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Title: SPECIFIC ENZYMIC INTERACTIONS RELATING TO THE

MECHANISM OF ACTION OF CERTAIN 2-CHLOROETHYL

NITROSOUREAS

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D. J. Reed

Certain 2-chloroethyl nitrosoureas have been demon-
strated 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-^{14}C]
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 gluta-thione 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]chclohexyl)-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
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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.

$$t_{1/2} = 0.693 \ k_{inact.}$$

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

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot I \overset{k_2}{\rightarrow} E\text{-}I \quad K_I = k_{-1}/k_1$$
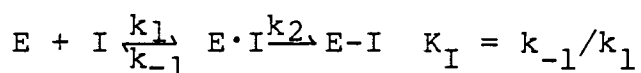
The dissociation constant K_I for the inhibitor can be considered as a measure of the affinity of the enzyme for the inhibitor. Substrate protection of an enzyme from inactivation is also evidence for an active-site-directed inhibitor. In addition, inactivation of an enzyme with radiolabeled inhibitor 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 anti-metabolites. 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 non-classical 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 sig-nificantly. 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 neoplas-tic 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.

$$\log (1/c) = -k_1 \pi^2 + k_2 \pi + k_3 \pi + k_4 E_S + k_5$$

The hydrophobic parameter π is the logarithm of n-octanol-water partition coefficient (P) of the compound. The electronic considerations are represented by σ, 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 lipophillic 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 inter-
mediate 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 con-
cern 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 crystalographic 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[195]. 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[195], 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.

$$GSSG + NADPH + H^+ \rightleftharpoons 2\ GSH + NADP^+$$
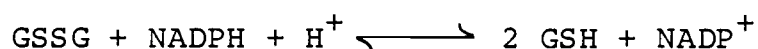
Ondarza et al. (1974) also proposed a glutathione reductase mediated reduction of acetyl-coenzyme A--glutathione mixed disulfide. A transhydrogenase activity has been ascribed to the reductase. In this reaction, thio-NADP$^+$ is the electron acceptor and a marked preference for NADPH over NADH is shown 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 disul-
fide. This was interpreted to represent the differing
positions of these thiols with respect to the substrate
binding site and the isoalloxazine ring. This was con-
firmed 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 com-
plex 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 pro-
tonated and dissociates from the enzyme as a product. The
Cys^{46} reforms the active site dithiol and the other gluta-
thione molecule is protonated as it leaves by the histidine
(Williams et al., Pai, 1978; Boggaram and Mannervick,

1978). 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).

$$H^+ + NADPH \xrightarrow{\text{glutathione}} GSSG \xrightarrow{\text{glutathione}} ROH + H_2O$$
$$NADP^+ \quad \text{reductase} \quad GSH \quad \text{peroxidase} \quad ROOH$$
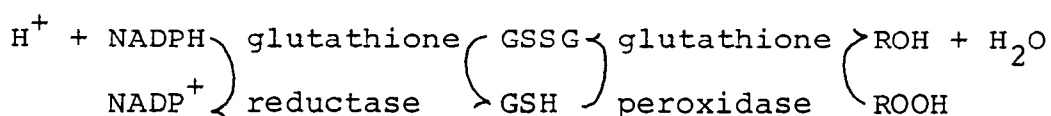
Glutathione reductase recycles glutathione disulfide after glutathione is oxidized during the glutathione peroxidase mediated reduction of peroxides (Srivastava and Beutler, 1969). These peroxides result from reactions that generate reactive oxygen species (Chance et al., 1979).

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 H_2O_2 respectively. Free radical scavengers, such as glutathione itself and α-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 clini-

cally 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 contro-

versy exists as to the role of these two reactive inter-

mediates 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 ribo-
nucleases 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 carbamy-
lation 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 con-
sideration 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 generaliza-
tions 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 unpre-
dicted 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 toxi-
city include alopecia, nausea, vomiting, stomatitis, leuko-
penia, 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 ex-
plain 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 prepara-
tions (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 grove 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 addi-
tion, 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).
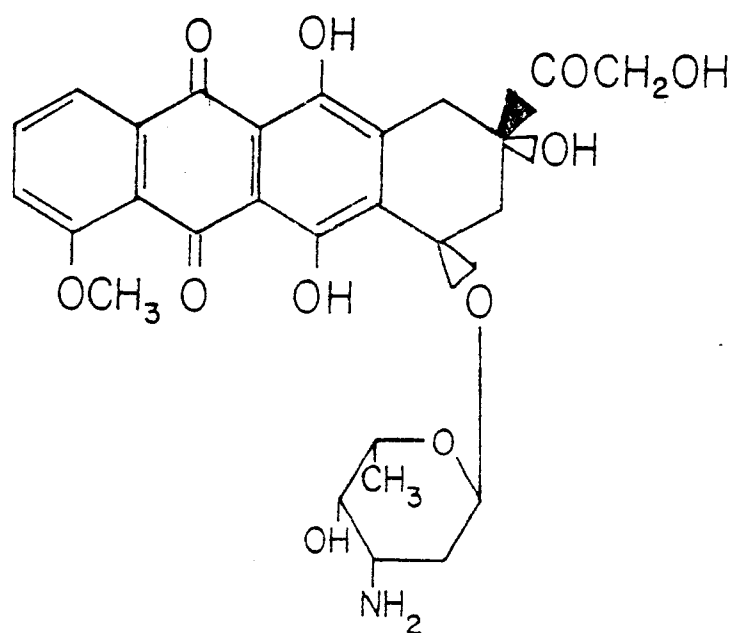
The work discussed in this thesis is primarily concerned with the oxidative aspects of ADR toxicity. Indeed, the ability of ADR to induce peroxidative damage may be an important underlying factor in ADR cardiotoxicity. Some ADR-mediated histological alterations in rat myocardium include degeneration 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).

