters indicate that the integrated amount of BCNU in the plasma accounts for more than 50% of the intact drug

in the body[47], serum reactions may be a major determinant of the in vivo biodisposition of chloroethylnitrosoureas.

B. Experimental Methods

1. Decomposition of BCNU in Human Serum

Human serum was prepared from freshly drawn blood obtained from volunteers. Blood was drawn and allowed to stand at room temperature for an hour, stirred against the test tube wall to loosen the clot and centrifuged at 2,000 rpm for 10 minutes. The serum on the upper layer was taken by a pipette. The pH of the serum was adjusted to 7.4 by adding 3 drops of 0.01N lactic acid to 3 ml of serum and gased under a stream of CO_2/air (5% CO_2) for one minute. The pH was checked by a pH meter. BCNU was dissolved in ethanol, an aliquot of the solution was added to 3 ml of serum at pH 7.4 to make ∿1mM solution. Aliquots of 100 µl were taken at 0, 15, and 30 min and added a known amount of cyclohexanol as internal standard, extracted with ether, and analyzed for the formation of chloroethanol. Aliquots of 400 µl were taken at the same time points and extracted with hexane to remove unreacted BCNU. The aqueous solution was added to two times the volume of methanol to precipitate protein, filtered and evaporated under nitrogen gas to dryness. A known amount of benzyl alcohol was added as internal standard, and 1 µl was injected into GC-MS system for the analysis of water soluble products. A selected ion monitoring gas chromatography-mass spectrometry system was used as described in the analytical section.

2. Decomposition of BCNU in Albumin

Bovine serum albumin (80 mg/ml) was mixed with an equal volume of phosphate buffer to obtain a protein concentration of 40 mg/ml, which is the approximate plasma albumin concentration. A 5 ml solution of bovine

solution containing 1 mM BCNU was incubated at 37°C. Aliquots were taken at different time points for the kinetic study of the disappearance of BCNU. The initial point was taken immediately after mixing and used as the zero time point. A known amount of D_8-BCNU was added to each aliquot and extracted with hexane..

Analysis of the products formed followed the same procedure as the reaction of BCNU in human serum. Formation of BCU was measured by HPLC. The reaction mixture was extracted by hexane and lyophilized. Methanol and a known amount of CCU were added to the residue and were centrifuged at 2,000 rpm for 2 minutes. The supernatant was removed by pipette and evaporated under nitrogen gas to a small volume. The formation of BCU was determined by HPLC.

3. Covalent Binding

BCNU-^{14}C (930 uM, labeled on both ethylene groups) was incubated with bovine serum albumin (800 uM) in 0.07 M phosphate buffer at pH 7.4, 37°C, for 30 minutes. An aliquot of the reaction mixture (1 ml) was extracted with hexane (2 x 3 ml) to remove unreacted BCNU. Protein was precipitated with methanol (5 ml) and the precipitated protein was washed with 40% methanol-water until no radioactivity was detected in the wash solution. The precipitated protein was dissolved in 3 ml of 0.1M NaOH. Total activity was computed from the hexane, precipitate, and combined methanol-water fractions. Radioactivity was counted on a Beckman LS-250 liquid scintillation counter; quench corrections were performed using the automatic external standard.

C. Results

The enhanced rate of chloroethylnitrosourea disappearance from serum indicated that some serum component catalyzed the decomposition of these

drugs. After passing serum through a 25,000 Dalton exclusion limit filter, the catalytic activity of serum was greatly diminished, which suggested that the activity was due to a macromolecule[47]. The washed serum proteins reconstituted in buffer have the same catalytic activity as whole serum which suggests that the reaction does not require cofactors[124]. The disappearance of BCNU in purified bovine serum albumin had a half life of 8 min. Table XVI lists the BCNU half lives in buffer, serum, serum ultrafiltrate, and serum protein reconstituted in buffer or serum ultrafiltrate [124]. The combination of these results suggests that BCNU disappearance may be catalyzed by rather nonspecific interactions between the chloroethylnitrosourea and serum proteins. Albumin is the major serum protein and constitutes 50-65% of all serum proteins. Albumin does not possess enzymatic activity but does have well characterized drug binding properties. Albumin could be the major macromolecule that catalyzes the reaction of BCNU. Since albumin has a much cleaner system than serum, the study of the protein catalyzed BCNU reaction was investigated in purified albumin solution.

The reaction of BCNU incubated in human serum was also investigated. The products found in BCNU-buffer reaction were obtained in low yield. The products formed during the incubation of BCNU (930 uM) and human serum (800 uM) at pH 7.4, 37^{o}C for 30 min were determined. After 93% reaction, 7% BCNU, 13% 2-chloroethanol, 7% acetaldehyde[64], 10% chloroethylamine were recovered as major products. Other products observed in aqueous reaction, such as 2-oxazolidone (< 2%) and BCU (< 3%) were formed in low yield. 2-(2-chloroethylamino)- 2 -oxazoline, a product formed by a competitive BCNU reaction pathway was not detected (< 0.5%). No other BCNU related products were detected using GC-MS and direct mixture CIMS analysis of lyophilized, protein free mixtures. All of the detected products are derived from

Table XVI BCNU half-lives at 37°C.

	Half Life (min)	pH
0.1 M phosphate buffer	63.9	7.34
Human serum	17.5	7.41
Serum ultrafiltrate[a]	40.0	7.56
Buffer + serum proteins[b]	12.0	7.32
Ultrafiltrate + serum proteins[b]	12.5	7.53

[a]Ultrafiltrate from an Amicon Centriflow filter centrifuged at 1000 x g for 1.25 hr at 0-4°C.

[b]Serum proteins retained by the Amicon filter were washed with 0.1 M phosphate buffer and centrifuged twice at 1000 x g for 1.25 hr at ambient temperature.

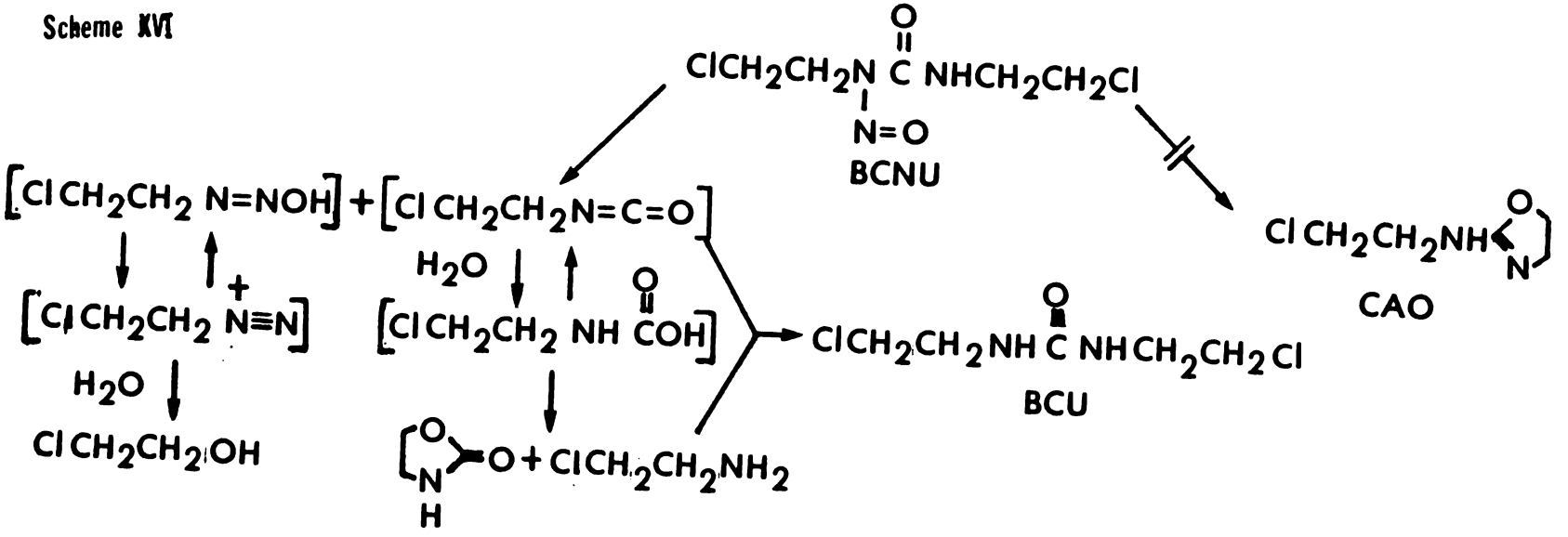
cleavage of BCNU to active azohydroxide or isocyanate intermediates (Scheme XVI). This appears to be the only reaction catalyzed by albumin, although the existence of other pathways are not excluded.

The low percentage of product recovery in this experiment may be due to the fact that 75% ($k_{albumin}-k_{buffer}/k_{albumin} \times 100$) of the reactive azohydroxide and isocyanates would be formed at the albumin surface. This would maximize the probability of covalent binding of these intermediates to the protein. The extent of covalent binding to protein was determined in a reaction of BCNU-^{14}C (930 uM) and albumin (800 uM) at pH 7.4, 37°C for 30 min. After 97% of BCNU had reacted, 23± 6% of the total label was bound to albumin. Incubation of a similar reaction mixture with salicylic acid (695 uM) reduced the percent of bound label to 12%. The total recovered and bound product accounts for 50-60% of starting BCNU. The unidentified product(s) is a nonvolatile, methanol soluble material.

