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Title: Biochemical Changes Associated with BCNU-Induced Pulmonary Toxicity



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Male Sprague Dawley rats received a single intraperitoneal dose of BCNU in experiments designed to measure biochemical changes associated with the pulmonary toxicity described for this agent. Changes in serum and lung lavage angiotensin converting enzyme (ACE) activity were measured to detect changes in pulmonary endothelial cell integrity. Alpha-1-proteinase inhibitor (PI) concentration and activity was monitored as well as changes in lavage elastase activity. Lung malondialdehyde and hydroxyproline concentrations were followed to detect peroxidative damage and collagen deposition. Lung catalase, glutathione concentration and glutathione shuttle enzyme activity was also monitored. Biochemical changes in response to BCNU was also followed in Fischer 344 rats.

Serum ACE activity was elevated 7 to 21 days postdosing. Significiant inhibition of lavage ACE activity was evident at 1 and 3 days. No significant increases in lavage ACE activity were detected. Lavage PI concentration increased 484% by 21 days, and lavage PI activity was decreased at 1 and 3 days. Serum PI activity decreased 80% by 28 days

postdosing. Increases in lavage PI concentration paralleled increases in lavage elastase activity. Lung malondialdehyde concentration increased in a biphasic manner and lung collagen content increased in a time dependent manner. Lung catalase activity was elevated at 3 days and depressed from 7 to 21 days. Glutathione peroxidase activity was elevated at 7 days and glutathione reductase activity was significantly decreased at 1 and 3 days. Oxidized glutathione concentration in the lungs of treated animals was increased at 72 hours. Treated Fischer 344 rats showed no changes in lavage elastase activity or serum PI concentration and activity.

The pulmonary biochemical changes induced by BCNU treatment indicate a biphasic response pattern. The early response is probably the direct result of BCNU while the delayed effects are consistant with the possible involvement of an inflammatory component in the development of BCNU-induced pulmonary fibrosis. The Sprague Dawley rats appear to be more sensitive than the Fischer 344 rats to the toxic effects of BCNU.

# Biochemical Changes Associated with BCNU-Induced Pulmonary Toxicity

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Eric J. Jarvi

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Dean, College of Pharmacy

Redacted for Privacy

Dean of Graduate School

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If you have a goal in life
that takes a lot of energy
that incurs a great deal of interest
and that is a challenge to you,
you will always look forward
to waking up to see what the new day brings.

If you find a person in your
life that understands you completely
that shares your ideas
and that believes in everything you do,
you will always look forward to the night
because you will never be lonely.

-Susan Polis Schultz

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## Chapter I. General Introduction

### A. Pathophysiology of Pulmonary Fibrosis

Any attempt to understand the fibrotic disease process requires some understanding of the pathogenesis of the disease. Fibrosis is generally felt to be a sequela to cellular injury (Fuller, 1985) and is characterized by an overaccumulation of a collagen network in the extracellular matrix. The normal extracellular matrix is composed of elastin, collagen and proteoglycans. The structure and function of any individual organ is based on the relative ratios and heterogeneity of these three components. An overaccumulation of collagen or a change in collagen distributuion changes the matrix structure in such a manner as to alter architecture and eventually impair organ function. The anatomical deposition of this collagen network occurs in three forms: (1) diffuse lesions as in interstitial fibrosis, (2) localized nodules as in silicosis, and (3) dense circumscribed areas as in pulmonary infarcts (Witschi et al., 1985).

Structurally a collagen molecule (tropocollagen) is a triple helix with a molecular weight of about 300,000 measuring 280 x 1.4 nanometers and displaying an axial periodicity of 64 nanometers. A nonhelical region (telopeptides) exists at the end of each chain which is important in cross linking and in the immunological specificity exhibited by each of the five types of collagen (Diegelmann and Lindblad, 1985). Collagen, the major structural protein responsible

for tensil strength in vertebrates, comprises 10-15% of total dry lung weight (Last, 1985). By contrast, normal mammalian dry liver contains only 1% collagen by weight. Determination of amino acid composition of each chain has revealed that every third residue is glycine. Proline and hydroxyproline constitutes 25% of each chain and hydroxylysine makes up 0.5%. Hydroxyproline and hydroxylysine are virtually specific for collagen. Hydroxyproline acts to stabilize the molecule and collagen actually degrades rapidly in vivo in its absence.