$$Cl-CH_2-CH_2-N-\overset{\overset{\textstyle O}{\|}}{C}-N-R \qquad R=$$

$$\underset{NO}{|} \qquad \underset{H}{|}$$

<u>BCNU</u> $-CH_2-CH_2-Cl$

<u>CCNU</u>
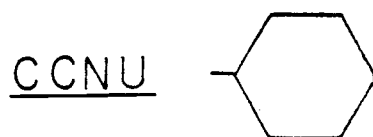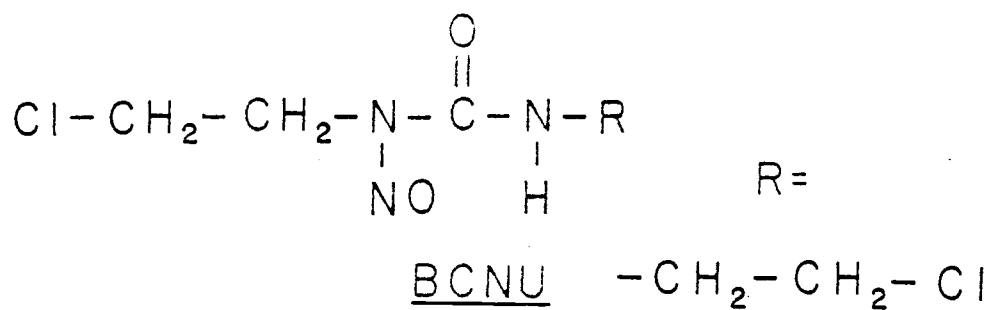
2-CHLOROETHYL NITROSOUREAS

ADRIAMYCIN

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 nitro-
soureas is being directed towards the alkylating and carba-
mylating 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-chloro-
ethyl 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 carbamyla-
tion 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}C with proteins
during degradation of [cyclohexyl-1-^{14}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 carba-
mylation 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-^{14}C]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-lol-xylene fluor. Counting efficiency was determined with [^{14}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 NaPO$_4$ buffer (pH 7.7) or 0.5 ml glass dis-
tilled water and incubated for 21-22 hr at 25°C. Cyclo-
hexyl isocyanate (0.40 μmole) was similarily 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 sub-
jected 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 Physics Autolab System I computing integrator.

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 (2.38×10^{-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 (2.38×10^{-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-^{14}C]CCNU labeling of chymotrypsin -
[Cyclohexyl-1-^{14}C]CCNU (0.850 µmoles) was added to buffered
solutions containing 0.476 µmoles chymotrypsin (2.38 x 10^{-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 chroma-
tography 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 et al. (1976). The samples were then
assayed for radioactivity and protein to determine the
moles bound [^{14}C]cyclohexyl moiety/mole enzyme.

[Cyclohexyl-1-^{14}C]CCNU labeling of partially inacti-
vated chymotrypsin - Buffered solutions containing 0.476
µmoles chymotrypsin (2.38 x 10^{-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-^{14}C]CCNU were added. The reactions were
allowed to proceed for 21-22 hr at 25°C. Enzymatic acti-
vity was determined prior to cyclohexyl isocyanate addi-
tion, 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-1-^{14}C]CCNU labeling of chymotrypsin in the presence of L-lysine - [Cyclohexyl-1-^{14}C]CCNU (0.850 μmoles) was added to buffered solutions containing 0.476 μmoles chymotrypsin (2.38 x 10^{-4} M0, and 0,2,10, and 20 μmoles 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 iso- cyanate reacts with chymotrypsin to inactivate the enzyme rapidly and irreversibly. A typical titration of chymotryp- sin 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 x 10^{-4} M chymotrypsin the

TABLE I: Titration of Chymotrypsin with Cyclohexyl Isocyanate. Cyclohexyl Isocyanate to Chymotrypsin Mole Ratio for 50% Inactivation.

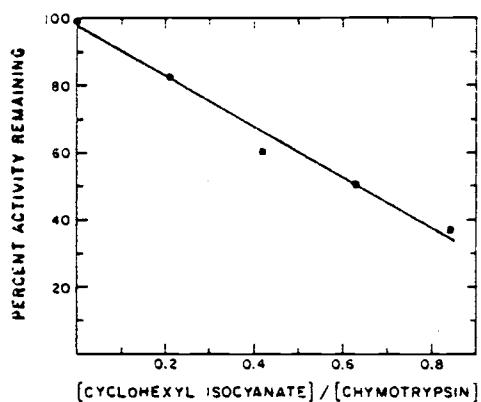| Enzyme Concn (M)	Cyclohexyl Isocyanate/Chymotrypsin
4.76×10^{-6}	7.96
1.19×10^{-5}	4.20
2.38×10^{-5}	3.25
4.76×10^{-5}	1.29
1.10×10^{-4}	0.90
2.38×10^{-4}	0.63

Figure 2. Titration of chymotrypsin (2.38×10^{-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.
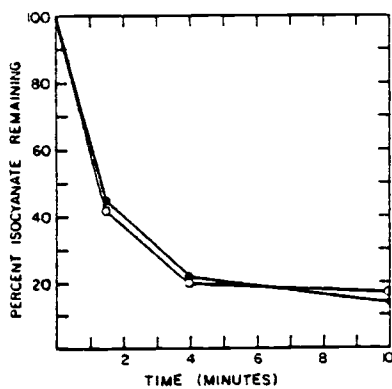

Figure 3. Degradation of octyl isocyanate (●), and cyclohexyl isocyanate (O) in 0.1 M Tris-HCl, pH 7.7, at 25°C.

