Certain 2-chloroethyl nitrosoureas have been demonstrated to be highly specific inactivators of chymotrypsin in vitro and glutathione reductase, both in vivo and in isolated rat hepatocytes. The inactivation of glutathione reductase in isolated hepatocytes resulted in an increased sensitivity toward an adriamycin induced oxidative challenge.

Prolonged incubation of 1-(2-chloroethyl)-3-([1-14C]cyclohexyl)-1-nitrosourea (CCNU) with chymotrypsin resulted in covalent modification and concomitant inactivation of chymotrypsin via degradation of the nitrosourea to form cyclohexyl isocyanate. Cyclohexyl isocyanate was shown to be an active-site-directed inactivator of chymotrypsin. L-Lysine did not protect the enzyme from inactivation by cyclohexyl isocyanate. Degradation of an excess of
1-(2-chloroethyl)-3-([1-\(^{14}\)C]-cyclohexyl)-1-nitrosourea in the presence of enzyme yielded 1.11 ± .07 mole of enzyme inactivated. Short-term incubation demonstrated that the nitrosourea neither inhibited nor protected the enzyme from cyclohexyl isocyanate inactivation. Treatment of chymotrypsin with less than stoichiometric amounts of cyclohexyl isocyanate or titration of the active site serine with phenylmethanesulfonylfluoride prior to \(^{14}\)C-labeling resulted in a proportional decrease in bound \(^{14}\)C cyclohexyl moiety.

In addition, the specific inactivation of yeast glutathione reductase by 2-chloroethyl isocyanate and cyclohexyl isocyanate derived from their respective 2-chloroethyl nitrosoureas has been demonstrated. Titration of the enzyme with 2-chloroethyl isocyanate or \(^{14}\)C labeling with 1-(2-chloroethyl)-3-([1-\(^{14}\)C]-cyclohexyl)-1-nitrosourea or 1,3-bis-(2-[\(^{14}\)C]chloroethyl)-1-nitrosourea (BCNU) resulted in a near stoichiometric inactivation and/or covalent labeling of the enzyme. In addition to BCNU and CCNU several other 2-chloroethyl nitrosoureas were capable of inactivation of not only purified glutathione reductase, but also the activity of this enzyme in cell-free extracts of murine lymphoma L5178Y ascites tumor cells and murine bone marrow. A positive correlation has been shown between the ability of certain 2-chloroethyl nitrosoureas to
inactivate glutathione reductase and the degree of myelo-
toxicity observed for these nitrosoureas.

Incubation of isolated rat hepatocytes with BCNU resulted in the selective and extensive (>90%) inactivation of hepatic glutathione reductase. BCNU also depleted intracellular glutathione by 70% but had no significant effect on cell viability or lipid peroxidation. Incubation of BCNU-treated hepatocytes with adriamycin (ADR) resulted in a decrease in cell viability concurrent with an increase in lipid peroxidation. These effects were not observed with untreated hepatocytes incubated with ADR. Glutathione depletion with diethyl maleate and incubation with ADR did not result in increased cellular damage or lipid peroxidation. Incubation of BCNU-treated hepatocytes with ADR in the presence of α-tocopherol resulted in a significant amount of protection from ADR damage.
Specific Enzymic Interactions Relating to the Mechanism of Action on Certain 2-Chloroethyl Nitrosoureas

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SPECIFIC ENZYMIC INTERACTIONS RELATING TO
THE MECHANISM OF ACTION ON CERTAIN
2-CHLOROETHYL NITROSOUREAS

I. INTRODUCTION

The main theme of this thesis is based on the concept of drug specificity on the molecular level. More precisely, this work is concerned with specific enzyme inactivation by certain antineoplastic 2-chloroethyl nitrosoureas. A better understanding of drug-mediated enzyme inactivation on the molecular level, particularly active-site-directed processes, may help explain the complex physiological effects of these drugs. It follows that this type of information should lead to more enlightened drug design and use. In chapter III, work is described in which chymotrypsin was used as a model system to demonstrate that a remarkable degree of specificity of enzyme inactivation can be shown for certain 2-chloroethyl nitrosoureas hitherto considered to be nonspecific in their molecular mode of action. The specific inactivation of a physiologically important enzyme, glutathione reductase, by 2-chloroethyl nitrosoureas is discussed in chapter IV. Chapter V discusses the physiological consequences of the nitrosourea-mediated inactivation of glutathione reductase.
II. BACKGROUND PERSPECTIVES

Active-Site Directed Enzyme Inactivation and Chemotherapy

The quintessential element of enzyme catalysis and indeed the main premise of this thesis rests on the work of Michaelis and Menten (1913). They proposed the formation of a reversible enzyme-substrate complex as a prerequisite to catalysis. This Michaelis complex is the first step in an enzyme catalyzed reaction and is one major factor contributing to the specificity of enzyme catalysis. Specificity may also be imposed during the catalytic steps following substrate binding. Observed rate enhancements can be explained in part by the formation of an enzyme-substrate complex. In such a complex, reactive groups of the substrate (or substrates) are held properly aligned with respect to the enzyme catalytic site (or each other) to facilitate the reaction. The binding of substrate to the enzyme active site is primarily driven by hydrophobic forces in aqueous solution. The phenomena, sometimes referred to as "hydrophobic bonding", represents an entropic contribution to catalysis (Jencks, 1969). The proper orientation of bound substrate is assured by steric factors and short-range attractive forces at the binding site. These short-range forces include hydrogen-bonding,
electrostatic interactions, and induced interactions such as van der Waals interactions (Jencks, 1969).

The abovementioned phenomena can also be applied to explain specific enzyme inactivation by irreversible inhibitors. An analogous Michaelis complex can form between inhibitor and enzyme. The same hydrophobic forces, short-range interactions, and steric factors can be invoked to explain the formation of this complex and the productive alignment of the inhibitor at the active site. However, the subsequent chemical steps result in a covalent modification of the enzyme, which renders the enzyme incapable of further catalysis. Thus irreversible inhibitors that "fit" into the substrate binding site can be expected to exhibit a high degree of specificity. This type of enzyme inhibition was first termed active-site-directed by Baker (1964) in his description of diisopropyl phosphofluoridate inactivation of cholinesterase. The effectiveness of an active-site-directed irreversible inhibitor depends primarily on the structural complementarity between the inhibitor molecule and the binding site at or near the catalytic site of the enzyme. In addition, proper alignment of the reactive groups of the inhibitor and enzyme which are involved in covalent modification can be attributed to the previously mentioned steric and short-range interactions. In essence, a reagent which in free solution is reactive but nonspecific can be made to exhibit a high degree of specificity.
given the presence of a structurally complementary enzyme binding site. These binding phenomena can also offer specificity by excluding a reactive molecule, thus not allowing covalent modification of the active site.

Kinetic investigation of active-site-directed inactivation can provide information as to the effectiveness of an inhibitor and also offer supportive evidence for the occurrence of an active site process. If one plots the log of enzyme activity remaining versus time for several different inhibitor concentrations, a series of half lives can be converted to inactivation rate constants.

\[ t_{1/2} = 0.693 \frac{k}{k_{\text{inact}}} \]

A plot of \(1/k_{\text{inact}}\) versus \(1/\text{inhibitor concentration}\) yields the limiting rate constant for inactivation and the dissociation constant of the \(E\cdot I\) complex (Walsh, 1979).

\[ E + I \xrightleftharpoons[k_1^{-1}]{k_2} E\cdot I \]

The dissociation constant \(K_I\) for the inhibitor can be considered as a measure of the affinity of the enzyme for the inhibitor. Substrate protection of an enzyme from inactivation is also evidence for an active-site-directed inhibitor. In addition, inactivation of an enzyme with radiolabeled inhibitor followed by denaturation and dialysis can demonstrate the covalent nature of the inactivation and indicate the stoichiometry of the inactivation. An
active site process would be expected to yield an inhibitor to active site ratio of one (Walsh, 1979).

It was Baker who first announced the chemotherapeutic potential of active-site-directed irreversible enzyme inhibition (Baker, 1967). He discussed the advantages of nonclassical antimetabolites over those of classical antimetabolites. The former group being a series of inhibitors that allow for large but effective structural changes and the latter group being very similar to substrates except for relatively slight structural alterations. In a nonclassical antimetabolite series the portion of the molecule that determines the selective binding to the target enzyme is unchanged. It is another portion of the molecule, not involved with target enzyme binding, that is altered significantly. Such structural alterations could endow the molecule with higher levels (i.e. tissue) of specificity (Baker, 1967). Ultimately, one would seek structural changes that would direct the drug exclusively to neoplastic tissue. It would then be possible, for example, to inactivate a crucial but ubiquitous enzyme in cancerous tissue while not affecting the enzyme in host tissue. Thus would the drug design goals discussed by Ariens (1971) then be met. These goals include the development of more potent antineoplastic analogues with differing tissue specificity and decreased host toxicity (i.e. lower ED$_{50}$ values).
The development of the various QSAR (quantitative structure-activity relationships) models (Hansch and Fujita, 1964; Free and Wilson, 1964; Bocek et al., 1964) was based on an attempt to increase the efficiency of drug design. A frequently used model is that of Hansch and Fujita (1964). This model expresses biochemical or biological response to a drug as a function of the hydrophobic, electronic, and steric properties of the drug.

\[
\log \left( \frac{1}{c} \right) = -k_1 \pi^2 + k_2 \pi + k_3 \eta + k_4 E_S + k_5
\]

The hydrophobic parameter \( \pi \) is the logarithm of n-octanol-water partition coefficient (\( P \)) of the compound. The electronic considerations are represented by \( \eta \), the sum of the Hammett substitution constants of the compound. \( E_S \) is the sum of the Taft steric constants. The other constants \( k_{1-5} \) are determined empirically from a test of several compounds in the series and are a measure of the relative importance of each parameter (Kozarich et al., 1979). The lipophilic properties (\( \log P \)) of drugs determine their distribution in the tissue. A large \( \log P \) value represents a compound which would rapidly partition into the lipid-rich biophases, the site of drug action. Thus, \( \log P \) values are of great importance in drug design (Cain, 1975).

The Hansch and Fujita model is somewhat limited by two major factors. The method requires the synthesis and testing of a substantial number of compounds to obtain
meaningful information. In addition, access to a computer is required to evaluate the data obtained. A more simplified version of this model, which compensates for these limitations, was designed by Topliss (1977). The pros and cons of these models have been discussed recently by Kozarich (1979). As will be discussed in the section on nitrosoureas, these methods are far from ideal.

**Chymotrypsin--A Model System**

Chymotrypsin was first isolated by Kunitz and Northrop (1933) and is probably the most studied, best understood enzyme at the present time. Chymotrypsin, which is often used in modern textbooks as an example to explain many enzyme related phenomena, has been recently reviewed by Blackburn (1976) and Blow (1976). The enzyme displays both esterase and amidase activities. The active form of the enzyme is derived from the trypsin-mediated cleavage of a single peptide linkage of the proenzyme, chymotrypsinogen. A detailed X-ray crystallographic study revealed that the zymogen contained a structurally intact catalytic site but lacked the hydrophobic substrate binding site (Freer et al., 1970). The enzyme displays classical Michaelis-Menten kinetics, is monomeric, and exhibits no allosteric properties.

The mechanism of chymotrypsin has been worked out in great detail. Observed biphasic kinetic curves alerted
investigators to the presence of an acyl intermediate, which has been isolated (Balls and Wood, 1956). This intermediate has been shown to be tetrahedral in form (Sweet et al., 1974). Another interesting mechanistic feature of chymotrypsin is the charge relay system utilized during enzyme catalysis. Blow et al. (1969), using an electron density map, demonstrated the structural basis for the charge relay system and speculated carefully about the activation of serine$^{195}$. Hunkapiller et al. (1973) using the α-lytic protease of Myxobacter 495, provided further proof as to how the charge relay system functions. The mechanism of Stroud et al. (1975) describes a concerted electronic shuttle and the involvement of the Asp$^{102}$, His$^{67}$, and Ser$^{195}$ residues. This mechanism explains the basis for the highly reactive Ser$^{195}$.

An aspect of chymotrypsin, which is of particular concern to this thesis, is the substrate binding site. The topography of this site has been precisely described by X-ray crystallography data (Blow, 1976). It is a narrow hydrophobic depression which very specifically defines the range of amino acid side groups it may accommodate. The range of side groups which can be accommodated correspond to the primary substrate specificity of the enzyme. The binding site also serves to orient properly the substrate so as to facilitate greatly the catalytic process (Steitz et al., 1969; Blow, 1976). The first active-site-directed
irreversible inhibitor described for chymotrypsin was N-tosyl-L-phenylalanine bromoketone. The phenylalanine moiety directed the reactive bromoketone to the catalytic site (Schoellmann and Shaw, 1962).