D. Discussion

BCNU has a much shorter half life in the presence of plasma protein or albumin. Other analogs of chloroethylnitrosoureas such as CCNU, MeCCNU have also shorter half lives in serum. Since there are many enzymes present in the plasma, enzymatic reactions could be the source of catalysis. However, the ability of albumin, human serum albumin or bovine serum albumin, to catalyze the decomposition of chloroethylnitrosoureas suggests that the observed rate increase is more likely to be due to a nonspecific macromolecule catalyzed reaction. This reaction involves an equilibrium between the chloroethylnitrosourea, protein, and a reactive nitrosourea-protein complex, which can decompose to give albumin and reactive products, 2-chloroethylazohydroxide and an isocyanate. Formation of these reactive species at the surface of the protein increases the probability of covalent

Scheme XVI


ClCH2CH2N C NHCH2CH2Cl
O
N=O
BCNU
[ClCH2CH2 N=NOH] + [ClCH2CH2N=C=O]
[ClCH2CH2 N≡N]
H2O
ClCH2CH2OH
H2O
[ClCH2CH2 NH COH]
O
ClCH2CH2NH C NHCH2CH2Cl
BCU
ClCH2CH2NH
CAO
O + ClCH2CH2NH2
H

bond formation with protein molecules. In the case of BCNU, 31% of the products formed at the protein surface become covalently bonded.

$$\text{CENU} + \text{Albumin} \rightleftharpoons (\text{CENU} \cdot \text{Albumin}) \rightleftharpoons \text{Products} + \text{Albumin}$$

Figure 28 shows the Lineweaver-Burk plots for BCNU, CCNU and MeCCNU. The Michaelis-Menten parameters V_{max} and K_m are listed in Table XVII. The rather high K_m value of 450-480 uM indicates that the albumin-CENU binding affinity is low. This drug-protein complex may be susceptible to competitive inhibition by compounds that have high albumin association constants. Salicylic acid and dodecanoic acid both reduced the decomposition reaction rate of BCNU in albumin solution to the decomposition rate observed in aqueous buffer[124]. Salicylic acid also reduced the covalent binding of BCNU to albumin.

Chloroethylnitrosoureas decompose in serum by two reaction pathways. The drug can decompose by a general base catalyzed reaction in aqueous media and by a protein catalyzed mechanism that involves the formation of a protein-drug complex prior to decomposition. The products of the protein mediated reaction are not necessarily the same as those described in aqueous degradation reactions. The products found in the reaction of BCNU with bovine serum albumin were basically those formed in aqueous buffer decomposition. The formation of BCU was less than that in phosphate buffer reaction because the concentration dependent bimolecular reaction leading to the formation of BCU would be competitively inhibited by the presence of protein molecules.

In contrast to enzyme reactions, the normal total serum protein (65-80 mg/ml) and albumin (35-50 mg/ml) concentrations are in excess of expected peak chloroethylnitrosourea serum concentrations (1-10 µg/ml). For example, peak patient BCNU plasma concentrations are near 30 uM[47] and albumin is present at a concentration of 515-740 uM. Under these condi-

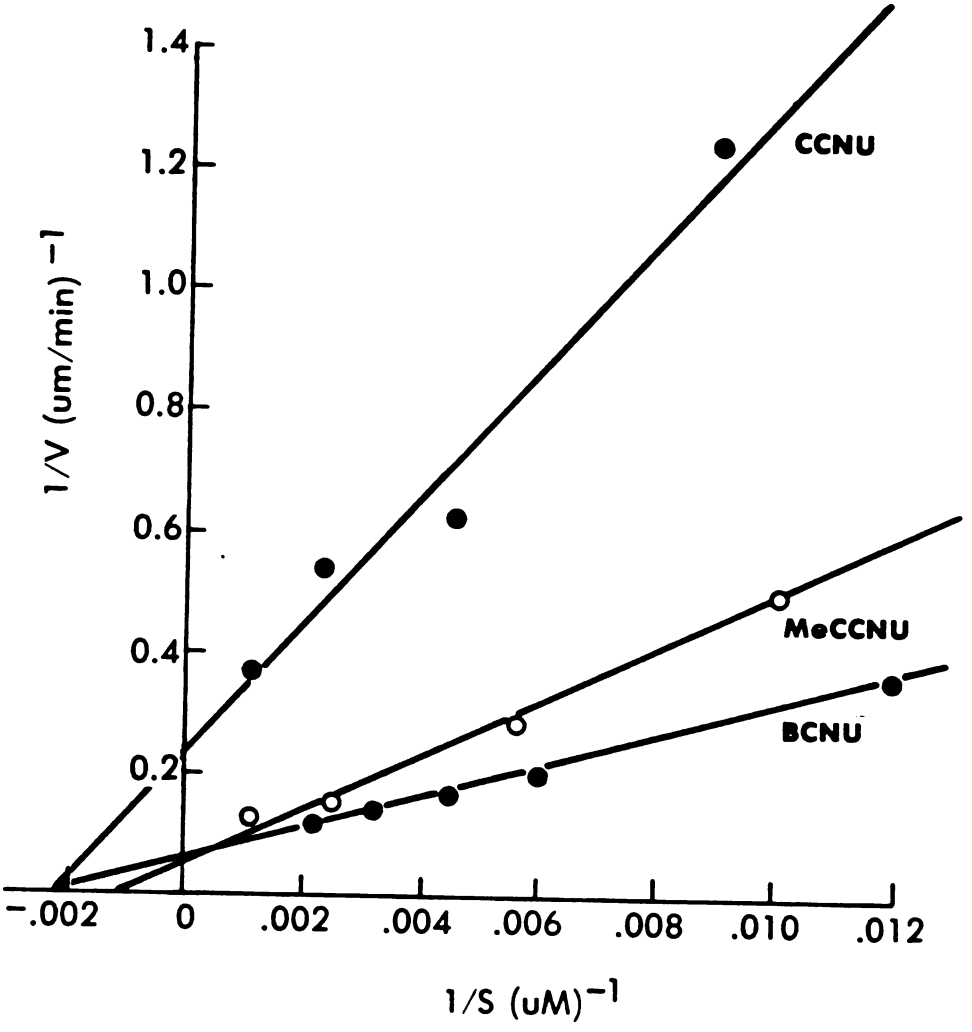


Figure 28. Lineweaver-Burk plot of albumin catalyzed disappearance of CCNU, MeCCNU, and BCNU.

Table XVI CENU first order disappearance rate constants and V_{max} and K_m parameters for albumin catalyzed disappearance.

	PCNU	BCNU	CCNU	MeCCNU
k_{buffer}, min^{-1} $(t_{½})^a$	0.027 (25)	0.014 (49)	0.013 (53)	0.014 (49)
k_{serum}, min^{-1} $(t_{½})^b$		0.046 (14)	0.023 (30)	0.024 (29)
$k_{albumin}$, min^{-1} $(t_{½})^c$	0.025 (27)	0.048 (14)	0.020 (34)	0.031 (22)
$k_{albumin} - k_{buffer}{}^d$	-0.002	0.034	0.007	0.017
V_{max} (μM/min)^e		17.5	4.1	19.2
K_m (μM)^e		448	462	855

[a]0.07 M phosphate buffer, pH 7.4, 37°C.

[b]Lipoprotein free human serum.

[c]The limiting rate constant observed at high albumin/CENU ratio, Figure 1.

[d]Rate constant of the protein catalyzed reaction at saturation.

[e]From Figure 2.

tions, protein catalyzed chloroethylnitrosoureas decomposition occurs at maximum velocity, is independent of changes in protein-concentration but is dependent on the concentration of chloroethylnitrosourea that is free to bind to protein.

The rate of chloroethylnitrosourea degradation in serum has been shown to be dependent on serum lipoprotein concentration and the chloroethylnitrosourea partition coefficient between serum and lipoprotein[123]. In the presence of these lipids, the chloroethylnitrosourea concentration in serum is reduced by partition into the lipoprotein core region. Chloroethylnitrosourea stability is increased because of the slow rate of nitrosourea degradation in the lipid media. The serum degradation rate constant can be described as the sum of the aqueous degradation rate and the maximum velocity of the protein catalyzed reaction.

Compounds that have a high binding affinity to albumin can also affect the rates of chloroethylnitrosourea disappearance. The rather weak and nonspecific association between a chloroethylnitrosourea and albumin can be inhibited by molecules that are not structurally similar to the chloroethylnitrosourea. Inhibition of chloroethylnitrosourea-albumin association reduces the contribution of the protein catalyzed reaction rate so that aqueous degradation becomes a major in vitro serum disappearance pathway. These results strongly suggest that the in vivo biodistribution and antitumor activity of BCNU, MeCCNU and, to a lesser extent, CCNU, may be affected by interreactions with drugs that have high albumin binding affinities.

Conversion of chloroethylnitrosourea to reactive products can affect the antitumor activity or toxicity of these drugs. Because the reactivity of the 2-chloroethylazohydroxide or 2-chloroethyldiazonium ion is thought to be very high, it is not probable that this intermediate will diffuse

from extracellular fluid to the nucleus of a tumor cell. Formation of the reactive species in serum would, therefore, reduce the concentration of parent drug that would be free to diffuse into the target cell and increase the amount of reactive, potentially toxic species formed in blood.

Chapter VI. METABOLIC REACTION OF CHLOROETHYLNITROSOUREAS

A. Introduction

Not much work has been done on the metabolism of chloroethylnitrosoureas perhaps because these compounds are not chemically stable. Most research on the metabolic reactions of chloroethylnitrosoureas has been centered on the biotransformation of CCNU to hydroxylated metabolites[94-102]. BCNU was reported to be metabolized to BCU[105] and possibly to a glutathione conjugate, although this compound has not been fully characterized[97]. However, the dramatic effect of phenobarbital on the antitumor activity of these compounds indicates that the metabolic reactions may be important. Phenobarbital pretreatment, as commonly used for enzyme induction, eliminated the antitumor activity of BCNU and reduced the activity of MeCCNU and PCNU (Table XVIII)[106].

Metabolic reactions of other alkylnitrosoureas have been reported. N-Butylurea has been shown to be a metabolite of the nitrosated compound[109]. N-Methyl-N'-nitrosoguanidine is denitrosated by an enzyme in the soluble portion of rat liver, kidney and stomach[125]; but the microsomal enzyme that catalyzes denitrosation of BCNU and MNU is clearly not related to this enzyme, which does not require NADPH or any other cofactors, while the metabolic reaction of BCNU requires NADPH[105].

The principal site for the metabolism of BCNU is the liver. Homogenates of mouse lung tissue had 30% of the activity of liver homogenates expressed on the basis of the amount of protein present in the assay[105]. There was no detectable activity (less than 5% of that in the liver) in homogenates of mouse kidney, spleen, brain, muscle, or intestine, or in mouse serum[105]. No in vivo metabolic studies have been conducted on BCNU. The in vitro metabolism of BCNU were carried out using rat liver homogenate in this study. However, extrapolation of in vitro results does not

Table XVIII Effects of 2 weeks of oral phenobarbital on the intracerebral 9 L antitumor activity of BCNU, CCNU and PCNU in rats.