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% ($^{\pm}$ 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. Incu-

bation 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 cyclo-

hexyl 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 iso-

cyanate formed during CCNU degradation has an opportunity

equivalent to that of added cyclohexyl isocyanate to in-

activate chymotrypsin. Consequently, the extent of enzyme

inactivation observed indicates that $70 \pm 12\%$ ($\pm$ 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,
$74 \pm 6\%$ ($\pm$ 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 iso-
cyanate 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 re-
sults is based on two assumptions: (1) that undegraded
CCNU does not effectively protect the enzyme from cyclo-
hexyl 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 sig-
nificant protection by CCNU would be evidenced by an in-
crease in this ratio. As shown in Figure 4, this ratio did
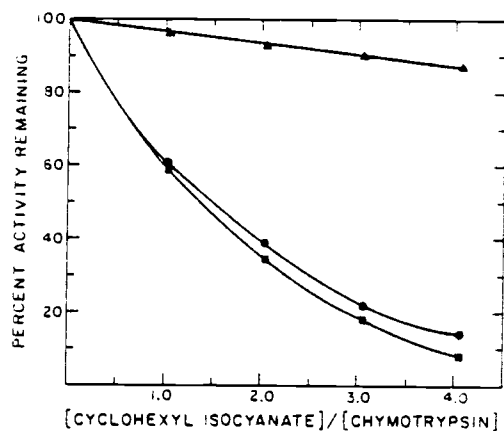not increase but was observed to decrease slightly. This

Figure 4. Titration of 4.76 x 10^{-5} chymotrypsin with 0.01 M cyclohexyl isocyanate in the presence of 1.25 x 10^{-4} M CCNU (■), and absence of CCNU (●). A control to measure the extent of enzyme inactivation by 1.25 x 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 cal-
culated quantity of the cyclohexyl isocyanate formed from
CCNU during the incubation period and (b) the observed
amount of inactivation in the CCNU control (▲, 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 modi-
fication 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 acti-
vity 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]-cyclo-
hexyl moiety. At these concentrations (3.12 x 10^{-4} M CCNU

and 2.38 x 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}C]cyclohexyl moiety to total enzyme in seven experiments was 0.70 ± .05. The ratio of bound label to CCNU-inactivated enzyme (total inactivated enzyme less acetone inactivation) was 1.11 ± .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}C labeling with [cyclohexyl-1-^{14}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}C]CCNU was incubated with chymotrypsin which had been previously inactivated to varying extents by unlabeled cyclohexyl isocyanate, the decrease in bound [^{14}C]-cyclohexyl moiety was found to be proportional to the extent of cyclohexyl isocyanate inactivation prior to ^{14}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}C] CCNU resulted in a value of 0.23 moles [^{14}C]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}C]CCNU. The decrease in the bound [^{14}C]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}C]CCNU contained 0.23 moles bound [^{14}C]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}C labeling is not unexpected, since the added [cyclohexyl-1-^{14}C]CCNU to enzyme ratio was 1.8 in these incubations. Brown and Wold (1973a) also observed 20% non-specifically bound [^{14}C]butyl isocyanate with chymotrypsin. PMSF has been demonstrated by Gold and Fahrney (1964) to react exclusively with the active site
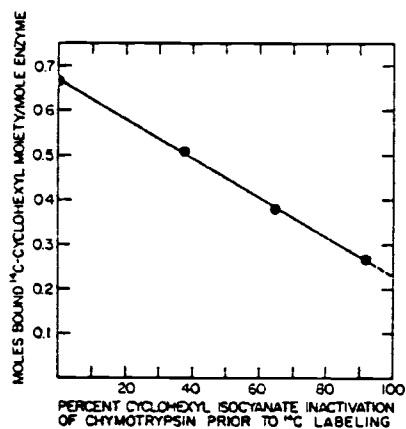
Figure 5. [chx-1-^{14}C]CCNU labeling of cyclohexyl iso-
cyanate inactivated chymotrypsin. Reaction mixtures con-
taining 2.38 x 10^{-4} M chymotrypsin were titrated to varying
extents of enzyme inactivation with cyclohexyl isocyanate.
The reaction mixtures were then incubated with 8.0 x 10^{-4} M
[chx-1-^{14}C]CCNU 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}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 carbamylimi-dazole 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}C]-butylcarbamylserine from chymotrypsin after treatment with [^{14}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 carbamyla-tion 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-^{14}C]CCNU labeling of chymotrypsin in the presence of L-lysine - [Cyclohexyl-1-^{14}C]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 4.0×10^{-4} M [cyclohexyl-1-^{14}C]CCNU was allowed to degrade for 21 hr in the presence of 2.38×10^{-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
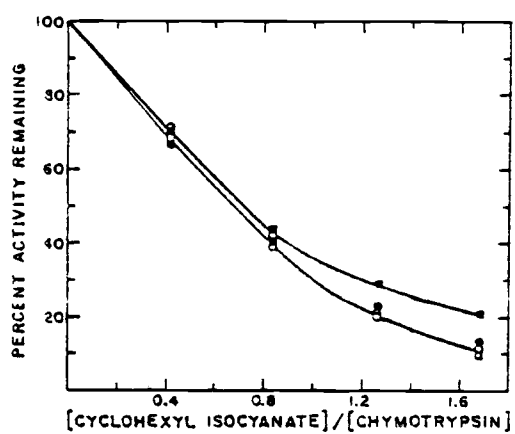
Figure 6. Chymotrypsin (2.38 x 10^{-4} M) was titrated with cyclohexyl isocyanate as described in the text (o). Similar titrations were carried in the presence of 1 mM (□), 5 mM (●), and 10 mM (■) L-lysine hydrochloride.