The biosynthetic pathway responsible for collagen synthesis is probably the same in all cells (Last, 1985) but has been best studied in fibroblasts. The intracellular events are initiated by the ribosomal synthesis of procollagen precursors. Next, hydroxylation of specific proline and lysine residues is performed by prolyl and lysyl hydroxylase, respectively. These enzymes are mixed function oxidases and require molecular oxygen, ferrous iron, ascorbic acid and alpha-ketoglutarate as cofactors. The third step involves glycosylation of specific hydroxylysine residues by the manganese-dependent galactosyl- or glucosyltransferase enzymes. Three precursor chains then roughly assemble and are extruded from the cell for extracellular processing to form tropocollagen. Procollagen peptidase cleaves the N- and C-terminal peptides from each chain paring the molecule down to its final length of 280 nanometers. The tropocollagen molecules then align end-to-end and side-to-side but staggered by 25% resulting in the classic 64 nanometer periodicity. Lysyl oxidase (cofactors; copper and pyridoxal phosphate) then cross links the separate tropocollagen molecules giving the fibril its tensil strength.

The fibrotic response to cellular injury is composed of three well defined phases. Initially there is cellular injury followed by an acute inflammatory reaction at the injury site. The third phase of the response is the repair process. The mediators of the inflammatory reaction either act as chemotactic factors (i.e., serum derived chemotactic factor, platlet derived growth factor, lymphocyte derived chemotactic factor, fibronectin and peptides derived from proteolytic digestion of structural proteins) or to increase vascular permeability (i.e., histamine, bradykinin, prostaglandin-E and slow reacting substance of anaphylaxis). Inflammation is characterized by an increase in neutrophils, eosinophils and mononuclear cells at varying rates and amounts, recruited in response to some of the chemical mediators. The repair phase is generally initiated by the recruitment of fibroblasts to the injury site. This is a limited recruitment step because fibroblasts are not found in general circulation but instead must be recruited from adjacent tissue sites. The cell population responsible for fibroplasia must proliferate and grow during the repair phase. Again, fibroblast proliferation is mediated by several known factors: platelet derived growth factor, a 60,000 dalton protein derived from T-lymphocytes, interleukin-1 and a 18,000 dalton factor derived from macrophages. Fibroblast collagen synthesis is then governed by two different sets of mediators comprising stimulator and inhibitor groups listed in Table 1.

Early provisional repair is intended to rapidly restore tissue continuity and provide a mechanical means for wound closure. It is at this point that the normal sequence of events can go awry leading to fibrosis. Under normal circumstances provisional repair is followed

Table 1: Effectors of Fibroblast Collagen Production*

| Stimulators | Inhibitors |
|---|---|
| ascorbic acid | interleukin 1 |
| T-lymphocyte factor | cAMP, beta-adrenergic agonists |
| fibroblast factor | glucocorticoids |
| collagen inducing factor | viral transformation |
| fibrogenic factor | amino terminal peptides |
| epidermal growth factor | alveolar macrophage factor |

*Source: Seyer, 1985

by a slower process of tissue involution, maturation and remodeling. This may lead to a complete resorption of the tissue or to a reorganization and regeneration of the tissue. Any failure to remodel the early components of provisional repair, a failure to regenerate the elastin portion of the extracellular matrix, or an inability to restore an epithelial cell layer following injury will result in disruption of the normal fibroblastic process. Instead, the provisional repair process continues which results in scarification. In its most severe forms, scarification is seen as adhesions, strictures, fibrosis, cirrhosis or sclerosis. It is now thought that a process such as fibrosis is characterized by increased amounts of interstitial collagen but may also result from a change in the composition of the collagen normally present.

Although the fibroblast is the major cell responsible for collagen synthesis, it is now known that a number of other cells can also synthesize collagen (Diegelmann and Lindblad, 1985). Indeed, cells from all three embryonic germ layers possess the necessary genetic code for synthesizing collagen. The differentiating feature appears to be that any one cell type is only capable of synthesizing a specific subset of the five immunologically recognizable forms of collagen. Collagen forms expressed by various cell types include: fibroblast (I, III, V), chondroblast (II), smooth muscle (I, III, IV, V), liver parenchyma (I, III, IV, V), granular pneumocytes (IV), epidermis (IV, V) and lens capsule (IV). The liver and the parenchyma of the lung, however, appear to be the most sensitive to the disruption of the repair pathway leading to fibrosis.