Drug	Dose (mg/kg)	T/C[b] after administration of phenobarbital at:		
		None	24 mg/kg	48 mg/kg
BCNU	14	213	100[c]	102[c]
CCNU	25	144	124	129
PCNU	14	215	165	141[d]

[a] 12 animals per group.

[b] T/C = (median day of death of treated x 100)/ median day of death of control.

[c] $P<0.001$ compared with control.

[d] $P<0.01$ compared with control.

always fit in in vivo systems, which involves more complexities encountered in whole animal metabolism.

B. Experimental Methods

1. The 9,000 x g Supernatant, 100,000 x g Supernatant, and 100,000 x g Microsomal Fraction, Preparation and Related Assay

In vitro metabolism studies have led to an understanding of tissues in which metabolic reactions take place, the steps involved in the metabolic sequence, the components of the enzyme system that catalyze these various steps, biochemical properties of the components and the mechanisms which control enzyme activity. The majority of in vitro studies use the liver homogenate fractions. The 9,000 x g supernatant fraction contains the membrane-bound enzymes including mixed-function oxidase, soluble enzymes, and the NADPH-generating system. The 100,000 x g supernatant contains only soluble enzymes and the NADPH-generating system. The 100,000 x g microsomal fraction contains the membrane bound enzymes of the endoplasmic reticulum.

The preparation of rat liver microsomal and supernatant fractions is conducted according to a method published by Fouts[126]. Fischer C-344 male rats are fasted overnight prior to removal of the livers. All of the preparative procedures are done in a cold room. Cold 0.01 M Sorensen's phosphate buffer[127] at pH 7.4 with 1.15% KCl was used as the homogenation medium. The buffer contains monophosphate (1/15 molar) and disodium phosphate (1/15 molar). The animal was stunned with a blow to the back of the head and sacrificed by decapitation.

Blood was drained and fur wetted with water to prevent hair from entering the abdominal incision. The liver is rapidly removed and placed in cold 1.15% KCl - 0.01 M Na/K-phosphate buffer at pH 7.4 and washed to

remove hemoglobin, blotted dry, and placed into a preweighed beaker containing 25 ml of cold buffer. The tissue is minced with a scissors. The minced tissue is placed into a cold Potter-Elvehjem glass homogenizing tube with a cold teflon pestle for about 1 minute. Warming of the tube during homogenization should be avoided.

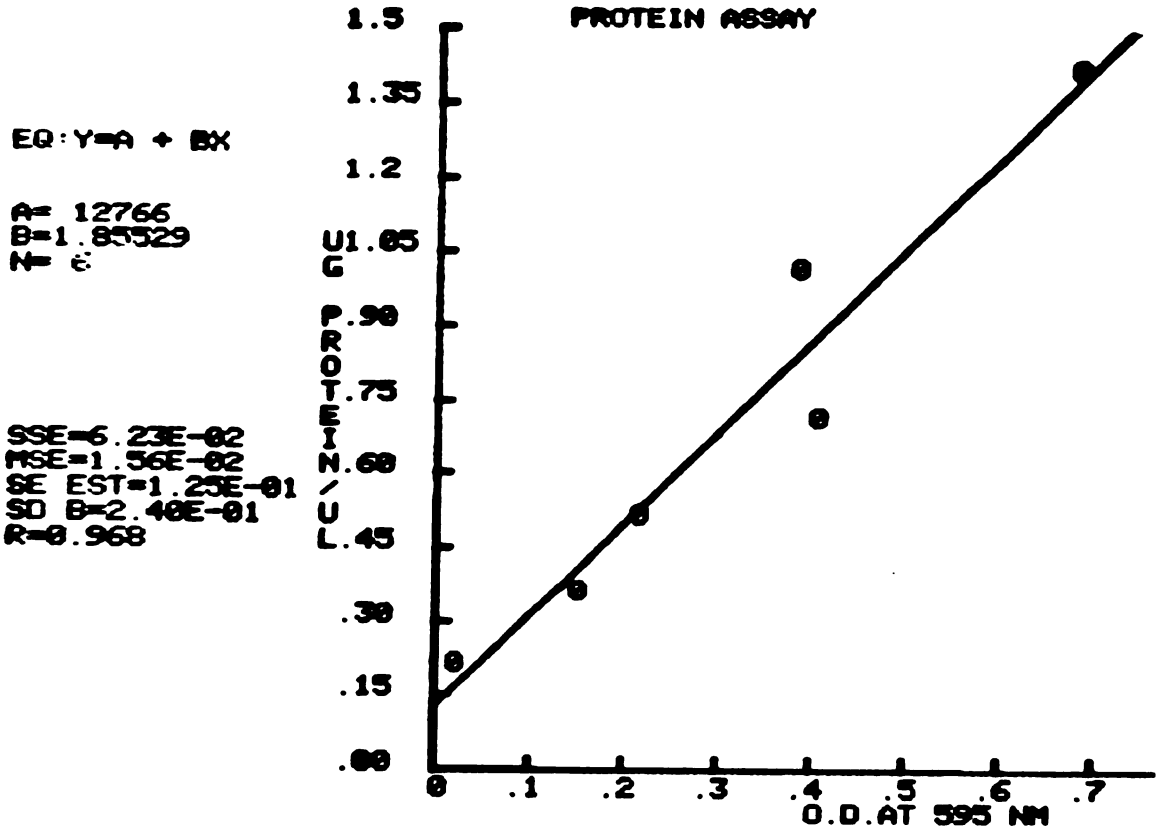
The homogenate is centrifuged at 9,000 x g (8700 rpm) in a Sorvall Superspeed RC2-B centrifuge for 15 minutes at $0^{o}C$. The supernatant is removed from below the white lipid upper layer and above the red-tan bottom pellet containing mitochondria and cellular debris. The supernatant is again centrifuged for 15 minutes. If more than 1 centrifuge tube is used, all of the tubes are pooled to minimize differences. This is the 9,000 x g supernatant fraction. To prepare the 100,000 x g supernatant and microsomal fractions, 10 ml of the 9,000 x g supernatant is placed into cold Spinco centrifuge tubes using cold buffer to completely fill the tubes. The tubes are then placed into a Beckman 50 Ti ultracentrifuge head and centrifuged at 100,000 x g (43,000 rpm) for 60 minutes at $2\text{-}5^{o}C$ in a Beckman L2-65B ultracentrifuge. The clear liquid is decanted and used as the 100,000 x g supernatant fraction. The microsomal pellet is resuspended in cold buffer and centrifuged for an additional 60 minutes. The clear supernatant is discarded and the pellet rehomogenized in 10.0 ml of buffer. This is the 100.000 x g microsomal fraction. Each 10 ml fraction represents 5 g of an initial 12.5 g of wet liver. The fractions may be used immediately or frozen at $-45^{o}C$ until use. The 100,000 x g microsomal pellet is stored as a pellet with 2 ml of buffer layered over it. The 9,000 x g supernatant fraction and 100,000 x g microsomal fraction can be stored for 1 month without substantial loss of p-nitroanisole O-demethylase activity. These stored fractions retain BCNU metabolizing ability.

The total protein in each liver homogenate fraction was determined

using the Bio-Rad Protein assay.[129] The assay is conducted on 0.1 ml of the homogenate prepared as described above. A 5.0 ml aliquot of freshly prepared dye reagent is added to each spectrometric tube. The dye reagent is prepared by diluting 20 ml of the dye concentrate with 80 ml of distilled water followed by vacuum filtering through a Whatman #1 filter paper. The tube is carefully vortexed to avoid excessive foaming. After 5 minutes to one hour, the absorbance of each sample is determined relative to an appropriate blank of buffer and dye in a Colman Jr. Spectrophotometer at 595 nm. Each sample is prepared in duplicate. A standard curve is generated using a Bio-Rad lyophilized protein standard at concentrations ranging from 0.35 to 1.36 μg/ l (Figure 29). The 9,000 x g, 100,000 x g supernatant, and 100,000 x g microsomal samples are prepared by diluting 100 μl of each homogenate solution described above with 500 μl of distilled water. After stirring or vortexing, 0.1 ml was used for the assay. This solution was then diluted 1:1 with distilled water and 0.1 ml of this dilution served as a second determination of the sample. Further dilutions were made if the absorbance at 595 nm is beyond the range of the standard curve. The blank contains 5.0 ml of dye and 0.1 ml of distilled water.

Enzyme activity of the liver microsomal enzymes was determined by assaying p-nitroanisole O-demethylase activity. This assay was performed using a modification of the procedure outlined by Zannoni.[130] O-Demethylation of p-nitroanisole to p-nitrophenol, which absorbs light at 420 nm, can be followed continuously.