Shotten and Watson (1970) had proposed that the substrate specificity of three serine proteases, chymotrypsin, trypsin, and elastase matched quite well with the X-ray crystallographic concept of their binding sites. Brown and Wold (1973a) reasoned that since structural work had been done primarily on inactive crystalline enzymes, a need existed to compare these binding sites as they exist in solution. They chose several alkyl isocyanates with varying alkyl chain lengths in order to probe the topography of these active sites. They postulated that the proper alkyl chain length bound in the hydrophobic substrate binding site would orient the reactive isocyanate in such a position as to enhance the carbamylation of the active site Ser<sup>195</sup>. The extent of the active-site-directed inactivation they observed matched that expected on the basis of known structural information. They also isolated the derivatized Ser<sup>195</sup>, thus further substantiating their hypothesis (Brown and Wold, 1973b). It is this work which prompted the investigation described in chapter III of this thesis. Chymotrypsin appeared to be the obvious model system with which to demonstrate specificity for certain nitrosourea-derived isocyanates.
Glutathione reductase--The
Mechanism of Action

Glutathione reductase has been isolated from many sources and in all cases contains a flavin adenine dinucleotide as a prosthetic group (Black, 1963). Glutathione reductase catalyses the reduction of the glutathione disulfide by NADPH.

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightleftharpoons 2 \text{GSH} + \text{NADP}^+
\]

Ondarza et al. (1974) also proposed a glutathione reductase mediated reduction of acetyl-coenzyme A -- glutathione mixed disulfide. A transhydrogenase activity has been ascribed to the reductase. In this reaction, thio-\text{NADP}^+ is the electron acceptor and a marked preference for NADPH over NADH is shown by the enzyme (Moroff et al., 1976). Spectral studies have been used to identify catalytic intermediates for the yeast enzyme. These intermediates include the oxidized enzyme, the two-electron reduced flavin, and a charge transfer complex between the isoalloxazine ring of the flavin and a thiolate anion at the active site (Massey and Williams, 1965; Kosower, 1966; Massey and Ghisla, 1974; Williams et al., 1978) of the yeast enzyme.

Recent structural work on both yeast and human erythrocyte enzymes has added to our understanding of the mechanism. The active site peptide has been characterized
and the presence of an active site thiol confirmed (Jones and Williams, 1975; Krohne-Ehrich et al., 1977). Williams et al. (1978) have demonstrated a differential activity of iodoacetic acid toward the thiols of the active site disulfide. This was interpreted to represent the differing positions of these thiols with respect to the substrate binding site and the isoalloxazine ring. This was confirmed on the erythrocyte enzyme by Schirmer's groups using amino acid sequence and X-ray data. They describe the thiol of Cys\(^{46}\) as proximal and the Cys\(^{41}\) as distal with respect to the flavin ring (Krohne-Ehrich et al., 1977; Schultz et al., 1978). Recently, an active site histidine has been identified, which appears to be mechanistically important (Boggaram and Mannerirk, 1978; Untucht-Grau et al., 1980). The following mechanism has been proposed to explain the abovementioned data. NADPH binds and reduces the FAD in a two electron process. A charge transfer complex forms between the proximal thios and the FAD and is stabilized by the protonated histidine. The Cys\(^{41}\) is activated by the protonated histidine and forms a disulfide with one glutathione moiety of the oxidized substrate, GSSG. Concurrently, the other glutathione moiety is protonated and dissociates from the enzyme as a product. The Cys\(^{46}\) reforms the active site dithiol and the other glutathione molecule is protonated as it leaves by the histidine (Williams et al., Pai, 1978; Boggaram and Mannervick,
An interesting aspect of this mechanism is the proposed activation of the Cys$^{41}$ or the distal active site thiol. This situation is similar to the activated serine of chymotrypsin and could in part explain the specificity observed in the BCNU-mediated inactivation of glutathione reductase, which is discussed in chapter IV of this thesis.

**Glutathione Reductase--A Protective Role During Oxidative Challenge**

Glutathione reductase exerts an important physiological role by its participation in the glutathione redox cycle. The glutathione redox cycle consists of the tripeptide glutathione, glutathione reductase, and at least two glutathione dependent peroxidase activities (Srivastava and Beutler, 1969; Hogberg et al., 1976; Sies et al., 1972; Burk et al., 1978).

$$\text{H}^+ + \text{NADPH}_{\text{glutathione}} \xrightarrow{\text{reductase}} \text{GSSG}_{\text{glutathione}} \xrightarrow{\text{peroxidase}} \text{ROH} + \text{H}_2\text{O}$$

Glutathione reductase recycles glutathione disulfide after glutathione is oxidized during the glutathione peroxidase mediated reduction of peroxides (Srivastava and Beutler, 1969). These peroxides result from reactions that generate reactive oxygen species (Chance et al., 1979).
The ground state of oxygen is the triplet state and as such is a diradical molecule, which can be reduced by one electron processes (Taube, 1965). In biological systems, a one electron reduced species of oxygen may be generated by the interaction of oxygen and reduced flavoproteins (Massey et al., 1969). A more reactive species of oxygen, the hydroxyl radical, may form via an ADP-ferrous ion mediated Haber-Weiss reaction (Haber and Weiss, 1934; King et al., 1975). In addition, hydroxyl radical generation by cytochrome p450 has been reported by Lai et al. (1979) while Torres et al. (1979) have reported protein-mediated hydroxyl radical generation. Hydroxyl radicals are extremely reactive and could possibly cause peroxidative damage to membrane lipids (Chance et al., 1979). Svingen et al. (1979) have proposed an elegant scheme for lipid peroxidation, which explains initiation and propagation. The initiation step involves the formation of lipid peroxides via the attack of a NADPH-dependent, ADP-perferryl-mediated superoxide anion upon membrane bound unsaturated lipids. This step is inhibited by superoxide dismutase and free radical scavengers. Once formed, the lipid peroxides are propagated by reaction of lipid hydroperoxides and chelated ferric ionx, including cytochrome p450.

Increased levels of reactive oxygen species could lead to lipid peroxidation, which has been associated with
membrane damage and cell lysis (Hogberg et al., 1975; Chance et al., 1979). Superoxide dismutase and catalase have a major role in the catabolism and thus offer protection from superoxide and \( \text{H}_2\text{O}_2 \) respectively. Free radical scavengers, such as glutathione itself and \( \alpha \)-tocopherol, form another defensive line against oxidative challenge (Kosower et al., 1965; Anundi et al., 1979).

Results of Burk et al. (1978) suggest that the glutathione redox cycle may play the major role in the removal of hydrogen peroxide and organic peroxides. The redox cycle may be impaired by a number of mechanism competing cellular processes. For example, depletion of glutathione by drug conjugation might impair the redox cycle's functioning and has been implicated in peroxidative damage (Anundi et al., 1979; Wendel et al., 1979). Glutathione may also be depleted by drug-mediated oxidation (Kosower et al., 1965). Impaired glutathione peroxidase or glutathione reductase activity have been shown to result in a susceptibility towards oxidative damage (Loos et al., 1976; Burk et al., 1979). The inactivation of glutathione reductase, and the subsequent impairment of the glutathione redox cycle, are a major point of interest in this thesis.

2-Chloroethyl Nitrosoureas--A Question of Specificity

Early work on nitrosoureas indicated that cytotoxicity of this class of drugs against L1210 cells was increased by
substituting a 2-chloroethyl moiety at the N-1 position (Johnston et al., 1963). The 2-chloroethyl nitrosoureas most common in clinical use today include 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), and 1-(2-chloroethyl-3-(trans-4-methylcyclohexyl)-1-nitrosourea (methyl-CCNU). These highly lipid soluble nitrosoureas readily cross the blood brain barrier (Oliverio, 1970) and have shown clinically promising results against various solid brain tumors (Carter et al., 1972) as well as various lymphomas (DeVita et al., 1965).

Reed et al. (1975) have proposed a mechanism for the decomposition of 2-chloroethyl nitrosoureas. The removal of the N-3 proton initiates the decomposition, which yields two potentially reactive species. One of these, a 2-chloroethyl diazene hydroxide, is thought to alkylate via a 2-chloroethyl carbonium ion (Reed et al., 1975). The other, an isocyanate, is a carbamylating agent. A controversy exists as to the role of these two reactive intermediates with respect to the antineoplastic and cytotoxic effects observed.

Studies by Cheng et al. (1972) using $^{14}$C in either the cyclohexyl moiety or the 2-chloroethyl moiety showed that carbamylation primarily involves proteins, while alkylation of both protein and nucleic acid occurred. Kohn (1977) has demonstrated crosslinking of DNA, which has been
attributed to the 2-chloroethyl moiety. Strand breakage of DNA has also been attributed to alkylation (Ewig and Kohn, 1977). It is now generally accepted that alkylation mainly exerts its effects on DNA and that carbamylation exerts its effects on proteins. Carbamylation has been implicated in inhibition of DNA synthesis (Wheeler and Alexander, 1974), possibly by inhibiting DNA polymerase (Wheeler and Bowdon, 1968). Kann et al. (1974a) have shown an inhibition of DNA repair by chloroethyl isocyanate. Carbamylation of ribonucleases may explain the interference of BCNU on RNA maturation (Kann et al., 1974b). In addition, Woolley et al. (1976) have demonstrated preferential carbamylation of histone H-1.

Wheeler et al. (1974) have attempted to correlate biological activities with chemical and physiochemical features of the nitrosoureas. They concluded that carbamylation is responsible for cytotoxicity and that alkylation is important for antineoplastic activity. Panasci et al. (1977) also attempted to correlate chemical and biological parameters. Their conclusions were similar to those of Wheeler. It must be pointed out that their criteria for carbamylating and alkylating activities, which are based on chemical reactivity in free solution, do not include consideration of selective binding to macromolecules. Since the interaction of a reactive moiety with the specific binding site in vivo (i.e. enzyme active site) could
drastically alter nitrosourea activity, these generalizations based on such analysis seem over simplified. Indeed, their correlations were far from linear. This point is further emphasized by the results of Montgomery et al. (1974). They examined eighty nitrosoureas by the QSAR method of Hansch and Fujita (1964). Their results were anomolous. As Kozarich et al. (1979) point out, an unpredicted interaction of certain analogues with specific receptors could render the predictive value of QSAR impotent.

At this point in time, it seems unwise to make generalized statements as to the importance or unimportance of either carbamylation or alkylation with respect to 2-chloroethyl nitrosourea activity. Yet it appears prudent to consider the novelty of each compound with respect to the possibility of a specific in vivo interaction. Such a possibility as an active-site-directed enzyme inactivation is the central point of this thesis.

Adriamycin--An Oxidative Challenge

The isolation of doxorubicin, more widely known as adriamycin (ADR), from a mutant strain of Streptomyces peucetius was first reported by Arcamone et al. (1969a). As such, ADR is classified as an antibiotic antineoplastic drug. Clinically ADR has proved to be a promising chemo-therapeutic agent in the treatment of a wide range of solid
tumors as well as hematologic malignancies. However, there is a significant, dose-limiting toxicity associated with ADR treatment. Physiological manifestations of ADR toxicity include alopecia, nausea, vomiting, stomatitis, leukopenia, and a life-threatening cardiotoxicity (Blum and Carter, 1974; Carter, 1975). An attempt is made later in this report (chapter V) to address some mechanistic aspects of ADR cardiotoxicity.

The work now discussed indicates that ADR has at least two possible molecular modes of action. ADR binds to DNA and is also capable of generating free radicals. These phenomena, either separately or in combination, may explain the antineoplastic and/or general toxic effects observed during ADR chemotherapy.

ADR has been shown to bind to double stranded DNA (Zunino, 1971), chromatin, and human chromosome preparations (Lin and van de Sande, 1975). In his review Henry proposes a model for the ADR-DNA binding complex. The salient features of this model include the intercalated aglycone and three hydrogen bonds formed between ADR in the major groove with phosphates of the DNA backbone (Henry, 1976). The binding of ADR to DNA in cell free systems and intact cells inhibits DNA and RNA synthesis by affecting DNA template activity (Momparler et al., 1976). In addition, ADR causes oxygen dependent DNA strand breaks (Lown et al., 1977), which may explain the chromosomal damage
observed in ADR treated cells (Vig, 1971). The mutagenic and carcinogenic aspects of the drug as well as its cytotoxicity could be a result of such chromosomal injury (Marquardt et al., 1976).