TABLE II: [Cyclohexyl-1-^{14}C]CCNU Labeling of Chymotrypsin in the Presence of L-lysine

| L-lysine Conc (mM) | Percent | | ^{14}[C]-CCNU Inactivated Enzyme[a] | ^{14}[C]cyclohexyl moiety bound/ mole total enzyme | ^{14}[C]cyclohexyl moiety bound/mole CCNU inactivated enzyme |
	Total Enzyme Inactivation[a]	Acetone Control Inactivation[a]			
0	30	26	54	0.70	1.10
1	39	24	65	0.61	0.93
5	88	27	52	0.65	1.04
10	95	21	64	0.80	0.94

[Cyclohexyl-1-^{14}C]CCNU (0.850 umoles) was added to buffered solutions containing 0.476 umoles chymotrypsin, (2.38 x 10^{-4}M) and 0,2,10 and 20 umoles L-lysine mono-hydrochloride. The solutions were incubated for 21 hr then treated and analyzed as described in the text.

[a]Values given as percent of total enzyme

well enough to affect the active-site-directed labeling of the enzyme.

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 iso-cyanate 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 cyclo-hexyl 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 ± .07) and (c) the

proportional decrease in ^{14}C labeling after PMSF modifica-
tion of the active-site serine.

The high degree of "productive" binding of the cyclo-
hexyl 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 effi-
ciency should be with n-alkyl chains of 8 to 9 carbons. It
was of interest to determine whether the nucleophilic en-
vironment of chymotrypsin in solution could alter the
effectiveness of cyclohexyl isocyanate as an active-site-
directed reagent. Thus "productive" binding of the cyclo-
hexyl side chain to the hydrophobic pocket of chymotrypsin
was measured against the rapid reaction of the cyclohexyl
isocyanate moiety with competing nucleophiles. Surprising-
ly, when using L-lysine as a competing nuclephile, a 70-
fold excess of lysine (Figure 6) was required to demon-
strate any decrease in the stoichiometric inactivation of
chymotrypsin (2.38 x 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 pro-
duct 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 impor-
tance in determining the carbamylation products formed dur-
ing 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-^{14}C]CCNU, unlabeled CCNU or cyclohexyl iso-
cyanate. They observed carbamylation as evidenced by ^{14}C
labeling and chromatographic R_f values of the reaction pro-
ducts. 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 [^{14}C]cyclohexyl isocyanate formed during
[cyclohexyl-1-^{14}C]CCNU degradataion. Again the effective-
ness of "productive" binding of the CCNU generated cyclo-
hexyl isocyanate with the enzyme binding pocket was such
that L-lysine concentration of up to 10 mM had essentially
no effect on the stoichiometric ^{14}C labeling and inactiva-
tionof chymotrypsin (2.38 x 10^{-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 transglutaminase (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 inter-
mediates generated during their degradation under physio-
logical 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 reason-
able to postulate the interaction of a 2-chloroethyl nitro-
sourea-generated isocyanates with an enzymatic active site
in vivo. Frischer and Ahmad (1977) observed reduced eryth-
rocyte 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 carba-
mylation of the NADPH-reduced GSSG-reductase by 2-chloro-
ethyl 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 alkyli-
socyanates 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] nitro-
soureas 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 nitrosoureas - 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-hydroxycyclohexyl)-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 isocyanate - 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

(1973a). Cyclohexyl isocyanate was also reacted with the enzyme at these concentrations.

Labeling of GSSG-reductase with [^{14}C] nitrosoureas - [Chx-1-^{14}C]CCNU (125 nmoles) or [chloroethyl-2-^{14}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}C] moiety/mole 55,000 MW monomer. The determination of this [^{14}C] labeling stoichiometry takes into consideration the symmetrical [^{14}C] label of the [chloroethyl-2-^{14}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 deter-
mined 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. Chloro-
zotocin, 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 inactiva-
tion. 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 titra-
tion 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 iso-
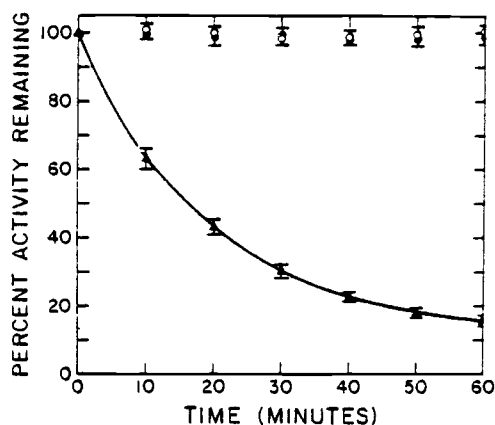cyanates. To confirm that the inactivation was due to

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Figure 7: BCNU inactivation of glutathione reductase. BCNU
(50 nmoles in anhydrous acetone) was added to solu-
tions containing 1.38 nmoles glutathione reductase
(5.48×10^{-6} M) in the presence of 1.58×10^{-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.