The assay is performed by using 2 Coleman 12 x 75 mm round-bottom cuvettes. The control tubes contain 0.8 ml of phosphate buffer, 0.15 ml of 0.1 M $MgSO_4$, 1.5 ml of p-nitroanisole (0.31 mg/ml), and 0.02 ml of the supernatant or microsome sample. The assay tubes contain the components in the control tubes except 0.75 ml of phosphate buffer, and 0.05 ml NADPH



Protein Concentration (ug/ml)

= [Optical Density at 595 nm] x [Slope of the standard curve]

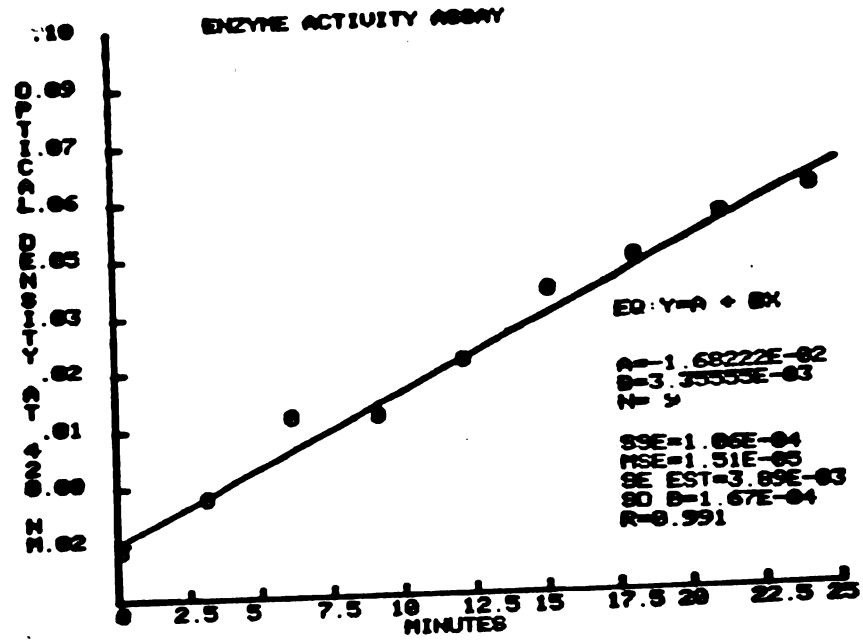
+ [Y-intercept of the standard curve]

Figure 29. Typical standard curve and equation used for the quantitation of the total protein in the liver homogenate fractions using the Bio-Rad protein assay and lyophilized protein standard.

(12.42 mg/ml) has been added. Before each sample time point, the Coleman Model 60 Jr. Spectrophotometer 420 nm absorbance is zeroed using the control tube. After the addition of NADPH, the tube was vortexed and "zero time" absorbance measurement was made. The tubes were incubated during the assay in a Dubnoff metabolic shaker at 37°C with shaking. The p-nitrophenol absorbance was measured every 3 minutes for 24-30 minutes. The absorbance of the product, 1 μg of p-nitrophenol, was determined under the assay conditions. p-Nitroanisole does not dissolve readily in buffer so the mixture must be heated at 45-50°C with shaking for over one hour or with sonication. This solution can be used for 8 hours. A 1 μg (0.003mM) sample of p-nitrophenol gives an absorbance of approximately 0.032. This value is used for each enzyme activity determination. A typical least squares linear regression analysis of these enzyme kinetic data is shown in Figure 30. Also shown is the equation used to calculate the enzyme activity expressed as μmoles of p-nitrophenol formed/hour/100 mg of total protein.

2. Kinetics of BCNU Metabolism in Rat Liver Microsomal Enzymes

The 100,000 x g supernatant and 100,000 x g microsomes were prepared as described above. The incubation mixture with 100,000 x g supernatant containing BCNU 540 μg in 80 μl ethanol (final concentration, 0.7 mM), 0.4 ml supernatant, 7.5 mg glutathione, 7.07 mg disodium EDTA, and phosphate buffer to a total volume of 3.6 ml, was incubated with shaking at 37°C for 40 minutes. The incubations with boiled supernatant or incubation without glutathione were used as control. Incubation with 100,000 x g microsomes contained BCNU 540 μg in 80 μl of ethanol (final concentration, 0.56 mM), microsomes 1.6 ml, 0.1 M $MgSO_4$ 0.1 ml, NADPH 7.5 mg, and phosphate buffer to a total volume of 4.5 ml. Incubation with boiled microsomes without NADPH was used as a control.



Liver microsomal p-nitroanisole O-demethylase activity (umoles of p-nitrophenol formed/hour/100mg total protein)

$$= \frac{[\text{Change in optical density/min}] \times [60 \text{ min/hr}] \times [100 \text{ mg total protein}]}{[\text{Optical density of p-nitrophenol}] \times [\text{MW p-nitrophenol}] \times [\text{amount of protein}]}$$

The optical density of p-nitrophenol = 0.032 under the experimental condition used.

The molecular weight of p-nitrophenol = 139.

Figure 30. Typical standard curve and equation for the spectrometric quantitation of the liver microsomal p-nitroanisole O-demethylase activity.

Aliquots of 200 µl were removed at 0, 5, 10, 15, 20, 30, 40 minutes. A known amount of BCNU-d_8 was added as internal standard and the mixture was extracted with ether (2 x 1 ml). The ether extract was evaporated to dryness by a stream of nitrogen prior to analysis. Selected ion monitoring chemical ionization mass spectrometry was used for the analysis.

3. Identification of Reaction Products

The incubation mixture containing BCNU with 100,000 x g microsomes is the same as those for kinetic studies. The mixture was incubated with shaking at 37°C for 30 minutes. Aliquots of 4 ml were extracted with hexane to remove unreacted BCNU and the aqueous solution was lyophilyzed. The residue was added to a small volume of water and centrifuged at 2,000 rpm for 2 minutes. The supernatant was used for the analysis of metabolite formation. The product, BCU was analyzed by HPLC with a C_{18}-uBondapak reverse phase column. The solvent system was 20% acetonitrile in water. The absorption at 205 nm was measured. The BCU peak was collected and checked by chemical ionization mass spectrometry.

4. BCU, a Metabolite of Chemical Decomposition Product?

BCU is a chemical decomposition product in the chemical reaction of BCNU with aqueous solution. As described in the previous section, BCU is formed by the reaction of chloroethylisocyanate and chloroethylamine, chemical decomposition reaction products. When a mixture of D_0/D_8-BCNU was used, an isotope ratio of BCU-$d_0/d_4/d_8$ of 1 : 2 : 1 was obtained.

A mixture of BCNU-d_0, 1.94 mg, and BCNU-d_8, 1.95 mg, was dissolved in 60 µl ethanol. A 20 µl aliquot of this solution was mixed with 3.2 ml microsomes, 6 mg NADPH in 2 ml buffer, 0.8 ml $MgSO_4$ (0.1 M) and 0.2 ml buffer to make a total volume of 6.2 ml, and the mixture was incubated with shaking at 37°C for 30 minutes. Incubation without NADPH was used

as control. Aliquots of 0.9 ml were taken at 10, 20, 30 minutes. The aliquots were extracted with 2 x 4 ml of ether. The combined ether extract was evaporated to dryness under nitrogen gas. The residue was dissolved in 30 μl of methanol, vortexed and 10 μl of the solution was injected into the HPLC. A C_{18}-uBondapak reverse phase column with 25% acetonitrile in water as solvent was used for separation. The peaks of BCU and BCNU were collected separately. The solutions collected from HPLC were extracted with ether, and analyzed by chemical ionization mass spectrometry. The peak height ratio was measured for BCU and BCNU and their isotope peaks.

5. Quantitative Analysis of the Metabolite BCU

The incubation containing BCNU 1.31 mg in 20 μl ethanol (final concentration, 0.99 mM), 3.2 ml microsomes, 6 mg NADPH, 0.8 ml 0.1 M $MgSO_4$, and phosphate buffer 2.2 ml to make a total volume of 6.22 ml, was incubated with shaking at 37°C, for 30 minutes. Aliquots of 250 μl were removed at 0, 5, 10, 15, 20, 30 minutes for the analysis of the disappearance of BCNU. Aliquots of 1 ml were taken at 0, 10, 20, 30 minutes. The aliquots were extracted with 3 x 3 ml ether, a known amount of CCU was added as internal standard and the combined ether extract was evaporated to a small volume. The samples were kept in dry ice until the analysis by HPLC at 205 nm wave length.

Incubations were conducted under nitrogen atmosphere and in open air. Formation of the metabolite, BCU was measured and compared. In the case of incubation under nitrogen gas, the incubation mixture without BCNU and NADPH was placed in a capped vial in ice bath and gased with nitrogen for 10 minutes. BCNU was added and, after thorough mixing, the first aliquot was removed as zero time point. NADPH was then added and the mixture was

incubated with shaking at 37°C and under a stream of nitrogen.

6. Secondary Metabolism of BCU by Rat Liver Microsomes

The incubation containing 292 µg BCU (1.02mM), 0.8 ml microsomes, 1.54 mg NADPH in 250 µl buffer, 0.2 ml $MgSO_4$ (0.1 M), and phosphate buffer 0.3 ml, was incubated at 37°C for 30 minutes. Aliquots of 200 µl were removed at 0, 10, 20, 30 minutes. The aliquots were extracted with 3 x 3 ml ether. A known amount of CCU was added as internal standard to the ether extract. This solution was evaporated under nitrogen gas and analyzed by HPLC.

C. Results

The rate of in vitro metabolism of BCNU in 100,000 x g microsomes was influenced by the content of lipid. Lipid materials have the effect of stabilizing BCNU because the drug is absorbed by lipid and is not free to undergo reaction with water or metabolism. The half life of BCNU with 100,000 x g microsomes (80 nmole/mg of mic. protein) was measured by Levin et al.[106] to be 24 minutes and V_{max} = 1.70 nmole/mg/min. At very low substrate concentration, 0.8 nmole/mg of mic. protein, the BCNU half life is 91 $\pm$ 7 minutes. The half life of BCNU in 100,000 x g microsomes observed during this work varied from 50 to 100 minutes. The BCNU/protein ratio was around 150 to 200 µmole/mg protein. The protein content of the microsomes ranged from 8 to 12 µg/µl. Lipid and protein content have opposite effects on the rate of reaction; enzyme activity might also change from one preparation to another. These factors make the kinetic study more complicated. Emphasis on the metabolism studies was then moved to the formation of metabolite(s).