The manifestations of the fibrotic process vary according to the affected organ. In the case of the lung, the proper performance of blood oxygenation requires a rhythmic expansion of the lung tissue to ventilate the alveoli and requires that alveolar oxygen be able to readily diffuse into the blood. The deposition of interstitial collagen thickens the alveolar wall altering ventilation and perfusion parameters in the lung. The lungs are stiffer, requiring more work to perform the task of ventilation. Oxygen must diffuse over a greater distance from alveolus to capillary blood. Physiologically the results are decreased compliance and impaired diffusion capacity. Deposition of collagen may not be confined to the interstitium but may involve epithelial and endothelial cells. Airways, arteries and veins are also involved in some forms of fibrosis. Continual disruption of alveolar structure leads to "end stage lung"; the loss of functional gas exchange units (Crystal et al., 1981). In an attempt to compensate for the continual loss of these exchange units, right side heart hypertrophy results from attempts to maintain cardiac output. Death is usually secondary to respiratory insufficiency or anoxic effects on the heart or brain.

Typical symptomatic indications of pulmonary toxicity, especially fibrosis, are manifested by dyspnea; a dry, hacking nonproductive cough; or acute respiratory distress. Pulmonary function findings are consistent with a restrictive ventilatory defect and include: decreased lung volume, diminished lung diffusing capacity, normal forced expiratory volume in one second/forced expiratory volume, mild resting arterial hypoxemia, mild hyperventilation and compensated respiratory

alkalosis. Radiographic studies show decreased lung volume and a diffuse reticulonodular infiltrate.

Pharmacological intervention, in an attempt to prevent fibrosis, has generally not been successful. Corticosteroid therapy has met with limited success in slowing the progression of idiopathic pulmonary fibrosis and occasionally even reverses the disease process (Crystal et al., 1981). The therapy used most often has been the oral administration of prednisone. Steroid intervention is most successful in the early stages of the disease when alveolitis is present (a chronic accumulation of inflammatory and immune effector cells within the alveolar structure) and before there is any derangement of the alveolar-capillary exchange unit. Alveolitis, however, is not generally associated with BCNU-induced pulmonary fibrosis. Nevertheless, steroidal and nonsteroidal anti-inflammatory agents have been used in an attempt to prevent pulmonary fibrosis (Last, 1985), especially in animal models. Methylprednisolone decreased the lung collagen synthesis rate in ozone and bleomycin induced fibrosis. Triamcinolone prevented increased lung collagen content in rats treated intratracheally with bleomycin. Indomethacin has also been shown to prevent bleomycin-induced increases in lung hydroxyproline concentrations in rats (Last, 1985). Penicillamine and colchicine have also shown antifibrotic activity in ozone exposed rats (Last, 1985).

It appears that the timing is critical in the administration of steroids because some studies have found they can improve or exacerbate fibrosis. It is generally believed the time dependent effects of steroid therapy are the result of the existing cell

population in the lung at the time of steroid treatment. Practical therapeutic measures have generally been more successful in animal models of fibrosis than in actual human cases.

Historically, the assessment of lung toxicity has relied on pulmonary function measurements and the histological examination of diseased tissue (Witschi, 1975). With the exception of lipid metabolism, little work had been done in the area of pulmonary biochemistry before 1975. Defining the mechanisms involved in pulmonary toxicity, and in particular pulmonary fibrosis, have not been easy because of the complexity of the lung system. Lung tissue is composed of over 40 different cell types and a number of biochemical and histologically demonstrable enzymes. The water content of lung is 80%, which is quite high compared with other tissues. Lung cells also have less cytoplasm, as reflected by the fact that lung has more DNA per unit weight but less RNA than other tissues. The lungs are not simply organs of passive gas exchange but contain active enzyme systems capable of metabolizing a number of different xenobiotics. The lungs are unique in their exposure to both inhaled and systemic toxicants and unique in their position for receiving the entire cardiac output. The lungs are also known to actively take up and concentrate various endogenous and exogenous substances (Boyd, 1980).