ENZYME CONCN (M)	2-CHLOROETHYL ISOCYANATE/ GLUTATHIONE REDUCTASE
4.5×10^{-6}	9.94
1.1×10^{-5}	5.52
2.8×10^{-5}	2.64
6.9×10^{-5}	1.15

Titration of glutathione reductase (4.5×10^{-6} M -
6.9×10^{-5} M in 0.1 M KPO_4 pH 7.6 with 0.2 M
KCl and 1 mM EDTA at 37°C) by the sequential addi-
tion 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 iso-
cyanates, [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 nitro-
soureas 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-chloro-
ethyl nitrosoureas which were found to inactivate the puri-
fied 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 iso-
cyanates were either added directly or generated in situ by
the degradation of BCNU and CCNU, respectively. These
results combined with the observed in vivo inactivation of
human erythrocyte GSSG-reductase by BCNU (Frischer and

TABLE IV [*chloroethyl*-2-^{14}C]BCNU AND [*chx*-1-^{14}C]CCNU LABELING OF GLUTATHIONE REDUCTASE

[^{14}C] NITROSOUREA	PERCENT OF TOTAL ENZYME			[^{14}C] MOIETY BOUND/MOL OF TOTAL ENZYME	[^{14}C] MOIETY BOUND/MOL OF NITROSOUREA INACTIVATED ENZYME
	Total Enzyme Inactivation	NADPH Inactivation	[^{14}C] Nitrosourea Inactivated Enzyme		
[*chloroethyl*-2-^{14}C] BCNU					
WITH NADPH	84	14	70	0.74	1.07
WITHOUT NADPH	0	–	0	0.19	–
[*chx*-1-^{14}C] CCNU					
WITH NADPH	95	12	83	1.16	1.41
WITHOUT NADPH	0	–	0	0.39	–

Table Ⅸ   Inactivation of Glutathione Reductase by Various Nitrosoureas

Nitrosourea	Concn (x 10^{-4} M)	t 1/2 (Min)	Percent inacti- vation at t 1/2	Carbamylating* Activity	Absolute Neutro- phile Count
CCNU	1.97	56	97	94[13]	25[13]
MeCCNU	1.97	58	96	91[16]	
BCNU	1.97	42	77	69[13]	25[13]
cis-2-OH-CCNU	9.72	48	3	1[18]	
trans-4-OH-CCNU	1.97	45	81	94[18]	
Chlorozotocin	9.72	22	0	4[13]	110[13]
GANU	9.72	7	2	63[13] .	113[13]
ACNU	9.72	24	30	3.5[12]	4[12]

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

* Percent of 2-chloroethylnitrosourea carbamylation of ^{14}C-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-reduc-
tase was compensated for by increased glutathione biosyn-
thesis. 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 carba-
mylation 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 demon-
strated that ACNU was capable of inactivating GSSG-reduc-
tase presumably via the isocyanate. Thus it seems unreason-
able to exclude the possibility of carbamylation by nitroso-
ureas solely on the basis of their low potential to chemi-
cally modify L-lysine. Clearly the structure of the iso-
cyanates' side chain determines the selectivity of the iso-
cyanate 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 iso-
cyanates as probes for the catalytic site of GSSG-
reductase.

V. PROTECTIVE ROLE OF THE GLUTATHIONE REDOX-CYCLE DURING OXIDATIVE CHALLENGE

Introduction

The antitumor benzanthraquinone, 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 un-checked 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 cyto-toxicity.