1,3-Bis(2-chloroethyl)urea was identified as the major product. Figure 31 shows the HPLC chromatograph of ether extract of BCNU metabolic reaction mixture with the CCU internal standard. With the solvent system

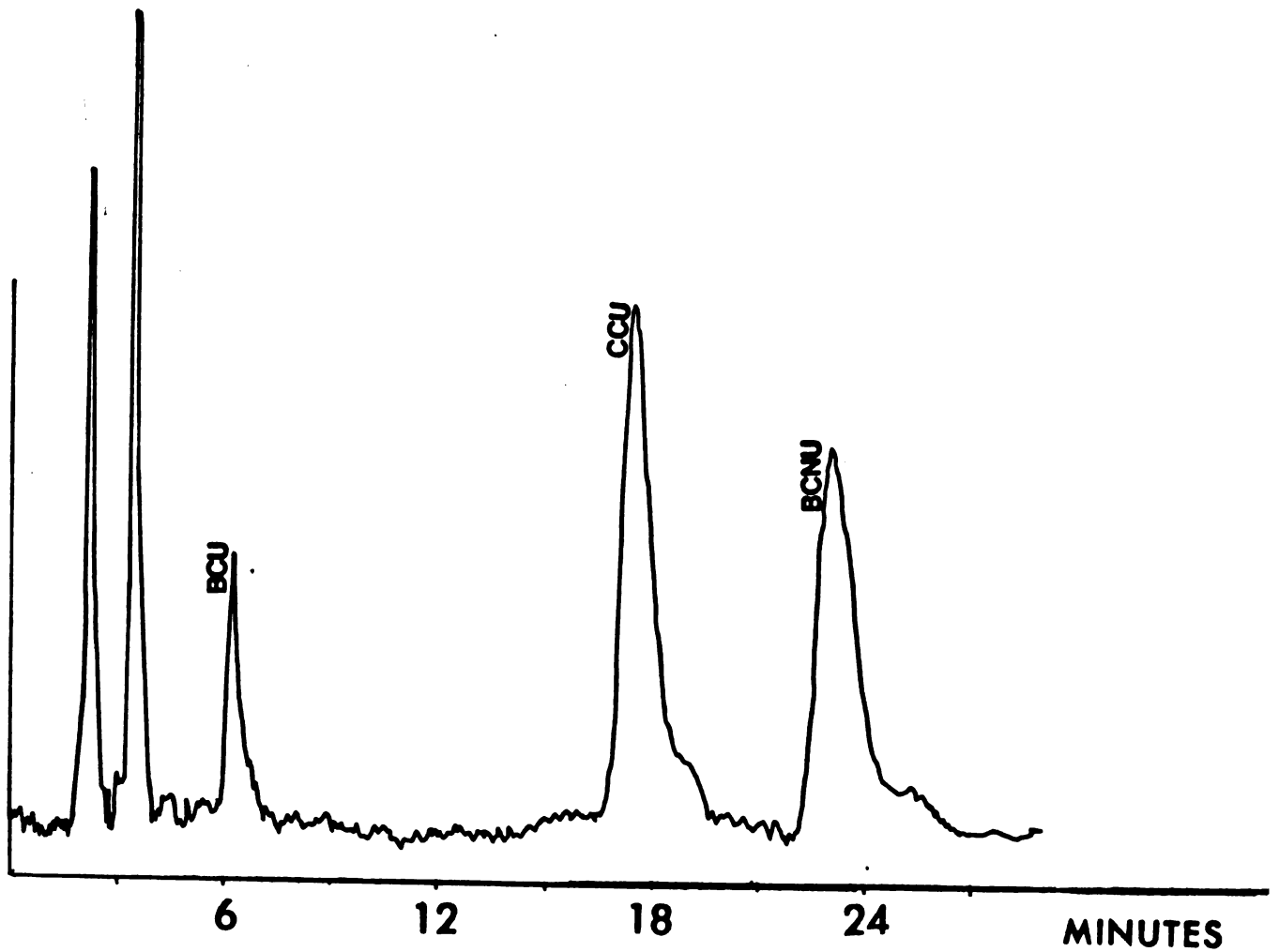


Figure 31. HPLC chromatograph of ether extract of BCNU metabolic reaction mixture with internal standard CCU.

of 25% acetonitrile in water and flow rate of 1 ml/min, BCU eluted at 6.3 minutes, CCU eluted at 17.7 minutes, and BCNU at 23.3 minutes after injection. The structure of BCU was further illustrated by the mass spectrum of the solution collected from HPLC.

The quantitative formation of BCU was followed by HPLC. Table XIX shows the disappearance of BCNU and the formation of BCU. After 30 minutes, 38% of starting BCNU was reacted and 16% of BCU was formed. This included BCU formed from chemical decomposition as 5% of BCU was observed in the control study with boiled enzyme preparation. It may be noted also that BCU formation in the control study did not increase throughout the reaction period. This probably was due to the secondary metabolic reaction of BCU or simple chemical reaction. A 15% loss of BCU was observed when BCU was incubated with microsomes at 37°C for 30 minutes. The difference between the experimental and control experiments can be accounted for by the metabolic reaction of BCNU. There was about 10% BCU formed from the metabolic reaction for 30 minutes (Figure 32).

Incubation of a mixture containing BCNU-d_0 and -d_8 clearly demonstrated that the formation of BCU with rat liver microsomes occurred through a mechanism other than chemical decomposition. Figure 33 shows the mass spectra of BCU formed in in vitro metabolism with 100,000 x g microsomes and unreacted BCNU, both separated and collected from HPLC. Figure 34 shows a mass spectrum of lyophilized aqueous solution from hexane extracted incubation mixture. Table XX shows the isotope ratio of BCU formed in in vitro metabolism, and that of unreacted BCNU. They were separated by HPLC column and the peaks were collected. There is no evidence of BCU-d_4 which suggests that the formation of BCU as a metabolite is not through the mechanism of recombination. The cleavage of the molecule as the first step in chemical reaction does not occur in the metabolic

Table XIX Disappearance of BCNU and formation of BCU in the metabolic reaction by rat liver microsomes.

time(min)	per cent of BCNU		
	BCU (microsomes)	BCU (boiled enzyme)	BCNU
0	12.8	7.1	100
5			82
10	18.1	6.4	74
15			72
20	18.5	6.1	65
30	16.0	5.3	62

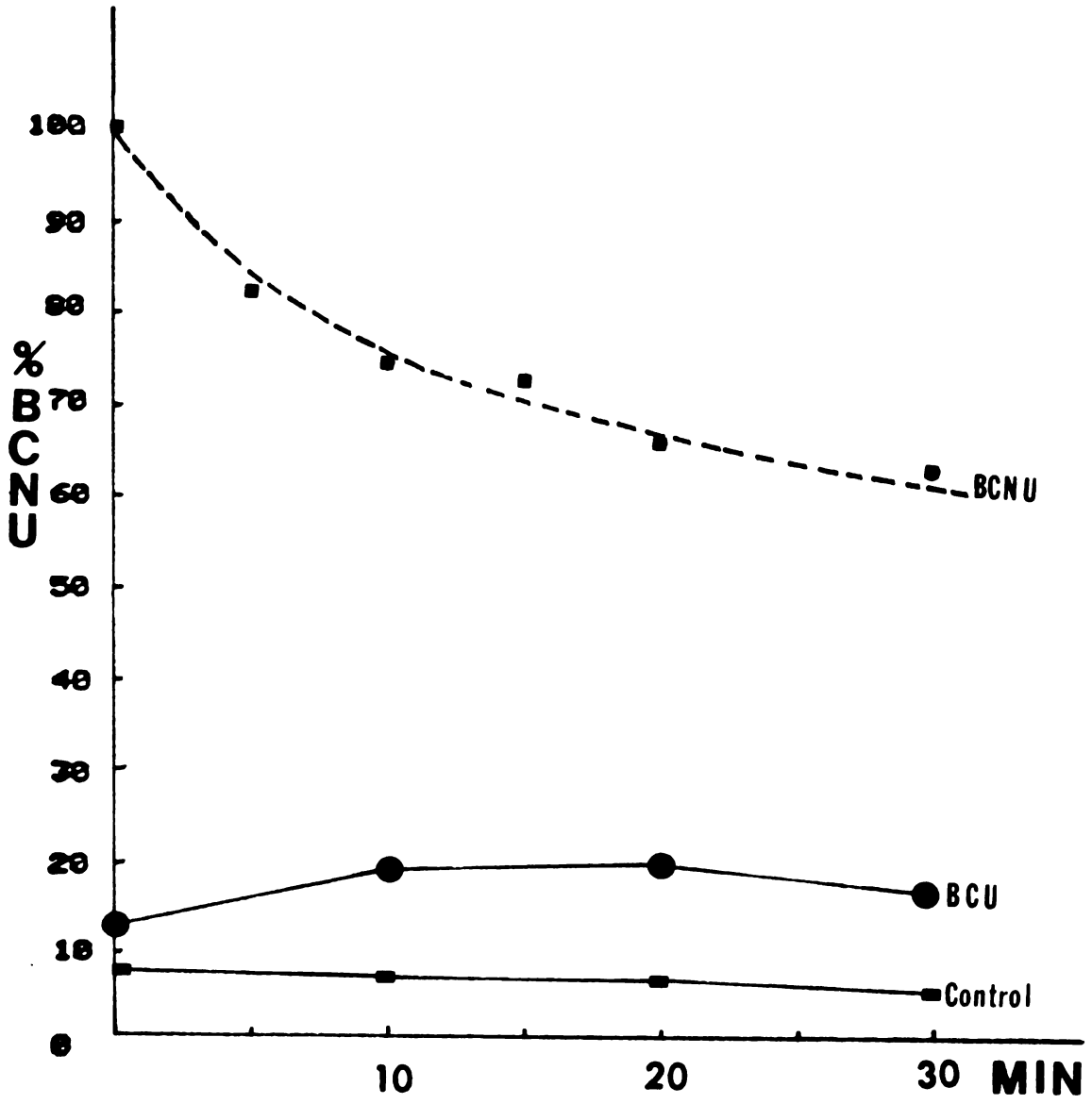


Figure 32. Disappearance of BCNU and formation of BCU in the metabolic reaction by rat liver microsomes.