The work discussed in this thesis is primarily concerned with the oxidative aspects of ADR toxicity. Indeed, the ability of ADR to induce peroxidative damage may be an important underlying factor in ADR cardiotoxicity. Some ADR-mediated histological alterations in rat myocardium include degeneration of mitochondria, dilation of sarcoplasmic reticulum, separation of myofibrils, and focal myocyte degeneration (Olson and Capen, 1978). In some human patients congestive heart failure resulted from ADR treatment (Lefrak et al., 1973). Recent investigations have attempted to define the molecular mechanism of an ADR mediated oxidative challenge. The reduction and subsequent autooxidation of the ADR semiquinone generates reactive oxygen species, which could be expected to induce lipid peroxidation (Bachur et al., 1979; Goodman and Hochstein, 1977; Thayer, 1977). Pietronigro et al. (1979) have demonstrated the spontaneous, pH dependent formation of the ADR semiquinone radical. Bachur et al. (1979) have observed the cytochrome p450 mediated univalent reduction of ADR, which also generates the semiquinone radical. Both groups confirm radical formation with EPR data. Of the two observed routes of radical formation, the latter seems more
likely in vivo. Myers et al. (1977) have linked lipid peroxidation and cardiotoxicity in ADR-treated mice. These authors and others have underscored the free radical nature of an ADR challenge by demonstrating the ameliorating effect of the free radical scavenger α-tocopherol in animal systems (Myers et al., 1977; Mimnaugh et al., 1979; Wang et al., 1980).
Figure 1. Chemical structures of importance
III. ACTIVE SITE SPECIFIC INACTIVATION OF CHYMOTRYPSIN BY CYCLOHEXYL ISOCYANATE FORMED DURING DEGRADATION OF THE CARCINOSTATIC 1-(2-CHLOROETHYL)-3-CYCLOHEXYL-1-NITROSOUREA

Introduction

Interest in the antitumor activity of certain nitrosoureas is being directed towards the alkylating and carbamylation intermediates formed during chemical degradation of the nitrosoureas (Montgomery et al., 1967, 1975; Reed et al., 1975; Colvin et al., 1974). Reed et al. (1975) have reported that alkylation by CCNU may occur via a 2-chloroethyl carbonium ion. In vivo, alkylation of thiols occurs producing thiodiacetic acid as a major urinary product after administration of CCNU (Reed and May, 1975).

Wheeler et al. (1975) have emphasized the carbamylation of both the epsilon amino group of lysine and the alpha amino groups of amino acids, peptides and proteins by nitrosoureas in vitro. The binding of $^{14}\text{C}$ with proteins during degradation of [cyclohexyl-1-$^{14}\text{C}$]CCNU has been shown to occur both in vivo and in vitro (Cheng et al., 1972; Schmall et al., 1973). Chromatographic evidence from in vitro experiments with proteins indicates cyclohexyl carbamylation of the lysine residue can occur to form $N^6$-cyclohexylcarbamyl-lysine (Schmall et al., 1973).

Brown and Wold (1973a, 1973b) have shown that butyl isocyanate and octyl isocyanate are active-site-directed
inactivators of elastase and chymotrypsin, respectively. The inactivation, which results from carbamylation of a serine hydroxyl, is highly dependent upon specific recognition of the proper alkyl chain structure by the hydrophobic binding pocket adjacent to the serine hydroxyl at the catalytic site of the enzyme (Brown, 1975).

This report describes the selective carbamylation by cyclohexyl isocyanate that results in a near stoichiometric inactivation of chymotrypsin. CCNU was found to inactivate the enzyme only after it had degraded to form cyclohexyl isocyanate. The presence of competing nucleophiles, particularly L-lysine, had no significant effect on the stoichiometry of cyclohexyl isocyanate inactivation of chymotrypsin. The rapidity of formation of the cyclohexyl isocyanate enzyme complex appears to account for the exclusive carbamylation of the enzyme in the presence of lysine and and other nucleophiles.

Materials and Methods

α-Chymotrypsin, three times crystallized, was obtained from Worthington Biochemical Co. All reagents and solvents were reagent grade and commercially available. Cyclohexyl isocyanate and n-octyl isocyanate, from Eastman, were redistilled and maintained anhydrous under nitrogen prior to use. CCNU and [cyclohexyl-1-14C]CCNU were supplied by the National Cancer Institute. Purity was greater than 98% as
determined by high pressure liquid chromatography (May et al., 1975).

**General conditions for chymotrypsin incubations** - Conditions used for chymotrypsin incubations throughout this study, unless otherwise noted, were 0.1 M Tris-HCl (pH 7.7) at 25°C and will be referred to as buffered solutions. All CCNU and cyclohexyl isocyanate additions were made as aliquots in anhydrous acetone (5-40 mM). Controls containing acetone, enzyme and buffer were run with all enzyme incubations. Acetone concentrations of 2% (v/v) or less did not result in enzyme inactivation.

**Assays** - Chymotrypsin activity was measured spectrophotometrically by following the increase in absorbance at 256 nm due to the hydrolysis of BTEE according to the method of Hummel (1959). The protein concentrations were routinely determined either by the absorbance at 280 nm using $E_{280}^{1%} = 20.4$ (Morimoto and Kegeles, 1967), or by the method of Lowry et al. (1951), and confirmed by dry weight. Radioactivity was determined with a Packard 2425 liquid scintillation spectrometer using Triton N-101-xylene fluor. Counting efficiency was determined with $[^{14}\text{C}]$ toluene internal standards. The degradation of isocyanates in buffered solution was followed by the conversion of the isocyanate to the corresponding benzyl urea with benzylamine as described by Brown and Wold (1973a). The isocyanate half-lives were calculated from these data.
Determination of half-life for CCNU degradation - CCNU (.100 μmoles) was added to buffered solutions containing 0 or .095 μmoles chymotrypsin (4.75 x 10^{-5} M). The decrease in the absorbance of CCNU at 230 nm (due to loss of the nitroso group) with time was followed using a Cary 15 spectrophotometer and the half-life determined from these data.

Degradation of CCNU and cyclohexyl isocyanate and the concurrent formation of cyclohexylamine - CCNU (0.25 μmoles) was added to either 0.5 ml 0.1 M Tris-HCl (pH 7.7), 0.5 ml 0.1 M NaPO₄ buffer (pH 7.7) or 0.5 ml glass distilled water and incubated for 21-22 hr at 25°C. Cyclohexyl isocyanate (0.40 μmole) was similarly treated. All incubation mixtures were reacted with 2,4-dinitrobenzene sulfonic acid (DNBS) according to a procedure of Smith and Jepson (1967). DNP-cyclohexylamine was synthesized by the same method, recrystallized, and used as a standard. The extracted 2,4-dinitrophenyl (DNP) derivatives were subjected to high pressure liquid chromatography (HPLC) on a 4mm x 250mm LiChrosorb column, 5μ particle size, using a Model 3500 Spectra Physics Liquid chromatograph equipped with a Model 770 spectrophotometric detector. The solvent was 2,2,4-trimethylpentane:dichloromethane:2-propanol (930:63:0.8), the flow rate 1.8 ml/min, and the elution time of DNP-cyclohexylamine 380 sec. The DNP-
cyclohexylamine peak was quantitated using a Spectra

**Titration of chymotrypsin with cyclohexyl isocyanate**

Chymotrypsin was titrated by the sequential addition of small increments of cyclohexyl isocyanate in anhydrous acetone to the same buffered solutions containing from $10^{-6}$ M to $10^{-4}$ M enzyme according to the procedure of Brown and Wold (1973a). Similar titrations were also performed on solutions containing 0.238 μmoles enzyme ($2.38 \times 10^{-4}$ M) and 0,1,5 or 10 mM L-lysine monohydrochloride.

**Incubation of chymotrypsin with CCNU**

CCNU (0.250 μmoles) was added to a buffered solution containing 0.238 μmoles of chymotrypsin ($2.38 \times 10^{-4}$ M). The reaction was incubated at 25°C for a period of time equivalent to six CCNU half-lives (21-22 hr). The enzyme was assayed at zero time and 21-22 hr using a dilution of sufficient magnitude (500-fold) to reverse any effects of a noncovalently bound inhibitor.

**Inactivation of chymotrypsin with cyclohexyl isocyanate in the presence of CCNU**

Chymotrypsin ($10^{-5}$ M-$10^{-4}$ M) was titrated with cyclohexyl isocyanate in the absence and presence of up to a 10-fold excess of CCNU. Controls for each incubation contained the same enzyme and CCNU concentration but no added cyclohexyl isocyanate. The controls were assayed at times corresponding to cyclohexyl isocyanate titration assay points (0,7,14,21 and 28 min).
[Cyclohexyl-1-\textsuperscript{14}C]CCNU labeling of chymotrypsin -

[Cyclohexyl-1-\textsuperscript{14}C]CCNU (0.850 µmoles) was added to buffered solutions containing 0.476 µmoles chymotrypsin (2.38 x 10\textsuperscript{-4} M). The incubations were allowed to proceed at 25°C for 21-22 hr. The enzyme activity was assayed at zero min and 21 hr. Aliquots (0.50 ml) were subjected to column chromatography on a 1 cm x 60 cm Sephadex G-25 column, eluted with 0.01 M Tris-HCl (pH 7.7) and/or dialyzed against 8 M urea in 0.1 M Tris-HCl (pH 7.7) at 4°C overnight, followed by extensive dialysis in 0.01 M Tris-HCl (pH 7.7) at 4°C. Some samples were dialysed against 5 M urea in 0.1 M ammonium bicarbonate (pH 9.0) according to the procedure used by Woolley \textit{et al.} (1976). The samples were then assayed for radioactivity and protein to determine the moles bound [\textsuperscript{14}C]cyclohexyl moiety/mole enzyme.

[Cyclohexyl-1-\textsuperscript{14}C]CCNU labeling of partially inactivated chymotrypsin - Buffered solutions containing 0.476 µmoles chymotrypsin (2.38 x 10\textsuperscript{-4} M) were titrated with varying amounts of cyclohexyl isocyanate. The solutions were incubated for 20 minutes at which time 0.825 µmoles [cyclohexyl-1-\textsuperscript{14}C]CCNU were added. The reactions were allowed to proceed for 21-22 hr at 25°C. Enzymatic activity was determined prior to cyclohexyl isocyanate addition, 20 min after cyclohexyl isocyanate addition, and at 21-22 hr. Aliquots (0.50 ml) were dialysed against 8 M urea in 0.1 M Tris-HCl (pH 7.7) at 4°C and then extensively
in 0.1 M Tris-HCl until dialysis buffer contained only background radioactivity. The amounts of $^{14}$C-label and of protein were then determined as described above, and the cyclohexyl/chymotrypsin molar ratio calculated. A similar experiment was performed using PMSF to inactivate the enzyme prior to $^{14}$C labeling.

[Cyclohexyl-$\text{L}^{14}$C]CCNU labeling of chymotrypsin in the presence of L-lysine - [Cyclohexyl-$\text{L}^{14}$C]CCNU (0.850 $\mu$moles) was added to buffered solutions containing 0.476 $\mu$moles chymotrypsin ($2.38 \times 10^{-4}$ M0, and 0,2,10, and 20 $\mu$moles L-lysine monohydrochloride (0,1,5 and 10 mM). The reaction mixtures were treated and analyzed as described above.

Results

Cyclohexyl isocyanate inactivation of chymotrypsin - Brown and Wold (1973a) have shown that n-alkyl isocyanates are active-site directed inactivators of chymotrypsin. Results of this study demonstrate that cyclohexyl isocyanate reacts with chymotrypsin to inactivate the enzyme rapidly and irreversibly. A typical titration of chymotrypsin with cyclohexyl isocyanate is shown in Figure 2. As the enzyme concentration was increased (Table I) the molar ratio of cyclohexyl isocyanate added to chymotrypsin to achieve 50% enzyme inactivation became nearly mole per mole. For example, at $2.38 \times 10^{-4}$ M chymotrypsin the
TABLE I: Titration of Chymotrypsin with Cyclohexyl Isocyanate
Cyclohexyl Isocyanate to Chymotrypsin Mole Ratio for 50% Inactivation.

<table>
<thead>
<tr>
<th>Enzyme Concentration (M)</th>
<th>Cyclohexyl Isocyanate/Chymotrypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>$4.76 \times 10^{-6}$</td>
<td>7.96</td>
</tr>
<tr>
<td>$1.19 \times 10^{-5}$</td>
<td>4.20</td>
</tr>
<tr>
<td>$2.38 \times 10^{-5}$</td>
<td>3.25</td>
</tr>
<tr>
<td>$4.76 \times 10^{-5}$</td>
<td>1.29</td>
</tr>
<tr>
<td>$1.10 \times 10^{-4}$</td>
<td>0.90</td>
</tr>
<tr>
<td>$2.38 \times 10^{-4}$</td>
<td>0.63</td>
</tr>
</tbody>
</table>
Figure 2. Titration of chymotrypsin (2.38 x 10⁻⁴ M in 0.1 M Tris-HCl pH 7.7) by the sequential addition of small increments of 0.01 M cyclohexyl isocyanate in anhydrous acetone.