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 hemacyto-meter 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 1.8×10^6 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 indi-
cated, 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 pres-
ence of 0.5% triton for 30 sec and clarified by centrifuga-
tion. The supernatants of both were than assayed indi-
vidually 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. Hepato-
cytes were either pelleted (80 g for 3 min), washed once
with saline, resuspended in 0.5-1.0 ml saline, and soni-
cated 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 NaN_3 (to inhibit catalase), 5 mM GSH, 20 units yeast glutathione reductase. The reaction was initiated by the addition of H_2O_2 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 NaN_3 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 iso-
lated 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 oxida-
tive 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 impor-
tance 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
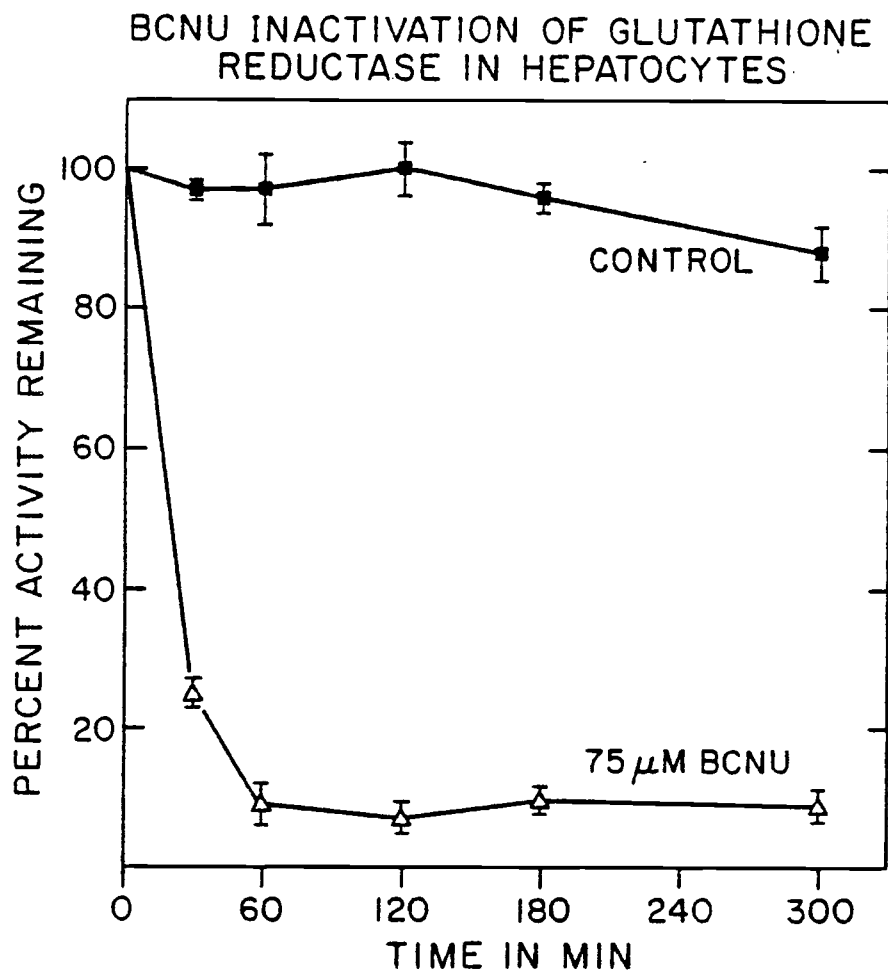
Figure 8. Hepatocyte suspensions (1.8×10^6 cells/ml) were treated either with 0.01 ml 95 mM BCNU in DMSO (Δ) or 0.01 ml DMSO ($\blacksquare$) and incubated as described in the Methods section. The final concentration of BCNU was 75 μM. Immediately prior to drug addition (0 min) and at intervals shown above, 1.0 ml aliquots were removed and glutathione reductase activity determined.
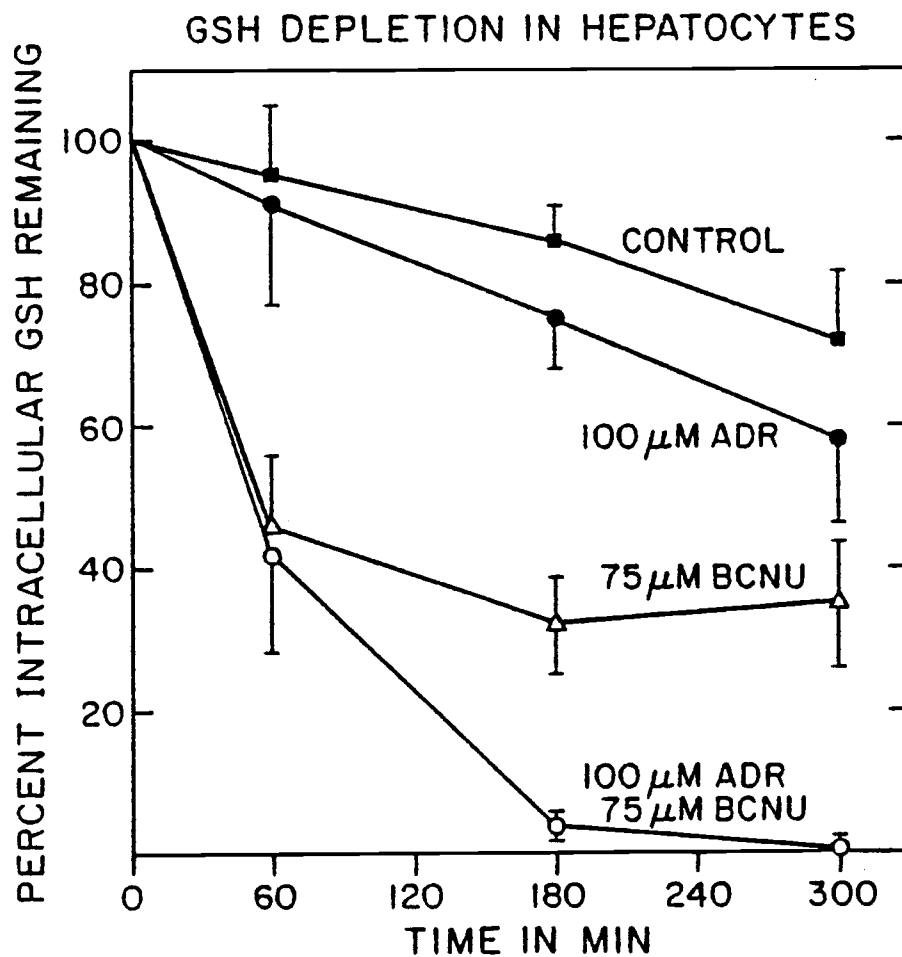
Figure 9. Hepatocytes (1.8 x 10^6 cells/ml) were incubated either with 100 µM ADR in the presence of 75 µM BCNU (0) and absence of BCNU (●) or with 75 µM BCNU in the absence of ADR (Δ). Controls contained 0.1% (v/v) DMSO and 2.0% (v/v) saline, but no ADR or BCNU (■). Aliquots (1.0 ml) were removed and 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-thiobarbi-
turic reactants. The increase was found to be dose depen-
dent with respect to ADR concentration. Neither BCNU or
ADR alone caused any measurable increase in lipid peroxi-
dation. Apparently either the ability of BCNU to inacti-
vate glutathione reductase, deplete intracellular GSH or
both could interfere with the ability of glutathione pero-
xidase to reduce effectively H_2O_2 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
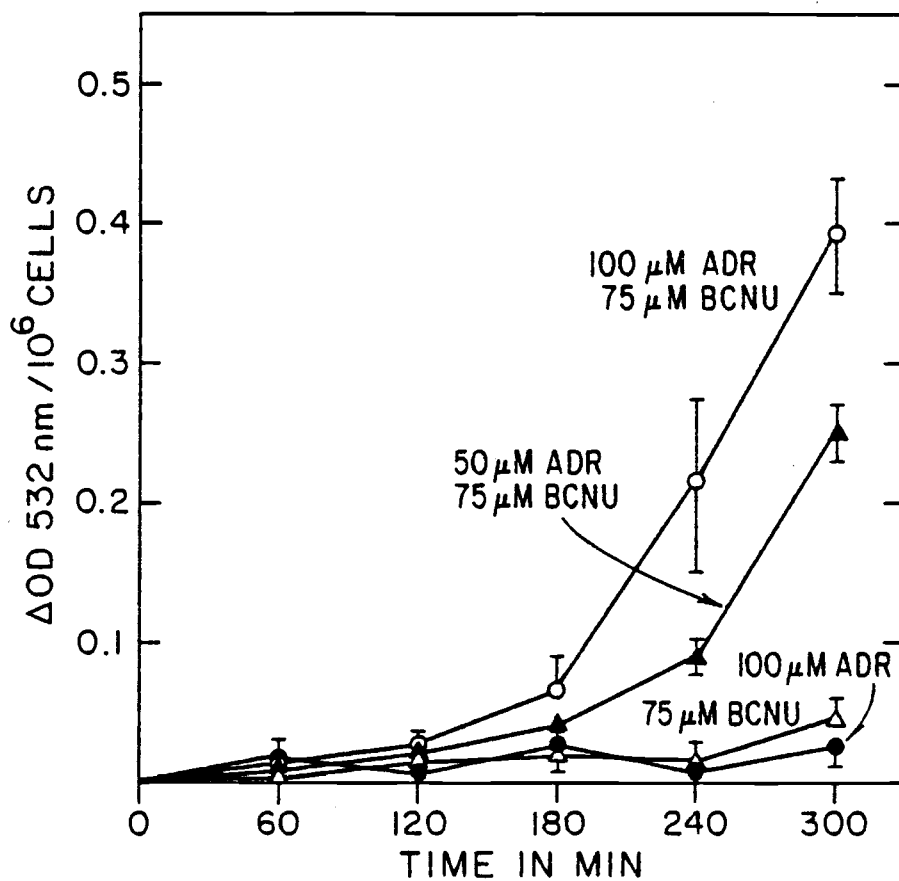
LIPID PEROXIDATION IN HEPATOCYTES