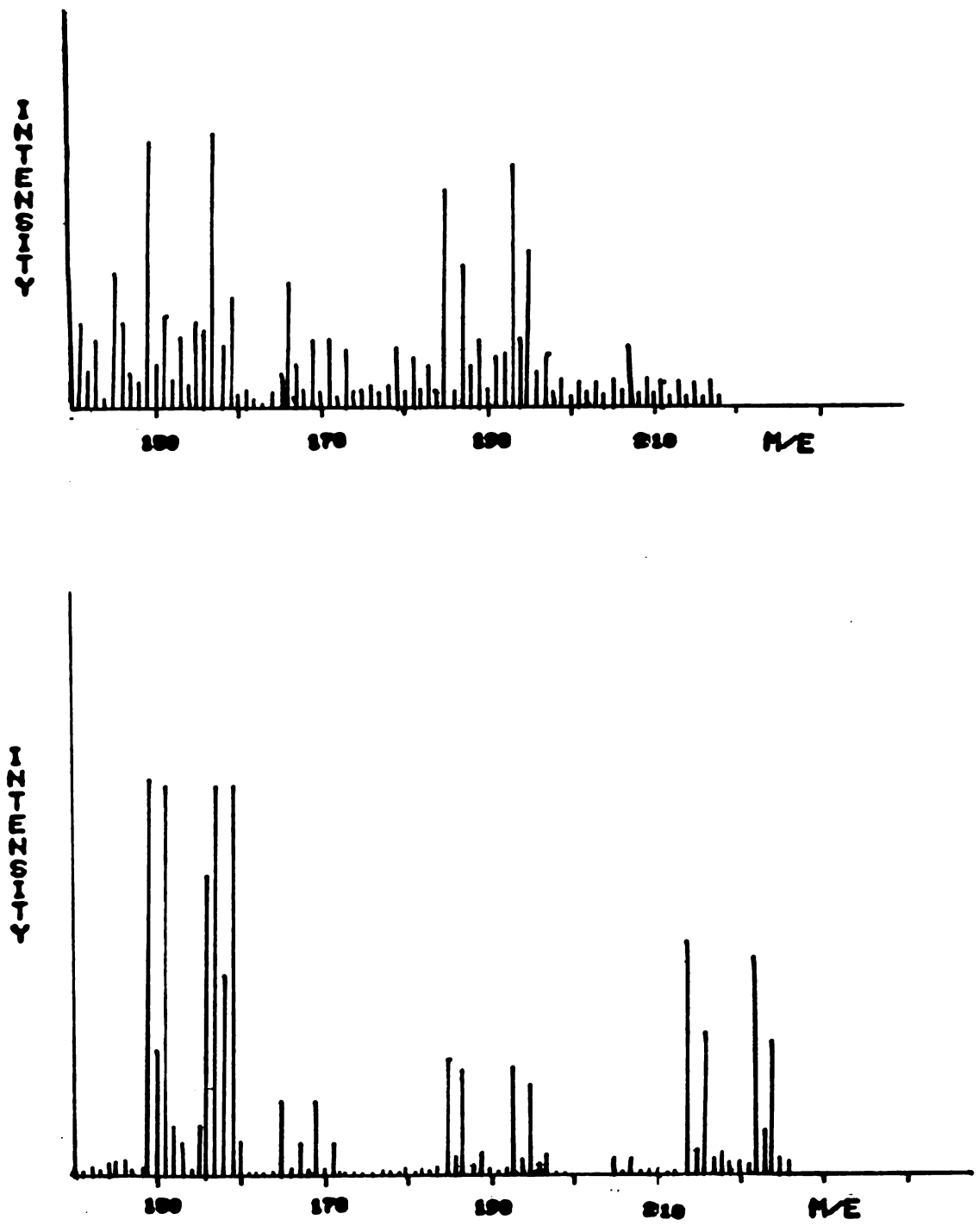


Figure 33. Mass spectra of BCU formed in vitro metabolism by rat liver microsomes (upper) and unreacted BCNU, both were collected from HPLC column.

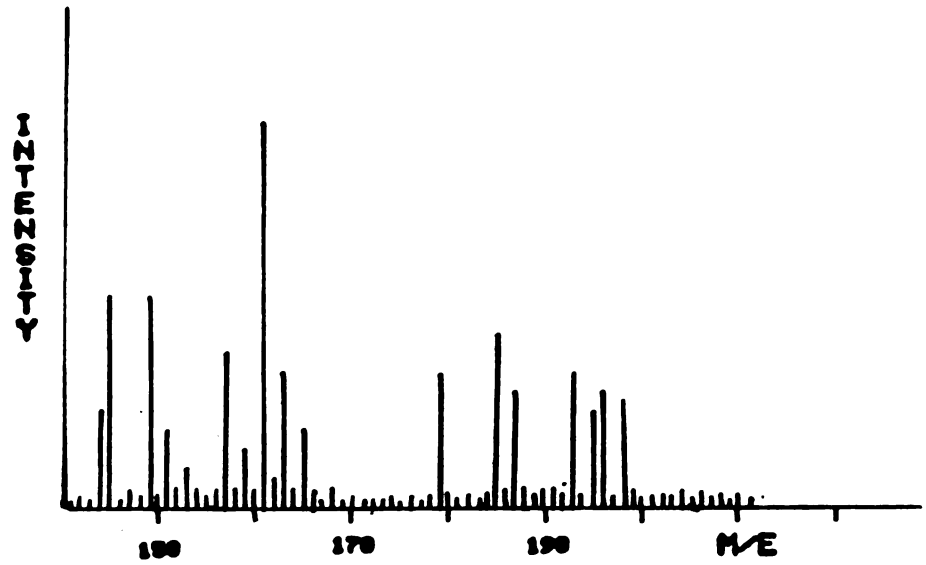


Figure 34. Mass spectrum of lyophilized aqueous solution from hexane extracted incubation mixture of BCNU with microsomes.

Table XX **Isotope ratios of BCU and unreacted BCNU from in vitro metabol metabolism of a d_0/d_8 BCNU with rat liver microsomes.**

	time	d_0	d_4	d_8
BCNU	20 min	1.03		1
	30 min	1.08		1
BCU	10 min	1.0		1
	20 min	1.0		1
	30 min	0.90		1

reaction.

The in vitro metabolism of BCNU with 100,000 x g microsomes was studied under different conditions. Oxygen was not required for the metabolic reaction. When the reaction was conducted under a nitrogen atmosphere, the formation of BCU is not significantly affected. Figure 35 shows that there is no significant difference on the BCU formation between these incubations under nitrogen or in open air. The formation of BCU from BCNU metabolism was calculated by taking the difference between the experimental and a control incubation. Table XXI lists the percent formation of BCU at different time points and different conditions.

Phenobarbital induced rat liver microsomes were also used for in vitro metabolism. Rats were treated with phenobarbital for 12 days before the liver was removed. Phenobarbital, 60 mg, was dissolved in 200 ml of drinking water. The dose of phenobarbital will be 6 mg/rat/day, assuming 20 ml of water was taken per day per rat. The enzyme activity measured as O-demethylation was increased 3 to 5 fold. Table XXII lists the BCU formation from the BCNU in vitro metabolism by phenobarbital pretreated rat liver microsomes. Also shown is the formation of BCU under nitrogen gas. There is a slight increase of BCU formation with pheobarbital pretreated rat liver microsomes, 36% increase for the incubations in open air, and 31% increase for the incubations under nitrogen. However, there was still no significant difference between the reactions under air or nitrogen. Figure 36 shows the amount of BCU formed by BCNU metabolism, in which BCU formed from chemical decomposition as compared with control group was excluded.

D. Discussion

Metabolic reaction of BCNU is different from that of CCNU or MeCCNU,

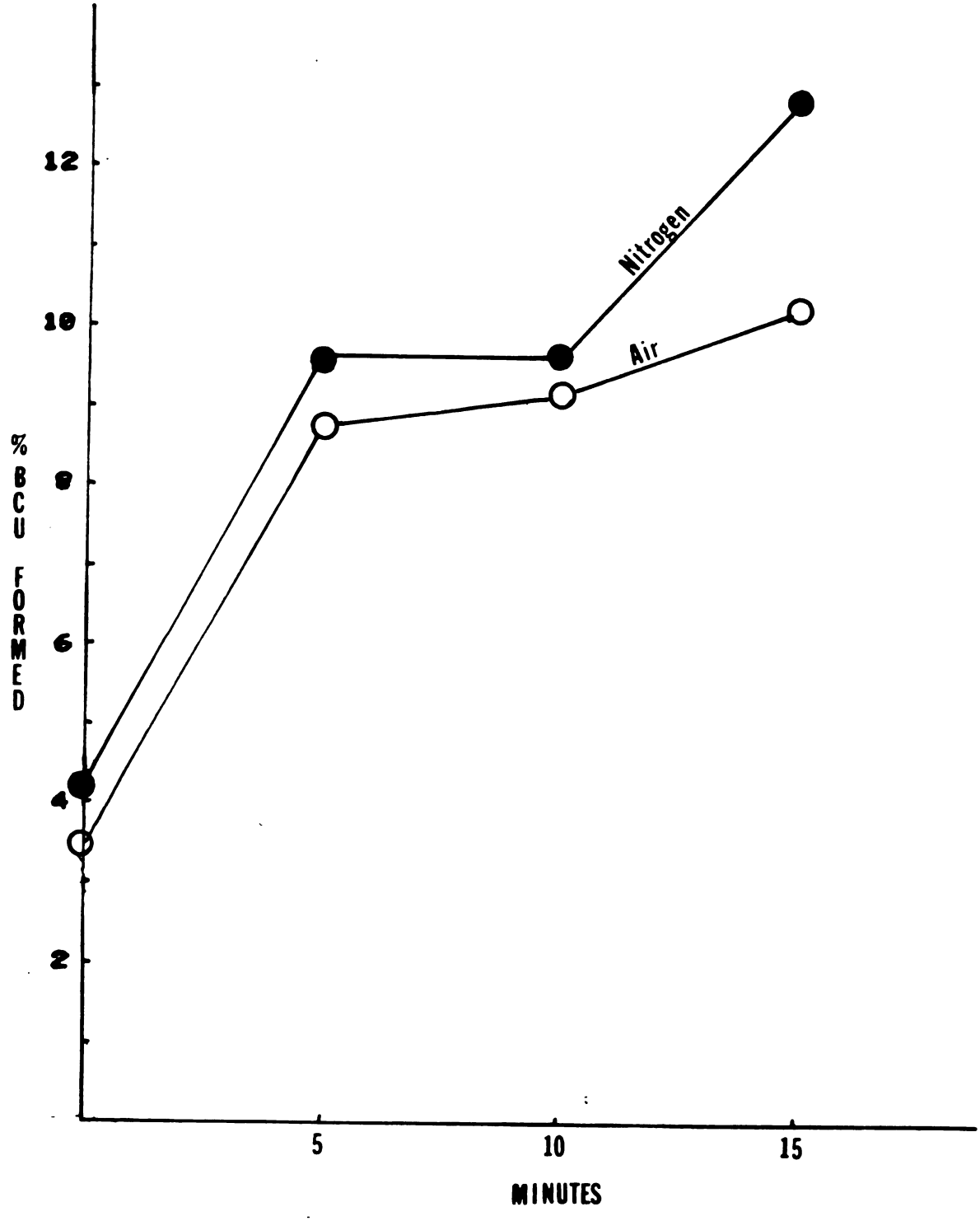


Figure 35. Formation of BCU from BCNU metabolism under nitrogen or in open air.