Figure 3. Degradation of octyl isocyanate (●), and cyclohexyl isocyanate (○) in 0.1 M Tris-HCl, pH 7.7, at 25°C.
cyclohexyl isocyanate to enzyme ratio for 50% inactivation was 0.63. This enzyme concentration effect is to be expected for a second order reaction that must compete with another rapid reaction which in this instance is that between cyclohexyl isocyanate and Tris ions. The lack of reaction with water was concluded from the absence of cyclohexylamine formation in Tris buffer.

**Cyclohexylamine formation during cyclohexyl isocyanate degradation** - CCNU degrades via a free cyclohexyl isocyanate which is then capable of reacting with nucleophiles. Thus it was deemed important to investigate the stability and reactivity of cyclohexyl isocyanate in aqueous solution. As Figure 3 indicates the half-life of cyclohexyl isocyanate and octyl isocyanate are both approximately one minute, as are the half-lives of several other alkyl isocyanates (Brown and Wold, 1973a).

Degradation of cyclohexyl isocyanate in 0.1 M Tris-HCl (pH 7.7) resulted in no detectable cyclohexylamine formation. Cyclohexyl isocyanate reacting with water would be expected to give a carbamic acid which decomposes to form cyclohexylamine. When degradation was allowed to proceed in 0.1 M phosphate buffer (pH 7.7) or in glass distilled water, the amount of cyclohexylamine formed was 44 ± 10% (± S.E.) and 72 ± 8% (± S.E.) respectively, of the theoretical yield. Thus it appears that Tris and phosphate ions are carbamylated to an extent that is reflected in the
relative nucleophilicity of these ions. The surprising degree to which chymotrypsin was inactivated by cyclohexyl isocyanate, even in the presence of competitive Tris ions (Tris/chymotrypsin ratio was 420) indicates the rapidity of "productive" binding of cyclohexyl isocyanate to the enzyme at a complementary binding pocket adjacent to the active site.

**Inactivation of chymotrypsin during incubation with CCNU, quantitation of cyclohexyl isocyanate formation** - Since CCNU degrades to form cyclohexyl isocyanate one might expect inactivation of chymotrypsin and that the extent of this inactivation would be proportional to the amount of CCNU degradation and thus to the amount of free isocyanate formed. The half-life of CCNU in buffered solution in the absence or presence of enzyme was the same, 3.6 hr. Incubation of chymotrypsin with CCNU for 21-22 hr resulted in near stoichiometric inactivation of the enzyme. It was noted that the length of the incubation determined the amount of inactivation whereas direct addition of cyclohexyl isocyanate resulted in almost immediate enzyme inactivation. As will be discussed in greater detail in a following section, evaluation of the enzyme inactivation data was based on the assumption that any cyclohexyl isocyanate formed during CCNU degradation has an opportunity equivalent to that of added cyclohexyl isocyanate to inactivate chymotrypsin. Consequently, the extent of enzyme
inactivation observed indicates that $70 \pm 12\%$ (± S.E.) of the CCNU degraded via a cyclohexyl isocyanate intermediate. When CCNU was allowed to degrade in glass distilled water for 21 hr followed by the quantitation of cyclohexylamine, 74 ± 6% (± S.E.) of the theoretical cyclohexylamine yield was found. These results demonstrate that CCNU degrades in such a manner as to generate a free cyclohexyl isocyanate intermediate and that the extent of this cyclohexyl isocyanate formation is at least 70% of the maximum possible yield.

**Titration of chymotrypsin with cyclohexyl isocyanate in the presence of CCNU** - Interpretation of the above results is based on two assumptions: (1) that undegraded CCNU does not effectively protect the enzyme from cyclohexyl isocyanate inactivation and (2) that undegraded CCNU does not inactivate the enzyme. The following experiment was performed to substantiate these assumptions.

Incubations containing from $10^{-5}$ M to $10^{-4}$ M enzyme were titrated with cyclohexyl isocyanate in the absence and presence of up to a 10-fold excess of CCNU. Since the cyclohexyl isocyanate/chymotrypsin ratio required for 50% enzyme inactivation is an indication of the effectiveness of cyclohexyl isocyanate to inactivate the enzyme, any significant protection by CCNU would be evidenced by an increase in this ratio. As shown in Figure 4, this ratio did not increase but was observed to decrease slightly. This
Figure 4. Titration of $4.76 \times 10^{-5}$ chymotrypsin with 0.01 M cyclohexyl isocyanate in the presence of $1.25 \times 10^{-4}$ M CCNU (■), and absence of CCNU (●). A control to measure the extent of enzyme inactivation by $1.25 \times 10^{-4}$ M CCNU was performed under similar conditions (▲). The control was assayed at time intervals (0, 7, 14, 21 and 28 min) corresponding to times of titration samplings. The reaction conditions and procedures are further described in the text.
result can be explained on the basis of (a) the small calculated quantity of the cyclohexyl isocyanate formed from CCNU during the incubation period and (b) the observed amount of inactivation in the CCNU control (A, Figure 4). These data indicate that CCNU must first degrade to form the isocyanate which then inactivates the enzyme, and that undegraded CCNU does not effectively protect the enzyme from cyclohexyl isocyanate inactivation under conditions used throughout this study.

Inactivation and labeling of chymotrypsin by 
[Cyclohexyl-1\(^{14}\)C]CCNU - [Cyclohexyl-1\(^{14}\)C]CCNU was used to accurately determine the extent of specific covalent modification of chymotrypsin by CCNU. [Cyclohexyl-1\(^{14}\)C]CCNU was allowed to degrade in the presence of 2.38 x 10\(^{-4}\) M chymotrypsin (CCNU/enzyme = 1.3) in buffered solution at 25°C for 21 hr, followed by measurement of enzymatic activity and removal of noncovalently bound \(^{14}\)C. It was noted that Sephadex G-25 chromatography was not sufficient to remove all non-covalently bound \(^{14}\)C-cyclohexyl moiety. When the column fractions containing label and protein were dialysed overnight against 8 M urea in 0.1 M Tris-HCl (pH 7.7) at 4°C, 30% of the \(^{14}\)C-label was subsequently removed. The remaining label was found to be stable to dialysis against 5 M urea in 0.1 M ammonium bicarbonate (pH 9.0) at 4°C and assumed to represent covalently bound \(^{14}\)C-cyclohexyl moiety. At these concentrations (3.12 x 10\(^{-4}\) M CCNU
and $2.38 \times 10^{-4}$ M chymotrypsin) 90% of the total enzyme was inactivated. The appropriate controls indicated that 60% of the total enzyme was inactivated by CCNU and 30% of the enzyme was inactivated by acetone (5% v/v) during the 21-22 hr incubation period. Brown and Wold (1973a) observed a similar degree of acetone inactivation and demonstrated that only active enzyme reacted specifically with octyl isocyanate. The molar ratio of bound $[^{14}\text{C}]$cyclohexyl moiety to total enzyme in seven experiments was $0.70 \pm 0.05$. The ratio of bound label to CCNU-inactivated enzyme (total inactivated enzyme less acetone inactivation) was $1.11 \pm 0.07$. This stoichiometry is in agreement with that obtained during added cyclohexyl isocyanate inactivation of chymotrypsin and indicates up to 20% non-specific carbamylation under these conditions.

$^{14}\text{C}$ labeling with [cyclohexyl-1-$^{14}\text{C}$]CCNU of chymotrypsin partially inactivated by cyclohexyl isocyanate and phenylmethanesulfonylfluoride - The rapid and stoichiometric inactivation of the chymotrypsin by cyclohexyl isocyanate strongly indicates that it covalently binds to the active site of the enzyme, which exhibits a high specificity for the cyclohexyl moiety. It was possible to demonstrate further that either cyclohexyl isocyanate formed during CCNU degradation or directly added cyclohexyl isocyanate both display a similar high degree of active site specificity during their inactivation of chymotrypsin.
When [cyclohexyl-1-\(^{14}\text{C}\)]CCNU was incubated with chymotrypsin which had been previously inactivated to varying extents by unlabeled cyclohexyl isocyanate, the decrease in bound \(\text{[}^{14}\text{C}\text{-cyclohexyl moiety}\) was found to be proportional to the extent of cyclohexyl isocyanate inactivation prior to \(^{14}\text{C}\) labeling (Figure 5). Linear regression analysis of these values and extrapolation to 100% inactivation with cyclohexyl isocyanate prior to addition of [cyclohexyl-1-\(^{14}\text{C}\)]CCNU resulted in a value of 0.23 moles \(\text{[}^{14}\text{C}\text{-cyclohexyl moiety}\) bound per mole of enzyme. A similar experiment was performed using PMSF to inactivate the enzyme prior to labeling with [cyclohexyl-1-\(^{14}\text{C}\)]CCNU. The decrease in the bound \(\text{[}^{14}\text{C}\text{-cyclohexyl moiety}\) was again observed to be proportional to the extent of enzyme inactivation prior to labeling. Enzyme incubations which had been 100% inactivated by PMSF and subsequently incubated with [cyclohexyl-1-\(^{14}\text{C}\)]CCNU contained 0.23 moles bound \(\text{[}^{14}\text{C}\text{-cyclohexyl moiety}\) per mole enzyme. This value and the extrapolation in Figure 5 suggest that approximately 20% of the covalently bound label was not at the active site. This extent of non-specific \(^{14}\text{C}\) labeling is not unexpected, since the added [cyclohexyl-1-\(^{14}\text{C}\)]CCNU to enzyme ratio was 1.8 in these incubations. Brown and Wold (1973a) also observed 20% non-specifically bound \(^{14}\text{C}\) butyl isocyanate with chymotrypsin. PMSF has been demonstrated by Gold and Fahrney (1964) to react exclusively with the active site...
Figure 5. [chx-1-14C]CCNU labeling of cyclohexyl isocyanate inactivated chymotrypsin. Reaction mixtures containing 2.38 x 10^{-4} M chymotrypsin were titrated to varying extents of enzyme inactivation with cyclohexyl isocyanate. The reaction mixtures were then incubated with 8.0 x 10^{-4} M [chx-1-14C]CCNU for 21.6 hr.
serine of chymotrypsin. Thus these results indicate that both cyclohexyl isocyanate and PMSF protect the active site from covalent labeling by [cyclohexyl-\(1^{-14}\text{C}\)]CCNU and that cyclohexyl isocyanate formed during CCNU degradation in-activated the enzyme by carbamylation of the active site. The involvement of the active-site serine is indicated by its role in catalysis and the stable nature of the observed covalent modification. Due to the lability of carbamylimidazole above pH 6 (Stark, 1965), the carbamylation of the active site histidine seems unlikely. Further support for the involvement of the active-site serine is given by the isolation of [\(14\text{C}\)]-butylcarbamylserine from chymotrypsin after treatment with [\(14\text{C}\)]-butyl isocyanate (Brown and Wold, 1973b).

Chymotrypsin inactivation by cyclohexyl isocyanate in the presence of L-lysine - Wheeler and coworkers (1974) have utilized the extent to which L-lysine is carbamylated by various nitrosoureas as an indication of the carbamylation potential of those nitrosoureas that are capable of degrading via isocyanates. Since chymotrypsin has shown its capability to compete for cyclohexyl isocyanate in a nucleophilic environment, it was of interest to determine the extent to which the presence of L-lysine could prevent enzyme inactivation by cyclohexyl isocyanate. Chymotrypsin was titrated as previously described by the sequential addition of small amounts of cyclohexyl isocyanate in the
presence of 0, 1, 5 and 10 mM L-lysine. Figure 6 summarizes the results of these incubations which show that the presence of L-lysine had no significant effect on inactivation by cyclohexyl isocyanate. Only 10 mM L-lysine had a slight effect on enzyme inactivation. This effect was not manifested until 60% of the enzyme had been inactivated and it resulted in only a 10% decrease in the extent of total enzyme inactivated. It must be emphasized that when 60% of the enzyme is inactivated the ratio of L-lysine to active enzyme is approximately 70. These data demonstrate that the binding of cyclohexyl isocyanate to the hydrophobic pocket of the enzyme and the subsequent carbamylation of chymotrypsin must be specific and very rapid in order to prevent any carbamylation of L-lysine.