Figure 10. Hepatocytes were incubated either with 50 µM
ADR plus 75 µM BCNU (▲), 100 µM ADR in the presence of
75 µM BCNU (0) 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.
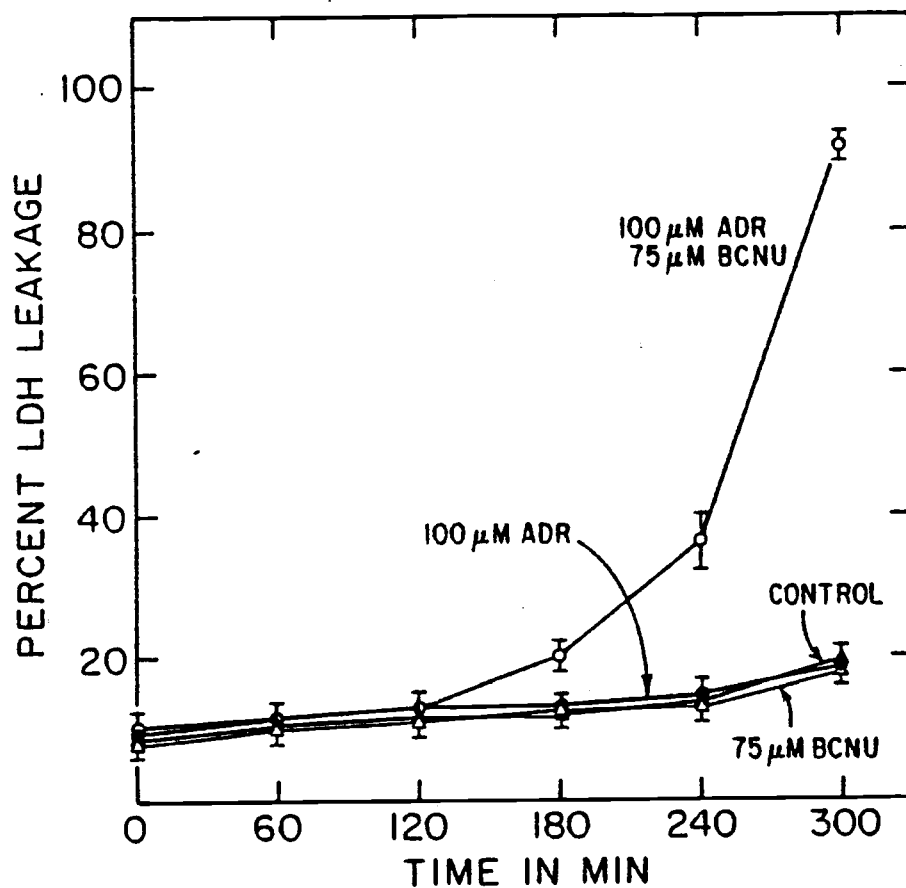
Figure 11. Hepatocytes were incubated either with 100 μM ADR in the presence of 75 μM BCNU (0) and absence of BCNU (●) or with 75 μM BCNU in the absence of ADR (Δ). Controls contained 0.1% (v/v) DMSO and 2.0% (v/v) saline (■). Cells were incubated and assayed for LDH leakage as described in the text.