Table XXI BCU formation from BCNU in vitro metabolism by rat liver microsomes.

Exp.	Time (min)	per cent BCU formed		
		air	nitrogen	control
I	0	4.3	3.4	
	3	6.7	8.4	
	6	6.9	9.0	
	9	7.7	8.6	
	15	9.5	10.5	
	20	6.7	10.8	
	30	6.6	10.2	
II	0	10.5	10.5	8.1
	5	21.8	16.3	8.5
	10	19.2	-	7.0
	15	18.6	22.7	6.4
III	0	8.8	9.7	10.9
	5	18.1	14.8	10.4
	10	24.4	10.9	12.1
	15	20.2	20.1	-
IV	0	16.7	20.2	9.2
	5	15.5	25.7	8.4
	10	12.5	18.6	8.4
	15	14.1	19.5	4.1

Table XXII **BCU formation from BCNU in vitro metabolism by phenobarbital pretreated rat liver microsomes.**

Exp	Time (min)	Per cent BCU formed		
		air	nitrogen	control
I	0	11.7	1.6	1.89
	5	23.6	11.1	1.4
	10	17.9	16.4	2.3
	15	21.8	19.7	2.0
II	0	1.6	0.99	0.99
	5	7.1	8.0	1.5
	10	7.8	12.5	0.7
	15	7.8	16.6	-

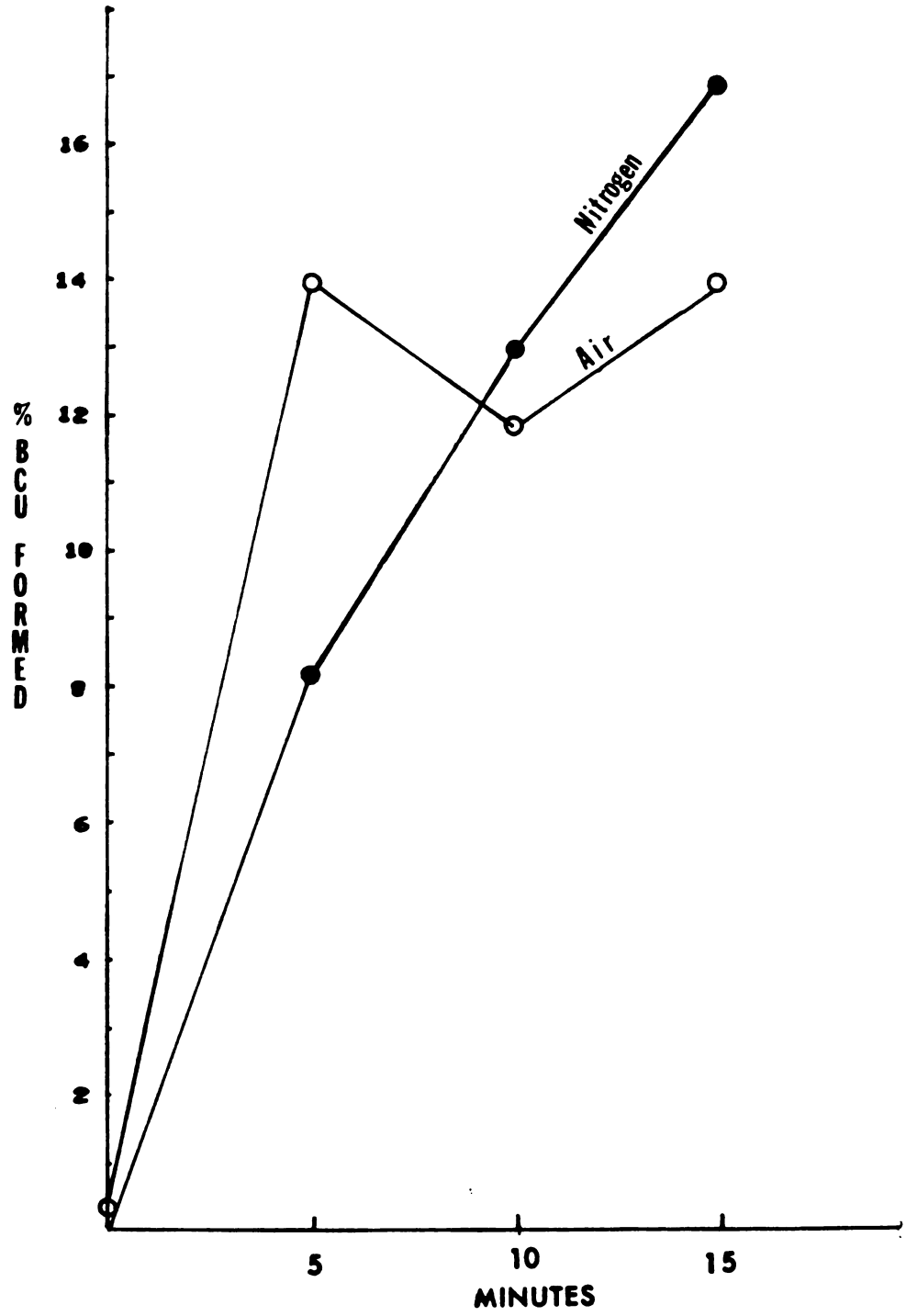


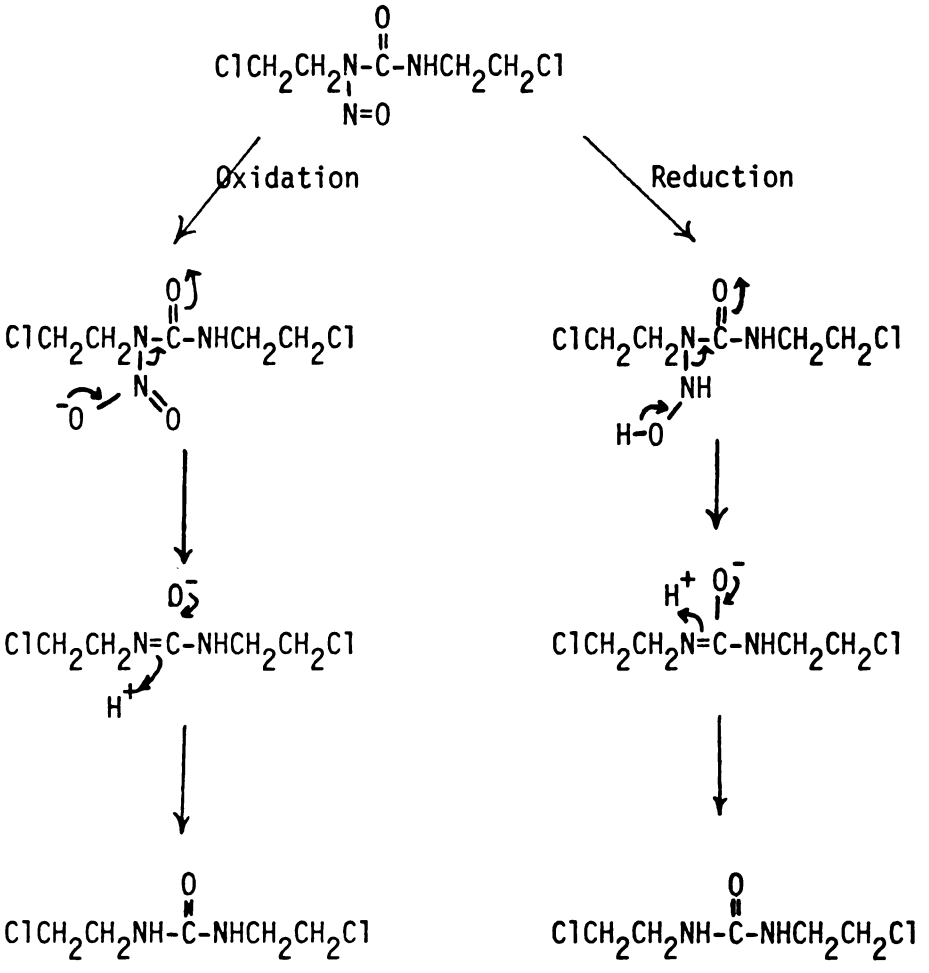
Figure 36. Formation of BCU from BCNU metabolism under nitrogen or in open air (phenobarbital pretreated rat liver microsomes).

which are metabolized to hydroxylated products. Denitrosation also occurs in the metabolic reaction of MeCCNU and accounts for 24% of the products recovered. It becomes a major metabolic pathway accounting for 40% of BCNU metabolized, in BCNU metabolism with 100,000 x g rat liver microsomes. Other metabolites have not been identified yet.

It is interesting to find that the metabolic reaction of BCNU to BCU undergoes denitrosation as indicated by the formation of BCU-d_0 and -d_8. In general, metabolic reactions of nitrogen-containing organic compounds undergo oxidation or reduction. For the oxidative metabolic reactions, amines can be oxidized to hydroxylamines, which can be further oxidized to nitroso compounds and finally to nitro compounds. N-oxidation of an amine to a N-oxide can also occur for quaternary amine[128]. The reverse reactions are also found as metabolic pathways, i.e., reduction of nitro compounds to nitroso compounds, further reduction to hydroxylamine and finally to amine[128]. The nitrosoureas would be expected to be either oxidized to a nitro compound or reduced to a hydroxylamine. Scheme XVII shows these two possible reaction mechanisms that may lead to the formation of BCU as the final product. If either of the pathways is the reaction mechanism, either oxygen or nitrogen would inhibit the reaction. As an oxidative metabolic reaction, oxygen is required, while as a reductive metabolic reaction the presence of oxygen will inhibit the reaction. However, the results obtained from the experiments indicated that the rate of BCU formation was not influenced by the presence of air or nitrogen. It could be explained that the reaction proceeds through either pathway dependent on the condition, or the reaction undergoes another totally different pathway not involving the oxidation-reduction mechanism.