[Cyclohexyl-1-14C]CCNU labeling of chymotrypsin in the presence of L-lysine - [Cyclohexyl-1-14C]CCNU labeling of the enzyme in the presence of L-lysine further demonstrated that the same high specificity and rapidity of binding exists for cyclohexyl isocyanate generated by the degradation of CCNU. When 4.0 x 10^{-4} M [cyclohexyl-1-14C]CCNU was allowed to degrade for 21 hr in the presence of 2.38 x 10^{-4} M chymotrypsin and 0, 1, 5 and 10 mM L-lysine, no significant alteration was observed in the extent of enzyme inactivation (Table II). The L-lysine appears to decrease the amount of non-specifically bound label but does not compete
Figure 6. Chymotrypsin (2.38 x 10^{-4} M) was titrated with cyclohexyl isocyanate as described in the text (o). Similar titrations were carried in the presence of 1 mM (□), 5 mM (●), and 10 mM (■) L-lysine hydrochloride.
<table>
<thead>
<tr>
<th>L-lysine Conc (mM)</th>
<th>Total Enzyme</th>
<th>Acetone Control</th>
<th>$^{14}$Cl-CCMU</th>
<th>$^{14}$Cl-cyclohexyl</th>
<th>$^{14}$Cl-cyclohexyl moles bound/mole CCMU</th>
<th>$^{14}$Cl-cyclohexyl moles bound/mole CCMU</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>36</td>
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</tr>
<tr>
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<td>67</td>
<td>0.65</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>55</td>
<td>21</td>
<td>64</td>
<td>0.80</td>
<td>0.74</td>
<td></td>
</tr>
</tbody>
</table>

$[^a]$ Values given as percent of total enzyme.

$[^a]$ CCMU (0.860 u mole) was added to buffered solutions containing 0.176 u moles chymotrypsin, (7.38 x 10$^{-4}$ M) and 0.1, 1.0 and 10 u moles L-lysine monohydrochloride. The solutions were incubated for 21 hr then treated and analyzed as described in the text.
Discussion

Cyclohexyl isocyanate has been shown to be an active site directed reagent that is capable of stoichiometric and irreversible inactivation of chymotrypsin. It has been demonstrated that CCNU degrades in such a manner as to form cyclohexyl isocyanate with a 70% yield as evidenced by active site specific inactivation of chymotrypsin and by cyclohexylamine yield in the absence of enzyme and any reactive nucleophile.

Brown and Wold (1973a) have postulated that octyl isocyanate inactivation of chymotrypsin proceeds in a two-step manner. The first step entails the binding of the n-alkyl side chain analogous to an enzyme-substrate complex formation. The second step has been demonstrated to involve the covalent linkage of the isocyanate group to the active site serine hydroxyl group (Brown and Wold, 1973b). Carbamylation of chymotrypsin by cyclohexyl isocyanate appears to proceed in a similar manner, as indicated by: (a) the almost identical enzyme titration data obtained with octyl isocyanate (Brown and Wold, 1973a) and cyclohexyl isocyanate (Table I); (b) the near stoichiometric inactivation and stable $^{14}$C labeling of chymotrypsin by [cyclohexyl-$^{1-14}$C]CCNU (ratio of $1.1 \pm .07$) and (c) the
proportional decrease in $^{14}$C labeling after PMSF modification of the active-site serine.

The high degree of "productive" binding of the cyclohexyl side chain to chymotrypsin was expected from previous studies on the hydrophobic pocket of this enzyme by other workers, especially the x-ray diffraction study of Brown (1975). He demonstrated that the limiting binding efficiency should be with n-alkyl chains of 8 to 9 carbons. It was of interest to determine whether the nucleophilic environment of chymotrypsin in solution could alter the effectiveness of cyclohexyl isocyanate as an active-site-directed reagent. Thus "productive" binding of the cyclohexyl side chain to the hydrophobic pocket of chymotrypsin was measured against the rapid reaction of the cyclohexyl isocyanate moiety with competing nucleophiles. Surprisingly, when using L-lysine as a competing nucleophile, a 70-fold excess of lysine (Figure 6) was required to demonstrate any decrease in the stoichiometric inactivation of chymotrypsin ($2.38 \times 10^{-4}$ M).

It might be assumed that degradation of cyclohexyl isocyanate in 0.1 M Tris buffer (pH 7.7) would lead to the formation of cyclohexylamine as the major degradation product due to the reaction of the isocyanate moiety with water and/or hydroxyl ion. However, this was not the case since there was a complete absence of cyclohexylamine in Tris-buffered incubation mixtures as determined by DNBS-
derivatization and HPLC analysis. In contrast a 72% yield of cyclohexylamine was observed in glass distilled water and a 44% yield in 0.1 M phosphate buffer. Therefore, the role of competing nucleophiles is of considerable importance in determining the carbamylation products formed during the degradation of CCNU and other nitrosoureas.

Wheeler et al. (1975) incubated amino acids, peptides and proteins in Tris and phosphate buffers with either [cyclohexyl-1-\textsuperscript{14}C]CCNU, unlabeled CCNU or cyclohexyl isocyanate. They observed carbamylation as evidenced by \textsuperscript{14}C labeling and chromatographic R\textsubscript{f} values of the reaction products. Since yields were not given, it is not possible to determine the degree to which the buffer ions successfully competed for reaction with cyclohexyl isocyanate. Thus, it was of interest to quantitate the extent of chymotrypsin inactivation by [\textsuperscript{14}C]cyclohexyl isocyanate formed during [cyclohexyl-1-\textsuperscript{14}C]CCNU degradation. Again the effectiveness of "productive" binding of the CCNU generated cyclohexyl isocyanate with the enzyme binding pocket was such that L-lysine concentration of up to 10 mM had essentially no effect on the stoichiometric \textsuperscript{14}C labeling and inactivation of chymotrypsin (2.38 x 10\textsuperscript{-4} M).

These studies indicate that even in a concentrated nucleophilic environment chymotrypsin successfully competes for the reactive cyclohexyl isocyanate generated by CCNU degradation. This ability to compete successfully involved
the rapid and "productive" binding afforded the enzyme by its hydrophobic binding pocket. A similar selective reaction with isocyanates has been shown to occur with trans-glutaminase (Gross et al., 1975) and alcohol dehydrogenase (Twu and Wold, 1973). The speculation that these and other such enzyme binding sites exist in vivo seems reasonable and indicates that the carbamylating activity of nitrosourea-derived isocyanates may be more specific than previously thought. Thus the structure of the isocyanates formed in vivo from CCNU and its monooxygenated metabolites (May et al., 1975) should be considered when attempting to elucidate the mechanism of the action of CCNU. These studies also indicate that attention should be directed toward the carbamylation of other amino acid side chains, analogous to the "activated" serine-195 of chymotrypsin, in addition to the carbamylation of lysine and N-terminal amino groups. Further work is now in progress to investigate the extent of protease inactivation and active-site-directed inactivation of other enzymes by those nitrosoureas currently being utilized as promising antineoplastic agents.
IV. INACTIVATION OF GLUTATHIONE REDUCTASE BY 2-CHLOROETHYL NITROSOUREA-DERIVED ISOCYANATES

Introduction

The 2-chloroethyl nitrosoureas are some of the most promising antineoplastic agents in use today. Interest is being focused on the alkylating and carbamylating intermediates generated during their degradation under physiological conditions. These studies have indicated (Colvin et al., 1974; Montgomery et al., 1967; Montgomery et al., 1975; Reed et al., 1975) that the carbamylating moieties generated are isocyanates. Certain alkyl isocyanates have been shown to be active-site directed inactivators of several enzymes (Babson et al., 1977; Brown and Wold, 1973a; Gross et al., 1975; Twu and Wold, 1973) and CCNU has been shown to be capable of stoichiometric inactivation of chymotrypsin by virtue of its cyclohexyl isocyanate intermediate (Babson et al., 1977). Thus it seemed reasonable to postulate the interaction of a 2-chloroethyl nitrosourea-generated isocyanates with an enzymatic active site in vivo. Frischer and Ahmad (1977) observed reduced erythrocyte GSSG-reductase activity in patients administered BCNU but saw no effect on the activity of nineteen other erythrocyte enzymes. This finding suggested that the myelosuppressive BCNU, via a 2-chloroethyl isocyanate, might be interacting with GSSG-reductase in a highly
specific fashion. This study describes the specific carbamoylation of the NADPH-reduced GSSG-reductase by 2-chloroethyl isocyanate and cyclohexyl isocyanate which resulted in the near stoichiometric inactivation of the enzyme. BCNU and several other 2-chloroethyl nitrosoureas were found to inactivate the enzyme, presumably after they had degraded to form their respective isocyanates. Of the 2-chloroethyl nitrosoureas used in this work those which inactivated GSSG-reductase also exhibit myelosuppressive activity. This work demonstrates the potential of alkylisocyanates as active site probes of glutathione reductase.

Materials and Methods

Reagents - Highly purified yeast GSSG-reductase was obtained from Sigma Chemical Co. All reagents and solvents were reagent grade and commercially available. Isocyanates from Eastman were redistilled and maintained anhydrous under nitrogen prior to use. Nitrosoureas and $^{14}$C nitrosoureas were supplied by the National Cancer Institute.

General conditions for GSSG-reductase incubations - Conditions used throughout this study for GSSG-reductase incubations were 0.1 M KPO$_4$ pH 7.6 with 0.2 M KCl and 1 mM EDTA and 37°C. Enzyme activity was determined by the method of Colman (1971). Protein concentrations were determined by the method of Lowry et al. (1951). The
quantitation of enzyme throughout this paper is based on
the monomer molecular weight of 55,000 daltons.

**Inactivation of GSSG-reductase by various nitro-
soureas** - Various nitrosoureas (50-250 nmoles) were added
to solutions containing GSSG-reductase (4.5 x 10^{-6} M - 6.9
x 10^{-5} M). Enzyme activity was determined after one half-
life of the nitrosourea added had elapsed or in the case of
BCNU every 10 min. The half-lives were determined by the
decrease in absorbance of the nitroso group in the above
incubation buffer, pH 7.6 at 37°C. The nitrosoureas used
in this study are abbreviated as follows: BCNU, 1,3-bis(2-
chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-(2-
cyclohexyl)-1-nitrosourea; MeCCNU, 1-(2-chloroethyl)-3-(4-
trans-methylcyclohexyl)-1-nitrosourea; cis-2-OH-CCNU, 1-
(2-chloroethyl)-3-(cis-2-hydroxycyclohexyl)-1-nitrosourea;
trans-4-OH-CCNU, 1-(2-chloroethyl)-3-(trans-4-hydroxycyclo-
hexyl)-1-nitrosourea; GANU, 1-(2-chloroethyl)-3-(β-D-

glucopyranosyl)-1-nitrosourea; chlorozotocin, 2-[3-(2-
chloroethyl)-3-nitrosoureido]-D-glucopyranose; ACNU, 1-
(4-amino-2-methylpyrimidin-5-yl) methyl-3-(2-chloroethyl)-
3-nitrosourea; and [Chx-1^{14}C]CCNU, 1-(2-chloroethyl)-3-
(1^{14}C-cyclohexyl)-1-nitrosourea.

**Titration of GSSG-reductase with 2-chloroethyl iso-
cyanate** - GSSG reductase (4.5 x 10^{-6} M - 6.9 x 10^{-5} M) was
titrated by the sequential addition of 2.5 mM 2-chloroethyl
isocyanate according to the procedure of Brown and Wold
Cyclohexyl isocyanate was also reacted with the enzyme at these concentrations.

**Labeling of GSSG-reductase with \(^{14}\text{C}\) nitrosoureas** - 
[Chx-1-\(^{14}\text{C}\)CCNU (125 nmoles) or [chloroethyl-2-\(^{14}\text{C}\)]BCNU (125 nmoles) was added to buffered solutions containing 7.2 nmoles (1.7 x \(10^{-5}\) M) GSSG-reductase in the absence and presence of 2.4 x \(10^{-4}\) M NADPH. The reactions were incubated at 37°C until approximately 90% enzyme inactivation was achieved. The incubates were diluted 25% with cold 8 M urea in 0.01 M KPO\(_4\) pH 7.6 at 4°C. The samples were then assayed for radioactivity and protein to determine moles bound \(^{14}\text{C}\) moiety/mole 55,000 MW monomer. The determination of this \(^{14}\text{C}\) labeling stoichiometry takes into consideration the symmetrical \(^{14}\text{C}\) label of the [chloroethyl-2-\(^{14}\text{C}\)]BCNU.

**Inactivation of L5178Y and expressed bone marrow cell by various nitrosoureas** - L5178Y cells grown in Gibco growth media or grown in vivo were harvested by centrifugation. Cells (1.3 x \(10^6\)/ml) were washed 2 x with saline, taken up in KPO\(_4\) pH 7.6 with 0.2 M KCl and 1 mM EDA, and sonicated 60s with a Kontes sonicator. The sonicate was spun at 39K in a Beckman Ti-50 rotor for 60', and the supernatants used for incubations. Murine bone marrow was expressed from femurs with saline and treated in a similar fashion as above. Various 2-chloroethyl nitrosoureas were incubated at concentrations of \(10^{-5}\) M - \(10^{-3}\) M with the
above supernatants. The GSSG-reductase activity was determined at 0 and 60 min. after addition of each nitrosourea.