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

Drug			Cell Viability[b]		Lipid Peroxidation[b]
ADR (μM)	BCNU (75 μM)	α-Tocopherol (75 μM)	% LDH[a] Leakage	% Trypan Blue Staining	ΔOD 532/10^6 Cells
Control	-	-	20 ± 2	22 ± 6	.01 ± .004
-	+	-	22 ± 4	21 ± 5	.039 ± .02
100	-	-	21 ± 4	22 ± 3	.03 ± .01
25	+	-	27 ± 4	29 ± 9	.085 ± .06
50	+	-	41 ± 8	48 ± 11	.179 ± .08
100	+	-	84 ± 12	90 ± 10	.499 ± .153
100	+	+	36 ± 11	42 ± 15	.135 ± .09

[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

Drug			% Intracellular GSH	% LDH Leakage	Lipid Peroxidation (ΔOD 532 nm/10^6 cells)
				Time in Min	
DEM (μM)	ADR (μM)	BCNU (μM)	180	300	300
Control			75 ± 3	20 ± 2	.010 ± 0.006
340	50	0	24 ± 6	19 ± 2	.010 ± 0.002
340	100	0	28 ± 3	18 ± 2	.040 ± 0.010
0	50	75	25 ± 1	47 ± 3	.140 ± 0.020
0	100	75	10 ± 4	85 ± 16	.538 ± 0.155
340	0	0	32	20 ± 1	.020 ± 0.006

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

	Enzyme Activity/10^6 Cells[a]			Percent Activity Remaining	
	zero-time	180 min. Control	ADR + BCNU[b]	180 min. Control	ADR − BCNU[b]
Glutathione reductase	.054 ± .010	.058 ± .010	.005 ± .001	109	9
Glutathione peroxidase	1.00 ± 0.10	1.07 ± .08	0.92 ± .08	107	92
Glutathione-S-transferase	648.6 ± 68.3	614.5 ± 82.0	539.0 ± 48.0	95	83
Catalase	24,556.0 ± 2039	20,416 ± 1726	21,052 ± 3926	83	86
Superoxide dismutase	22.8 ± 2.6	19.3 ± 4.3	18.8 ± 3.5	84	82

[a]Activities given in enzyme units as described in the test.

[b]Incubations 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 pre-
viously 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 gluta-
thione redox-cycle which also includes glutathione peroxi-
dases and GSH. The reductase reduces glutathione disul-
fide which is formed during the glutatione peroxidase
reduction of H_2O_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 mem-
brane damage and eventual cell lysis (Chance et al., 1979).
Catalase and superoxide dismutase comprise another line of
defense against the reactive oxygen species H_2O_2 and O_2
respectively. Sagone and Burton (1979) observed an in-
crease in the oxidation of [^{14}C]-1-glucose and [^{14}C]-
formate upon administration of ADR to BCNU-treated and
G6PD-deficient erythrocytes. Normal erythrocytes showed
increased [^{14}C]-1-glucose oxidation only after BCNU treat-
ment. The oxidation of [^{14}C]-1-glucose and [^{14}C]-formate
was taken as a measure of HMPS activity and H_2O_2 genera-
tion, respectively. They concluded that BCNU impaired the
ability of erythrocytes to reduce ADR-generated H_2O_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 H_2O_2 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 cardio-toxicity 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 chemo-therapy involving both ADR and BCNU.

In summary, we have shown that ADR act as an effective oxidative agent to hepatocytes in which glutathione reduc-tase has been inactivated by BCNU. BCNU-treated hepato-cytes 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 inacti-
vation 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 gluta-
tione 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 gluta-
thione 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 deter-
minant 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 pro-
tective 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 endo-
genous 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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