A 9,000 x g supernatant fraction was prepared from 9L tumor cells and tested for the metabolic activity against BCNU. No metabolic activity

Scheme XVII


ClCH2CH2N-C-NHCH2CH2Cl
N=O
Oxidation
Reduction
ClCH2CH2N=C-NHCH2CH2Cl
ClCH2CH2NH-C-NHCH2CH2Cl

was observed in the reaction with BCNU. The metabolic reaction probably takes place before the drug gets into the tumor cell, and may be metabolized in liver or other tissues. Since phenobarbital pretreatment eliminates the antitumor activity of BCNU against intracerebral inoculated 9L tumor cells in rats, that suggests the importance of the metabolic transformation. In vitro metabolism of BCNU demonstrated that phenobarbital increases the metabolic formation of BCU, which is a deactivated product. How does phenobarbital affect the antitumor activity of BCNU? An effort was made to analyze BCU formation in the urine of normal rats and phenobarbital pretreated rats after ip BCNU. This information would contribute to the understanding of the in vivo biotransformation of BCNU and the mechanism of BCNU deactivation by phenobarbital. Mass spectrometry and HPLC were used however, these analytical techniques do not have sufficient sensitivity to analyze BCU in the presence of large quantities of impurities present in urine extracts.

Chapter VII. CONCLUSION

Chloroethylnitrosoureas react in aqueous solution with an initial cleavage to yield alkylating and carbamoylating intermediates. The alkylating intermediate is thought to be responsible for antitumor activity. Chemical decomposition products, such as 2-chloroethylamine, 2-chloroethanol, acetaldehyde, 2-(2-chloroethylamino)-2-oxazoline, 2-oxazolidone, 1,3-bis(2-chloroethyl)urea, 1-(2-chloroethyl)-3-(2-hydroxyethyl)urea, are formed from BCNU in phosphate buffer at pH 7.4 and 37°C.

Studies with deuterated BCNU showed that the formation of BCU was formed from the reaction of the intermediate chloroethylisocyanate and a product chloroethylamine instead of a denitrosation reaction. Formation of BCU is concentration dependent. Oxazolidone and chloroethylamine are generated from the same intermediate, chloroethylcarbamate. Intramolecular cyclization of chloroethylcarbamate gives 2-oxazolidone, which cannot occur in the case of CCNU. However, formation of part of 2-(2-chloroethylamino)-2-oxazoline was formed directly from BCNU in addition to the pathway of cyclization of BCU.

The low yield of 2-(2-chloroethylamino)-2-oxazoline and the derivation of other reaction products from the chloroethyldiazonium ion and isocyanate species indicate that this initial cleavage reaction is the major chloroethylnitrosourea decomposition pathway in aqueous solution. Less than 5% of starting chloroethylnitrosourea has been found to react by another route in these studies that account for 80-85% of starting material.

Chemically, BCNU is usually given by iv infusion. Pharmacokinetic studies showed that this drug disappear rapidly from plasma in vivo. This rapid clearance may be due , at least in part to a plasma protein catalyzed decomposition on BCNU. The half-life of BCNU in serum is much shorter than in aqueous buffer. In additions, a high percentage of covalent binding was

observed. This probably results from the formation of reactive intermediates on the protein surface by the protein-substrate complex implicated in the catalyzed reaction mechanism. This catalysis by plasma protein can be competitively inhibited by highly protein binding agents like salicylic and dodecanoic acids.

Plasma lipoproteins, on the other hand have been found to stabilize chloroethylnitrosoureas. This is probably due to the partition of the lipophilic chloroethylnitrosourea into the lipoprotein core. The chemical and physical interactions that have been an identified role in the biodistribution of chloroethylnitrosourea are shown in Figure 37.

In addition to the above interactions, metabolic denitrosation has been identified as a factor that affecting the pharmacokinetics and chemotherapeutic activity of BCNU. Phenobarbital induced metabolism in rat eliminates the antitumor activity of BCNU, reduces the activity of MeCCNU, and PCNU but does not affect CCNU, presumably because this compound is metabolized to active hydroxylated products. BCU was identified as a metabolite of BCNU in vitro metabolism by rat liver microsomes. The formation of BCNU takes place via a different mechanism than in aqueous chemical decomposition. Results from metabolic studies with a mixture of BCNU and BCNU-d_8 indicated that the mechanism was denitrosation process. The details of the mechanism are still not clear. The percent yield of BCU is about 40% of the BCNU metabolized in the in vitro metabolism. Phenobarbital pretreatment causes a 30% increase in BCU formation from rat liver microsomal metabolism. This is consistent with the increase in BCNU clearance observed in phenobarbital pretreated rats and deactivation of BCNU by this agent as the denitrosation of BCNU will result in a loss of activity.

BCNU and other chloroethylnitrosoureas have simple chemical structures,

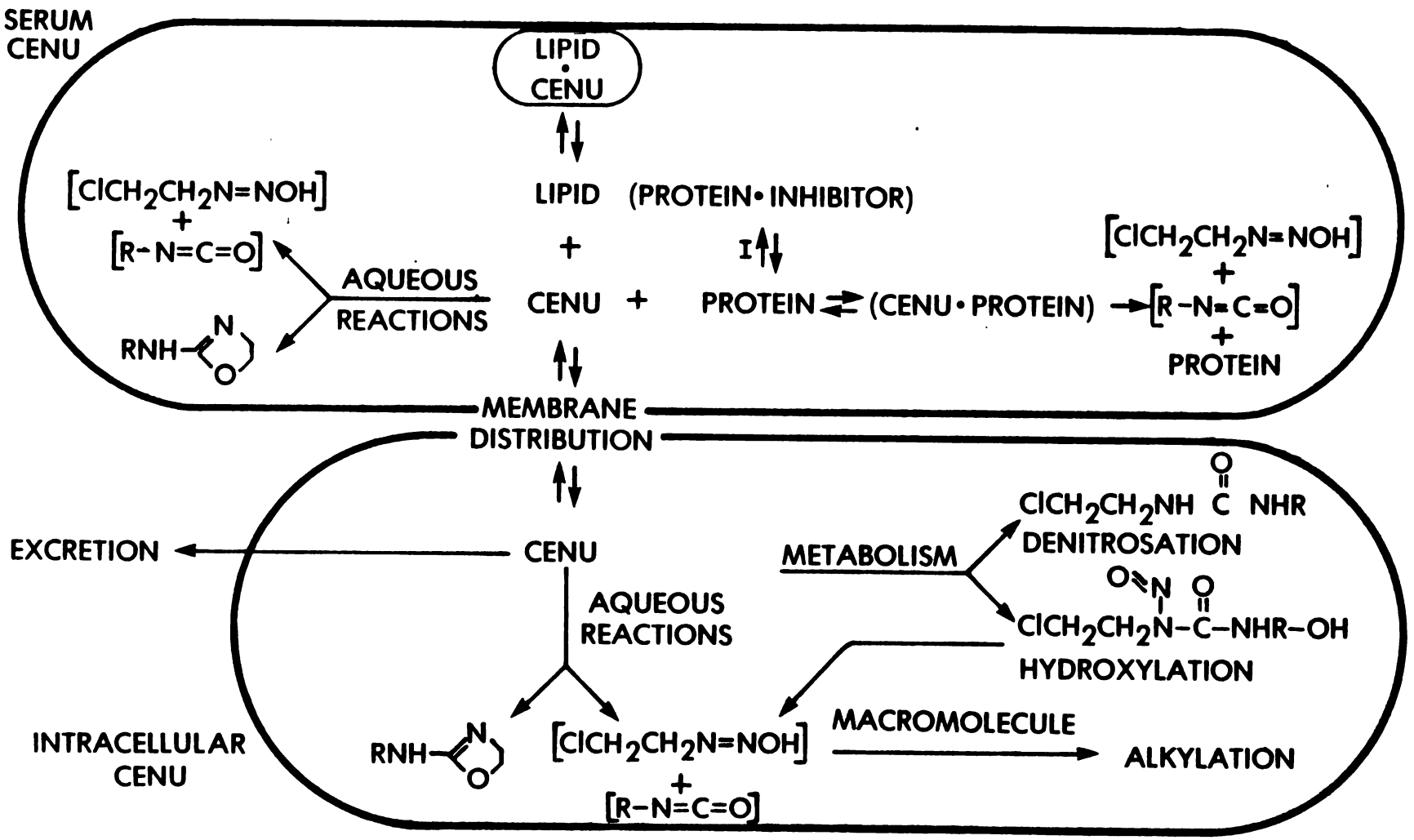


Figure 37. Chemical-biological interactions of serum and intracellular chloroethylnitrosoureas

but the chemical and enzymatic reactions of these drugs are complicated. The lipophilicity of these drugs enable them to cross the blood brain barrier. However, these drugs have to survive from all the chemical and enzymatic reactions before entering the target cells. In biological systems, the chemical decomposition intermediates react with macromolecules to initiate antitumor and toxic effects. It is significant to note, however, that vitually all of the stable reaction products are known to be toxic or are structurally related to toxic substances. These products will contribute to the toxic manifestations of chloroethylnitrosoureas to the extent that they are formed in vivo. The ease with which formation of the aminooxazoline ring may be reversed by chloride ion suggests that this product may covalently bind to more nucleophilic functions present in the biological system.

Drug interactions may be expected between chloroethylnitrosoureas and highly protein binding drugs, that displacement of protein binding may occur and lead to a slower decomposition rate in the blood. The administration of chloroethylnitrosoureas with phenobarbital, which is usually taken as an anticonvulsant in brain tumor patients, was shown in animal test to alter the antitumor activity of these drugs. Further studies are necessary to define the magnitude and pharmacological significance of these interactions in human.

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