**Results**

BCNU (50 nmoles) when incubated with NADPH-reduced GSSG-reductase (1.38 nmoles) inactivated the enzyme. The inactivation was time dependent. No enzyme inactivation was observed when BCNU was incubated with GSSG-reductase in the absence of NADPH (Figure 7). In separate experiments BCNU, CCNU, MeCCNU, trans-4-OH-CCNU and to a lesser extent ACNU inactivated NADPH-reduced GSSG-reductase but had no effect on enzyme activity in the absence of NADPH. Chlorozotocin, cis-2-OH-CCNU, and GANU did not inactivate the NADPH-reduced enzyme significantly (Table V). Since these 2-chloroethyl nitrosoureas all generated 2-chloroethyl carbonium ions and the enzyme inactivation was dependent on nitrosourea degradation, the isocyanates generated were assumed to be responsible for the observed enzyme inactivation. To determine this, 2-chloroethyl isocyanate or cyclohexyl isocyanate were incubated with NADPH-reduced GSSG-reductase; immediate enzyme inactivation ensued. As Table III indicates, the 2-chloroethyl isocyanate titration of GSSG-reductase became more stoichiometric as the enzyme concentration was increased. The oxidized form of the enzyme was not inactivated by either of these isocyanates. To confirm that the inactivation was due to
Figure 1: BCNU inactivation of glutathione reductase. BCNU (50 nmoles in anhydrous acetone) was added to solutions containing 1.38 nmoles glutathione reductase (5.48 x 10^{-6} M) in the presence of 1.58 x 10^{-4} M NADPH (▲), and absence of NADPH (●). Controls contained acetone, NADPH, and enzyme, but no BCNU (○).

### TABLE III

TITRATION OF GLUTATHIONE REDUCTASE WITH 2-CHLOROETHYL ISOCYANATE. 2-CHLOROETHYL ISOCYANATE TO GLUTATHIONE REDUCTASE MOLE RATIO FOR 50% INACTIVATION.

<table>
<thead>
<tr>
<th>ENZYME CONCN (M)</th>
<th>2-CHLOROETHYL ISOCYANATE/GLUTATHIONE REDUCTASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5 x 10^{-6}</td>
<td>9.94</td>
</tr>
<tr>
<td>1.1 x 10^{-5}</td>
<td>5.52</td>
</tr>
<tr>
<td>2.8 x 10^{-5}</td>
<td>2.64</td>
</tr>
<tr>
<td>6.9 x 10^{-5}</td>
<td>1.15</td>
</tr>
</tbody>
</table>

Titration of glutathione reductase (4.5 x 10^{-6} M - 6.9 x 10^{-5} M in 0.1 M KPO₄ pH 7.6 with 0.2 M KCl and 1 mM EDTA at 37°C) by the sequential addition of 2.5 mM 2-chloroethyl isocyanate in anhydrous acetone. Values are given as mole ratios for 50% glutathione reductase inactivation.
stoichiometric carbamylation by nitrosourea derived isocyanates, [chx-1-^{14}C]CCNU and [chloroethyl-2-^{14}C]BCNU at a 17-fold molar excess were incubated separately with GSSG-reductase in the absence and presence of NADPH. These additions of radiolabeled 2-chloroethyl nitrosoureas to the reduced enzyme led to the time-dependent inactivation and concurrent \[^{14}C\] labeling of GSSG-reductase. In the absence of NADPH there was no inactivation of enzyme and \[^{14}C\] labeling was quite low (Table IV). The amount of \[^{14}C\] moiety bound per mole of nitrosourea-inactivated enzyme was stoichiometric. The eight 2-chloroethyl nitrosoureas listed in Table V were incubated for 1 hr with sonicates of L5178Y and murine bone marrow cells and the GSSG-reductase measured at 0' and 60'. Those 2-chloroethyl nitrosoureas which were found to inactivate the purified yeast enzyme also inactivated GSSG-reductase in these sonicates.

Discussion

2-Chloroethyl isocyanate and cyclohexyl isocyanate have been shown to be near stoichiometric inactivators of GSSG-reductase. Inactivation was observed when these isocyanates were either added directly or generated \textit{in situ} by the degradation of BCNU and CCNU, respectively. These results combined with the observed \textit{in vivo} inactivation of human erythrocyte GSSG-reductase by BCNU (Frischer and
**TABLE IX  [chloroethyl-2-^{14}C]BCNU AND [chx -1-^{14}C]CCNU LABELING OF GLUTATHIONE REDUCTASE**

<table>
<thead>
<tr>
<th>[^{14}C] NITROSOUREA</th>
<th>PERCENT OF TOTAL ENZYME</th>
<th>[^{14}C] MOIETY BOUND/MOL OF TOTAL ENZYME</th>
<th>[^{14}C] MOIETY BOUND/MOL OF NITROSOUREA INACTIVATED ENZYME</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Enzyme Inactivation</td>
<td>NADPH Inactivation</td>
<td>[^{14}C] Nitrosourea Inactivated Enzyme</td>
</tr>
<tr>
<td>[chloroethyl-2-^{14}C]BCNU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WITH NADPH</td>
<td>84</td>
<td>14</td>
<td>70</td>
</tr>
<tr>
<td>WITHOUT NADPH</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>[chx -1-^{14}C]CCNU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WITH NADPH</td>
<td>95</td>
<td>12</td>
<td>83</td>
</tr>
<tr>
<td>WITHOUT NADPH</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>
### Table V: Inactivation of Glutathione Reductase by Various Nitrosoureas

<table>
<thead>
<tr>
<th>Nitrosourea</th>
<th>Conc. (x 10^{-4}) M</th>
<th>t 1/2 (Min)</th>
<th>Percent inactivation at t 1/2</th>
<th>Carbamylating Activity</th>
<th>Absolute Neutrophile Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCMU</td>
<td>1.97</td>
<td>56</td>
<td>97</td>
<td>94 (13)</td>
<td>25 (13)</td>
</tr>
<tr>
<td>MeCCNU</td>
<td>1.97</td>
<td>58</td>
<td>96</td>
<td>91 (16)</td>
<td></td>
</tr>
<tr>
<td>BCNU</td>
<td>1.97</td>
<td>42</td>
<td>77</td>
<td>69 (13)</td>
<td>25 (13)</td>
</tr>
<tr>
<td>cis-2-OH-CCNU</td>
<td>9.72</td>
<td>48</td>
<td>3</td>
<td>1 (18)</td>
<td></td>
</tr>
<tr>
<td>trans-4-OH-CCNU</td>
<td>9.72</td>
<td>45</td>
<td>81</td>
<td>94 (18)</td>
<td></td>
</tr>
<tr>
<td>Chlorozotocin</td>
<td>9.72</td>
<td>22</td>
<td>0</td>
<td>4 (13)</td>
<td>110 (13)</td>
</tr>
<tr>
<td>GAMU</td>
<td>9.72</td>
<td>7</td>
<td>2</td>
<td>63 (13)</td>
<td>113 (13)</td>
</tr>
<tr>
<td>ACNU</td>
<td>9.72</td>
<td>24</td>
<td>30</td>
<td>3.8 (12)</td>
<td>4 (12)</td>
</tr>
</tbody>
</table>

* Percent of control peripheral blood neutrophile count on Day 3, WBC at nadir.

* Percent of 2-chloroethyl nitrosourea carbamylated of 14C-lysine.
Ahmad, 1977) suggest that this highly specific inactivation may have important in vivo consequences. Loos et al. (1976) have shown that the lack of erythrocyte GSSG-reductase was compensated for by increased glutathione biosynthesis. However, an oxidative challenge resulted in a severe hemolytic crisis resembling the myelosuppression which accompanies administration of certain 2-chloroethyl nitrosoureas. It is interesting to note that of the nitrosoureas examined, those which inactivated the enzyme also exhibit myelosuppressive activity. The observed stoichiometric inactivation of GSSG-reductase indicates that this and other active site-specific inactivations of enzymes by nitrosourea-derived isocyanates warrant further investigation.

Alkyl isocyanates have been demonstrated to be active-site-directed inactivators of chymotrypsin (Babson et al., 1977; Brown and Wold, 1973), alcohol dehydrogenase (Twu and Wold, 1973), and transglutaminase (Gross et al., 1975). In the case of chymotrypsin, Brown and Wold have suggested a two-step process. Octyl isocyanate first binds by virtue of its n-alkyl side chain, analogous to the formation of a Michaelis complex, followed by carbamylation of the active site serine (Brown and Wold, 1973b).

To date the rationale for determining carbamylating potential of 2-chloroethyl nitrosoureas has been the extent to which L-lysine is carbamylated (Wheeler et al., 1975).
The presence of structurally complementary binding sites for isocyanates in vivo would preclude their random carbamylation of nucleophiles. L-lysine concentrations up to 10 mM in vitro had no effect on the active site directed inactivation of chymotrypsin by CCNU (Babson et al., 1977). The intramolecular carbamylation which occurs during degradation of ACNU could be responsible for the low extent to which ACNU carbamylates L-lysine (Tanaka et al., work cited by [Nagourney, et al., 1978]). Yet it was demonstrated that ACNU was capable of inactivating GSSG-reductase presumably via the isocyanate. Thus it seems unreasonable to exclude the possibility of carbamylation by nitroso-ureas solely on the basis of their low potential to chemically modify L-lysine. Clearly the structure of the isocyanates' side chain determines the selectivity of the isocyanate for a complementary binding site and therefore determines its role in vivo. The specificity of these isocyanates suggest their potential as active site probes. Work is now in progress to assess the value of these isocyanates as probes for the catalytic site of GSSG-reductase.
V. PROTECTIVE ROLE OF THE GLUTATHIONE REDOX-CYCLE DURING OXIDATIVE CHALLENGE

Introduction

The antitumor benzantrahquinone, adriamycin (ADR), has been proven to be effective in the treatment of a broad spectrum of neoplastic diseases (Blum and Carter, 1974; Carter, 1975; Lenaz and Page, 1976). However, its clinical use has been complicated by a dose-limiting, cumulative cardiomyopathy (Blum and Carter, 1974; Lefrak et al., 1975; Minow et al., 1975; Minow et al., 1977). Lipid peroxidation has been reported to be associated with ADR cardiotoxicity (Myers et al., 1977). Formation of an ADR semiquinone either spontaneously (Pietronigro et al., 1979) or enzymatically and its subsequent redox cycling to generate reactive oxygen species could explain the observed lipid peroxidation (Bachur et al., 1977; Goodman and Hochstein, 1977; Thayer, 1977). α-Tocopherol, a free radical scavenger, has been shown to reduce the cytotoxicity of ADR (Myers et al., 1977; Mimnaugh et al., 1979; Wang et al., 1980). In addition, the depletion of intracellular glutathione levels has been considered as a factor contributing to ADR toxicity (Olsen et al., 1977; Sagone and Burton, 1979; Wang et al., 1980). These findings suggest that ADR cytotoxicity may be the result of a free radical challenge which overwhelms
the antioxidant protective systems of cells including the glutathione redox-cycle.

Several studies have demonstrated the protective nature of the glutathione redox-cycle, which consists of glutathione in conjunction with glutathione reductase and at least two glutathione-dependent peroxidase activities (Christopherson, 1968; Srivastava and Beutler, 1969; Hogberg et al., 1975; Sies et al., 1972; Burk et al., 1978). Lipid peroxidation and cell damage could result from a failure to catabolize effectively hydroperoxides (Chance et al., 1979). The glutathione redox-cycle along with catalase and superoxide dismutase have been implicated in the catabolism of reactive oxygen species which if unchecked are capable of lipid peroxidative damage (Christopherson, 1968; O'Brien and Little, 1969; McCay et al., 1976; Hogberg, 1978; Chance et al., 1979).

The purpose of this study is to examine the toxicity of ADR from the standpoint of an oxidative challenge and to determine the extent of the protection afforded by the glutathione redox cycle, catalase, superoxide dismutase, and α-tocopherol during such an ADR challenge. We have used isolated hepatocytes as a model system to investigate, in particular, the role of glutathione reductase during drug-induced oxidative challenges. This was accomplished by selectively inactivating glutathione reductase with BCNU, thus allowing a direct assessment of the importance
of this enzyme (Reed and Babson, 1980). Additionally, the recent clinical use of ADR and BCNU in combination chemotherapy (Presant et al., 1978; Presant et al., 1979; Hall et al., 1979) heightened our interest in the possibility of synergistic effects of these drugs with respect to cytotoxicity.

Materials and Methods

Hepatocyte isolation - Hepatocytes were isolated from fed male Sprague-Dawley rats of 200-250 g body weight as previously described (Reed and Orrenius, 1977). Both the pre-perfusion and perfusion solutions were buffered at pH 7.4 with .01 M hepes. The total length of the perfusion was routinely less than 15 min. Aliquots of freshly isolated cells were immediately counted with a hemacytometer in 0.14% trypan blue solution containing 2% bovine serum albumin. Lactate dehydrogenase leakage (LDH) was used to determine cell viability. Cell suspensions with leakage of 9% or less were routinely used for incubations.

General conditions for hepatocyte incubations - Hepatocyte suspensions of $1.8 \times 10^6$ cells/ml were prepared in Fischer's medium (Fischer and Sartorelli, 1964) which was devoid of sulfur amino acids, containing 10% (v/v) sterile fetal calf serum, and buffered at pH 7.4 with .01 M hepes. Incubations were performed in 25 ml Erlenmyer flasks under 95% $O_2$/5% $CO_2$ at 37°C in a gyratory shaker.
BCNU, DEM, and α-tocopherol additions were made as aliquots in DMSO. Adriamycin additions were made in saline. Controls with either DMSO or saline additions were also performed. Zero-time points were taken just prior to drug additions. At times indicated, 1.0 ml aliquots were removed for determination of cellular glutathione (GSH) levels, LDH leakage, trypan blue staining, lipid peroxidation and/or various enzyme activities. Controls indicated that DMSO additions had no effect on the parameters examined herein. All results are expressed as the mean and standard deviation values of at least three separate hepatocyte isolations.

**Quantitation of intracellular glutathione levels** - Hepatocytes and media, separated as described above, were analyzed for GSH by the HPLC method of Reed et al. (1980). The method involves conversion of free thiols to S-carboxymethyl derivatives, reaction of amino groups with 1-fluoro-2,4 dinitrobenzene and separation of the resulting derivatives by reverse phase ion exchange high pressure liquid chromatography.

**Determination of lipid peroxidation** - Lipid peroxidation was monitored by measuring the formation of 2-thiobarbituric reactants as described by Fong et al. (1973). The data are presented as the change in absorbance at 522 nm with respect to zero-time absorbance.
Lactate dehydrogenase leakage - At the times indicated, two 0.5 ml aliquots were removed. The medium was separated from cells in one aliquot by centrifugation (80 g for 3 min). The other aliquot was sonicated in the presence of 0.5% triton for 30 sec and clarified by centrifugation. The supernatants of both were then assayed individually by a modified procedure of Lindstrom et al. (1978) with either a Beckman TR Analyzer or a Cary 15.

Determination of other enzyme activities - Aliquots (1.0 ml) were removed just prior to drug addition (0 min) and at times indicated after addition of drugs. Hepatocytes were either pelleted (80 g for 3 min), washed once with saline, resuspended in 0.5-1.0 ml saline, and sonicated with a Kontes sonicator at power setting 4 for 30 sec or allowed to settle and sonicated directly in media. Samples (5 to 50 μl) were then assayed for various enzyme activities as described below.

Glutathione reductase - Glutathione reductase activity was determined by monitoring the oxidation of NADPH at 340 nm in a Cary 15 spectrophotometer at 25° using the method of Worthington and Rosemeyer (1974). The assay mixture contained 0.2 M KCl, 1 mM EDTA, 1 mM oxidized glutathione in 0.1 M potassium phosphate buffer, pH 7.0, and was initiated by the addition of NADPH to a final concentration of 0.1 mM.
Glutathione peroxidase - Enzyme activity was determined at 25° by a modified procedure of Paglia and Valentine (1967). Cell sonicate samples were added to assay solutions containing 0.3 mM NADPH, 3.8 mM NaN₃ (to inhibit catalase), 5 mM GSH, 20 units yeast glutathione reductase. The reaction was initiated by the addition of H₂O₂ to a final concentration of 70 μM. The oxidation of NADPH was monitored at 340 nm.

Catalase - Catalase activity was determined by the method of Beers and Sizer (1952) in which the reduction of hydrogen peroxide is followed spectrophotometrically at 240 nm at 25°. The change in absorbance which was inhibited by 3.8 mM NaN₃ was ascribed to catalase mediated reduction.

Superoxide dismutase - Superoxide dismutase activity was determined by the inhibition of nitroblue tetrazolium reduction by superoxide according to the method of Winterbourn et al. (1975).

Glutathione-S-transferase - Glutathione-S-transferase activity was determined by the method of Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene as the substrate.

Collagenase, Type IV for isolation of hepatocytes, was purchased from Sigma Chemical Co. HPLC grade solvents were purchased from either Burdick and Jackson Laboratories Inc. or J. T. Baker Chemical Co. All reagents were reagent grade and commercially available. BCNU and ADR were supplied by the National Cancer Institute.
Results

Our previous observations demonstrated that BCNU is a highly specific inactivator of glutathione reductase (Babson and Reed, 1978). This stoichiometric inactivation of the enzyme in vitro led us to believe that BCNU might be employed to inactivate selectively the reductase in isolated hepatocytes. As Figure 8 indicates, 75 μM BCNU inactivated greater than 90% of the hepatic glutathione reductase within 60 min. This level of inactivation was maintained throughout the remainder of the 5 hr incubation. BCNU (75 μM) had no significant effect on the hepatocytes as determined by either trypan blue staining, LDH leakage, or lipid peroxidation, the criteria used to assess oxidative damage. However, in the absence of supplemented sulfur-containing amino acids, the intracellular GSH level was decreased 65% by BCNU when compared to the initial GSH level in control cells (Figure 9). Thus the inactivation proved selective enough to permit examination of the importance of the glutathione reductase and the glutathione redox-cycle during an ADR induced oxidative challenge.

ADR was used to challenge both normal and BCNU-treated hepatocytes. Figure 9 illustrates the time-dependent effects of ADR and BCNU alone or in combination on the intracellular GSH levels of isolated hepatocytes. ADR (100 μM) only slightly reduced intracellular GSH levels when compared to corresponding control values. Previous values
Figure 8. Hepatocyte suspensions (1.8 x 10^6 cells/ml) were treated either with 0.01 ml 95 mM BCNU in DMSO (Δ) or 0.01 ml DMSO (■) and incubated as described in the Methods section. The final concentration of BCNU was 75 μM. Immediately prior to drug addition (0 min) and at intervals shown above, 1.0 ml aliquots were removed and glutathione reductase activity determined.
Figure 9. Hepatocytes (1.8 x 10^6 cells/ml) were incubated either with 100 μM ADR in the presence of 75 μM BCNU (○) and absence of BCNU (●) or with 75 μM BCNU in the absence of ADR (△). Controls contained 0.1% (v/v) DMSO and 2.0% (v/v) saline, but no ADR or BCNU (■). Aliquots (1.0 ml) were removed and assayed for intracellular GSH. Incubation and assay conditions are described further in the Methods section.
for GSSG efflux (Reed and Beatty, 1978) when compared to our control values indicated that glutathione synthesis is minimal in the medium devoid of sulfur-containing amino acids. BCNU (75 μM) rapidly decreased GSH levels to 45% of initial values within 60 min. The level of GSH decreased to and remained at 35% of initial control values for the rest of the incubation. In combination 75 μM BCNU and 100 μM ADR decreased GSH levels the same extent as 75 μM BCNU alone by 60 min. However the level dropped markedly to 5% of initial values by 180 min with no GSH detectable at 300 min.

Figure 10 shows that the combination of 75 μM BCNU and ADR (50 or 100 μM) increased hepatocyte lipid peroxidation as monitored by the absorbance at 532 nm of 2-thiobarbituric reactants. The increase was found to be dose dependent with respect to ADR concentration. Neither BCNU or ADR alone caused any measurable increase in lipid peroxidation. Apparently either the ability of BCNU to inactivate glutathione reductase, deplete intracellular GSH or both could interfere with the ability of glutathione peroxidase to reduce effectively H₂O₂ and organic peroxides. Such reactive species could be responsible for the observed lipid peroxidation.

Lipid peroxidation has been implicated as the cause of membrane damage during oxidative stress (Bidlack and Tappel, 1973). Figure 11 indicates that BCNU and ADR in
Figure 10. Hepatocytes were incubated either with 50 μM ADR plus 75 μM BCNU (▲), 100 μM ADR in the presence of 75 μM BCNU (○) and absence of BCNU (●), or 75 μM BCNU in the absence of ADR (Δ). Samples (1.0 ml) were removed and assayed for lipid peroxidation as described in the text.
Figure 11. Hepatocytes were incubated either with 100 μM ADR in the presence of 75 μM BCNU (○) and absence of BCNU (●) or with 75 μM BCNU in the absence of ADR (△). Controls contained 0.1% (v/v) DMSO and 2.0% (v/v) saline (■). Cells were incubated and assayed for LDH leakage as described in the text.
combination induce membrane damage as evidenced by the increased LDH leakage. This increase corresponds in time with the increase observed for lipid peroxidation (Figure 10). Again ADR (100 μM) or BCNU (75 μM) alone did not increase LDH leakage over that of control incubations. Thus an ineffective glutathione redox-cycle could be responsible for the hepatocytes' increased susceptibility to an ADR mediated oxidative challenge.

To determine whether the increase in cell damage and lipid peroxidation were actually due to ADR, several concentrations of ADR (25, 50, 100 μM) were incubated with hepatocytes in the presence of 75 μM BCNU. The increase in lipid peroxidation, trypan blue staining, and LDH leakage proved to be dose dependent with respect to ADR concentration (Table VI). The increases in both indicators of cell viability, LDH leakage and trypan blue staining, were essentially the same at each ADR concentration used. Values were approximately 28%, 45%, and 87% with 25, 50, and 100 μM respectively. The increase in lipid peroxidation corresponds well with the cell viability criteria. The dose dependence of these phenomena strongly indicate that ADR is responsible for their increase. The free radical nature of the ADR challenge is underscored by the observed protection by α-tocopherol. Both cell damage and lipid peroxidation due to 100 μM ADR plus 75 μM BCNU were significantly decreased by 75 μM α-tocopherol (Table VI).
Table VI
BCNU-ADR Mediated Effects in Hepatocytes

<table>
<thead>
<tr>
<th>Drug</th>
<th>Cell Viability(^b)</th>
<th>Lipid Peroxidation(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% LDH(^a) Leakage</td>
<td>% Trypan Blue Staining</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ADR (µM)</th>
<th>BCNU (75 µM)</th>
<th>α-Tocopherol (75 µM)</th>
<th>Control</th>
<th>-</th>
<th>-</th>
<th>20 ± 2</th>
<th>22 ± 6</th>
<th>.01 ± .004</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>22 ± 4</td>
<td>21 ± 5</td>
<td>.039 ± .02</td>
</tr>
<tr>
<td>25</td>
<td>+</td>
<td>-</td>
<td>21 ± 4</td>
<td>22 ± 3</td>
<td>.03 ± .01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>+</td>
<td>-</td>
<td>27 ± 4</td>
<td>29 ± 9</td>
<td>.085 ± .06</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>+</td>
<td>-</td>
<td>41 ± 8</td>
<td>48 ± 11</td>
<td>.179 ± .08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>+</td>
<td>+</td>
<td>84 ± 12</td>
<td>90 ± 10</td>
<td>.499 ± .153</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>+</td>
<td>+</td>
<td>36 ± 11</td>
<td>42 ± 15</td>
<td>.135 ± .09</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Lactate dehydrogenase (LDH) leakage at 0 time was 9 ± 2%.

\(^b\)Values were obtained after 5 hr incubation.
The free radical damage attributed to ADR required the co-condition of glutathione reductase inactivation and/or the reduction of intracellular GSH levels, both achieved by BCNU treatment. ADR or BCNU alone did not markedly affect the abovementioned criterion. An attempt was made to determine if the BCNU-mediated inactivation of glutathione reductase per se was primarily responsible for the increased hepatic sensitivity to ADR and not simply the decreased intracellular GSH level. When 340 μM DEM is used to deplete intracellular GSH to 25% of initial values at 180 min, no lipid peroxidation or cell damage is observed with either 50 μM or 100 μM ADR after 5 hr. Whereas 50 μM ADR in the presence of 75 μM BCNU results in a similar decrease of GSH content (75%) by 180 min, accompanied by significant lipid peroxidation and cellular damage. It appears that low levels of intracellular GSH (25% of initial control values) in the presence of glutathione reductase activity is sufficient to protect hepatocytes from an ADR challenge. However, given these same low levels in the absence of the recycling activity of the reductase, the cells cannot cope with the ADR challenge. This suggested that the reductase plays a key role in the cellular defense against oxidative stress (Table VII).

There was also the possibility that the observed damage was the result of the inactivation of other enzymes with a protective capacity. To determine whether or not
Table VII
Effect of Glutathione Depletion on ADR-Treated Hepatocytes

<table>
<thead>
<tr>
<th>Drug</th>
<th>% Intracellular GSH</th>
<th>% LDH Leakage</th>
<th>Lipid Peroxidation (AOD 532 nm/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time in Min</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>DEM (µM)</td>
<td>ADR (µM)</td>
<td>BCNU (µM)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>75 ± 3</td>
<td>20 ± 2</td>
<td>.010 ± 0.006</td>
</tr>
<tr>
<td>340</td>
<td>50</td>
<td>0</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>340</td>
<td>100</td>
<td>0</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>0</td>
<td>50</td>
<td>75</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>0</td>
<td>150</td>
<td>75</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>340</td>
<td>0</td>
<td>0</td>
<td>32</td>
</tr>
</tbody>
</table>
this was the case, the activities of enzymes known to afford cellular protection from oxidative challenges were measured. As Table VIII indicates, catalase, glutathione peroxidase, superoxide dismutase and glutathione-S-transferase activities were not significantly affected by either ADR, BCNU or a combination of both. These data further demonstrate the importance of glutathione reductase and the glutathione redox-cycle during a drug-induced oxidative insult. This data also implies that tissues with an impaired glutathione redox-cycle may be particularly susceptible to the oxidative aspect of an ADR challenge.

Discussion

A dose-dependent decrease in cell viability accompanied by increased lipid peroxidation resulted from ADR treatment of BCNU-modified hepatocytes. Since BCNU selectively inactivated glutathione reductase, a key protective role of the glutathione redox-cycle is suggested by our results.

The molecular mechanism of ADR toxicity has drawn much interest over the past several years. The view that the ability of ADR to generate reactive oxygen species thus causing cell damage is becoming more widely accepted. Pietronigro et al. (1979) have shown the spontaneous pH-dependent formation of ADR semiquinone radicals. This
Table VIII
Effect of ADR-BCNU on Certain Hepatic Enzyme Activities

<table>
<thead>
<tr>
<th>Enzyme Activity/10^6 Cells^a</th>
<th>Percent Activity Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>zero-time</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>.054 ± .010</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>1.00 ± 0.10</td>
</tr>
<tr>
<td>Glutathione-S-transferase</td>
<td>648.6 ± 68.3</td>
</tr>
<tr>
<td>Catalase</td>
<td>24,556.0 ± 2039</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>27.8 ± 2.6</td>
</tr>
</tbody>
</table>

^aActivities given in enzyme units as described in the test.

^bIncubations contained 100 μM ADR and 75 μM BCNU.
route of radical formation could be expected to occur to only a limited extent at physiological pH. A more likely route of semiquinone radical formation in vivo has been suggested by Bachur et al. (1979). They demonstrated the involvement of a univalent reduction of ADR by purified NADPH-dependent cytochrome p-450 reductase. Once formed, the semiquinone radical is then capable of reducing molecular oxygen, thus generating toxic oxygen species. This redox-cycling has been suggested by several groups as the probable cause of oxidative damage to the cell (Bachur et al., 1979; Goodman and Hochstein, 1977; Sato et al., 1977; Thayer, 1977). The free radical nature of ADR toxicity is further supported by the observed lipid peroxidation associated with ADR cardiotoxicity and the protection afforded by α-tocopherol, a free radical scavenger (Myers et al., 1977; Mimnaugh et al., 1979; Wang et al., 1980).

Our results using BCNU-treated hepatocytes support these findings. ADR in combination with BCNU caused an increase in cellular damage which was accompanied by lipid peroxidation. The observed damage was dose-dependent with respect to ADR. α-Tocopherol greatly inhibited the ADR damage. Interestingly, no ADR toxicity was observed in the absence of BCNU. Since glutathione reductase is specifically inactivated by BCNU (Frischer and Ahmad, 1977; Babson and Reed, 1978), the importance of this enzyme in the defense against oxidative insult is implied by our results.
Additionally, Loos et al. (1976) have shown that lack of glutathione reductase activity rendered human erythrocytes susceptible to drug-induced oxidative insult. We have previously demonstrated a positive correlation between the ability of several nitrosoureas to inactivate glutathione reductase and their myelotoxicity (Babson and Reed, 1978).

Glutathione reductase is a component of the glutathione redox-cycle which also includes glutathione peroxidases and GSH. The reductase reduces glutathione disulfide which is formed during the glutathione peroxidase reduction of \( \text{H}_2\text{O}_2 \) and organic peroxides (Srivastava and Beutler, 1969). If not catabolized effectively, these reactive species derived from molecular oxygen are capable of initiating lipid peroxidation which could result in membrane damage and eventual cell lysis (Chance et al., 1979). Catalase and superoxide dismutase comprise another line of defense against the reactive oxygen species \( \text{H}_2\text{O}_2 \) and \( \text{O}_2 \) respectively. Sagone and Burton (1979) observed an increase in the oxidation of \([^{14}\text{C}]-1\)-glucose and \([^{14}\text{C}]-\text{formate}\) upon administration of ADR to BCNU-treated and G6PD-deficient erythrocytes. Normal erythrocytes showed increased \([^{14}\text{C}]-1\)-glucose oxidation only after BCNU treatment. The oxidation of \([^{14}\text{C}]-1\)-glucose and \([^{14}\text{C}]-\text{formate}\) was taken as a measure of HMPS activity and \( \text{H}_2\text{O}_2 \) generation, respectively. They concluded that BCNU impaired the ability of erythrocytes to reduce ADR-generated \( \text{H}_2\text{O}_2 \) and
this impairment was similar to that in ADR-treated G6PD-deficient erythrocytes. However, they made no attempt to assess the protective role of the glutathione redox-cycle with respect to peroxidative damage. It was our intent to evaluate the importance of known protective mechanisms during an ADR challenge, in particular the role of the glutathione redox-cycle, with respect to the prevention of peroxidative damage. Our results indicate that the other protective enzyme activities we examined, glutathione peroxidase, glutathione-S-transferase, catalase and superoxide dismutase were not significantly affected by ADR or BCNU. Only glutathione reductase was significantly inactivated (>90%) by BCNU but not by ADR. We conclude that the primary protection from ADR-induced peroxidative damage is the glutathione redox-cycle. Frischer and Ahmad (1977), Sagone and Burton (1979) and ourselves observed BCNU related glutathione depletion. Is the ADR induced damage just a result of glutathione depletion or is the inactivation of the reductase required? Data in Table II indicate that when hepatic GSH levels are lowered by DEM to levels seen in BCNU treated hepatocytes (25% of control), ADR causes no visible oxidative damage. Thus at very low GSH levels normal levels of glutathione reductase activity appears to recycle effectively glutathione disulfide formed by the glutathione peroxidase reduction of H₂O₂ and organic peroxides.
Although it is imprudent to extrapolate directly to clinical situations, our results do raise questions of clinical importance. For example, do biochemical defects or differences in tissues of various organs which impair the glutathione redox-cycle imply an increased sensitivity towards ADR and other oxidative drugs? Interestingly, the heart tissue of mice which exhibited ADR-induced cardiotoxicity contained relatively low levels of catalase and glutathione peroxidase activity (Wang et al., 1980). Certainly the results of Sagone and Burton (1979) and those of ourselves should be considered during combination chemotherapy involving both ADR and BCNU.

In summary, we have shown that ADR act as an effective oxidative agent to hepatocytes in which glutathione reductase has been inactivated by BCNU. BCNU-treated hepatocytes appear to be a promising model system with which to study the mechanism of oxidative drugs. A key protective role of glutathione reductase and the glutathione redox-cycle during an ADR-induced oxidative challenge has been demonstrated.
VI. CONCLUSIONS AND COMMENTS

The commonly accepted concept that 2-chloroethyl nitrosoureas are nonspecific in their mode of action should be carefully re-evaluated in light of the results presented in this study. Although detailed mechanisms of action of these drugs are not known, it seems overly simplistic to think of them as merely nonspecific alkylating agents. It is now evident that nitrosourea-derived isocyanates not only carbamylate proteins randomly at the ε-amino groups of lysine residues but are also able to interact in a highly specific manner with certain proteins. This statement is based not only on the observed active-site-directed inactivation of purified chymotrypsin and glutathione reductase in vitro but is supported additionally by the selective inactivation observed for glutathione reductase in BCNU-treated isolated hepatocytes. The question now arises that if 2-chloroethyl carbamylation can exhibit such specificity, why cannot the alkylation observed for these drugs also be expected to display some preference for selectively defined molecular target sites.

The present controversy regarding the relative contribution of either alkylation or carbamylation to the antineoplastic properties of 2-chloroethyl nitrosoureas is not resolved by the data in this thesis. Quite the contrary, the controversy is heightened and the situation becomes
more complicated as simplistic solutions fade. As previously discussed, the criteria used to determine carbamylating activity makes any extrapolation to cell systems rather tenuous. In fact, our examples indicate that carbamylating activity and cytotoxicity such as myelosuppression do not always correlate. Interestingly, the carbamylating activity also fails to predict the ability of certain 2-chloroethyl nitrosoureas-derived isocyanates to inactivate glutathione reductase. Thus the question arises as to whether intramolecular carbamylation precludes the intermolecular carbamylation of a specific enzyme active site. This does not appear to be the case with respect to ACNU.

The ability of certain 2-chloroethyl nitrosourea-derived isocyanates to inactivate glutathione reductase does correlate well, given the relatively small sampling of drugs, with the clinically observed myelosuppression of these compounds. The inactivation of the glutathione redox-cycle would cause a cell type to rely on other protective mechanisms to deal with an oxidative challenge. Various tumor cell lines are deficient in catalase activity and have low glutathione resynthesis rates. BCNU-treated hepatocytes which are not synthesizing glutathione are susceptible to an ADR challenge, while those that can synthesize glutathione are not greatly affected by ADR (Babson, unpublished results). Could this obvious metabolic
weaknesses of the tumor cells be exploited? Is the inactivation of glutathione reductase per se solely a basis for host toxicity or in the case of a metabolically susceptible tumor cell, is it the basis for antineoplastic activity? Despite the general opinion, specific carbamylation may not be all that bad.

The results with respect to the importance of glutathione reductase during oxidative challenge seem quite clear. The inactivation of this enzyme under normal circumstances, even when glutathione levels are low, does not drastically effect the well being of the hepatocytes. However, the system can be overwhelmed by an oxidative challenge such as that caused by ADR. Based on the criteria used to monitor cytotoxicity, the ADR challenge appears to be free radical in nature. Additional support for this conclusion is the observed protection afforded by α-tocopherol.

The depletion of certain drugs of intracellular glutathione is believed to be a major aspect of their toxicity. The evidence presented herein questions this statement. Indeed, the cells appear to sustain a substantial loss of glutathione and suffer no observable ill effects indicating that any endogenous oxidative challenge appears not to be substantial enough to cause immediate cellular damage. However, problems arise when glutathione levels drop below 10% of control levels. This could be taken to indicate that glutathione depletion, in the presence of a
resynthesis capability, may not always be the main determinant of drug toxicity. Indeed the protective mechanism of the glutathione redox-cycle appears capable of operating at levels of glutathione which are much lower than those normally present in the cell. Therefore, it appears that it is not necessarily the depletion of glutathione per se but the inactivation of glutathione reductase which rendered the hepatocytes susceptible to the exogenous drug mediated oxidative insult. In addition, since no other major protective enzyme was inactivated by BCNU and ADR treatment, including glutathione peroxidase, glutathione reductase inactivation and the resultant susceptibility to a free radical challenge demonstrates the enzyme's importance. It follows that the glutathione redox-cycle itself is the major protective mechanism during drug-mediated oxidative stress.

The potential of the BCNU-treated hepatocytes as an experimental tool can be gleaned from results of Chapter V. The BCNU-treated cells show no significant increase in the criteria used to measure oxidative stress and thus seem an appropriate model system to use to examine questions about peroxidative damage. One important problem to be assessed is the question of endogenous oxidative stress. BCNU-treated hepatocytes would be more sensitive to the endogenous generation of reactive oxygen species. Improved measurements of these products might be possible. It would
be interesting to measure the extent to which catalase and superoxide dismutase offer protection. The careful use of other specific inhibitors could determine the extent to which various oxidative systems contribute to the endogenous challenge. For example, cytochrome P450 is thought to contribute significantly to reactive oxygen generation. It could be specifically inactivated in the BCNU-treated hepatocytes and various oxidative criteria examined. In addition, various drugs, which are believed to work via an oxidative mechanism including paraquat and acetaminophen, could be tested in these sensitized hepatocytes.

It is necessary to seek a more efficient method to aid in the design and evaluation of chemotherapeutic drugs for clinical use. However, current QSAR methods have their limitations. They may be misleading or unreliable in situations where selective interactions occur. Therefore, it seems premature to simplistically characterize 2-chloroethyl nitrosourea-derived isocyanates as agents which mediate host toxicity but not antineoplastic effects. It may be more prudent to design isocyanates which could be directed toward specific in vivo targets of consequence in neoplastic tissue.
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