Because of this complexity any biochemical measurement only indicates an overall change occurring in some but not all cell types. Conversely, certain metabolic pathways are associated with specific cell populations and may tell us nothing about toxic effects on other cell types. Therefore, measuring changes in any single biochemical parameter is not specific and uniequivocal proof of lung toxicity.

B. Agents Known to Induce Pulmonary Fibrosis

It is of particular interest that a wide variety of agents by different routes of exposure and putatively working through different mechanisms of action, can all lead to the development of fibrosis. The list of chemicals reputed to cause pulmonary fibrosis contains both drug related and environmental agents including: BCNU (Aronin et al., 1980), bleomycin (Adamson, 1976), busulfan (Leake et al., 1963), cadmium chloride (Niewoehner and Hoidal, 1982), methotrexate and cyclophosphamide (Sostman et al., 1977), hexamethonium (Heard, 1962), pentolinium (Hildeen et al., 1958), paraquat (Schoenberger et al., 1984), ozone (Menzel, 1970), nitrogen dioxide (Ichinose and Sagai, 1982), oxygen (Chvapil and Peng, 1975), and a variety of inorganic and organic dusts (Crystal et al., 1981). The mechanism of pulmonary toxicity has been investigated for a few of these agents: bleomycin, paraquat and oxygen in particular. Despite the extensive studies using these three agents, there is still uncertainty over a definitive mechanism for each compound. An understanding of the speculated mechanisms is useful background for studies on BCNU-induced pulmonary toxicity.

Bleomycin induced fibrosis is perhaps the most widely studied because of the high incidence, rapidity and reproducibility of the intratracheal (i.t.) administration model for the disease (Newman et al., 1980). Fibrosis also has been demonstrated in an intraperitoneal (i.p.) dosing model (Tom and Montgomery, 1980). Bleomycin, in the presence of $Fe^{2+}$ and $O_2$, is capable of generating free radicals ultimately resulting in DNA strand scission (Phan et al., 1983). Bleomycin first chelates with a ferrous ion ($Fe^{2+}$). The bithiazole group

of bleomycin intercalates with DNA bringing the complexed $Fe^{2+}$ in close proximity to the deoxyribose moiety. This leads to an oxidation of $Fe^{2+}$ to $Fe^{3+}$ and the oxidized $Fe^{3+}$-bleomycin complex dissociates from DNA. A subsequent reduction of $Fe^{3+}$ permits a recycling of the $Fe^{2+}$-bleomycin complex to interact with DNA again. Each oxidation cycle through DNA can result in a hydrogen extraction at C4 of the deoxyribose molecule leading to strand scission and the release of a thymine residue. This mechanism coupled with low bleomycin hydrolase activity in the lung (Adamson, 1976; Lazo and Humphreys, 1983; Lazo et al., 1984) is probably responsible for the high incidence of pulmonary toxicity. Bleomycin hydrolase normally inactivates bleomycin by hydrolyzing the amide group of beta-aminoanaline amide in the bleomycin core.

Paraquat toxicity appears to result from an entirely different set of circumstances. Paraquat actively accumulates in type II epithelial cells by a high affinity, energy dependent, sodium independent, saturable transport system (Bend et al., 1985). It is proposed that paraquat is reduced in the presence of NADPH (Bus et al., 1976). Oxidation of the reduced paraquat utilizes molecular oxygen yielding oxidized paraquat and superoxide radicals. Paraquat is then recycled for a subsequent reduction. Superoxide radicals nonenzymatically degrade to singlet oxygen capable of reacting with unsaturated lipids to form lipid hydroperoxides. Lipid hydroperoxides, in the presence of transition metal ions, decompose to lipid free radicals responsible for initiating the chain reaction process of lipid peroxidation. The entire lipid peroxidation process results in labilization of biomembranes with obvious adverse effects on the cell.

Oxygen-induced lung toxicity offers yet another mechanism leading to pulmonary fibrosis (Chvapil and Peng, 1975). Chronic exposure to either low or high oxygen concentrations results in significant increases in total pulmonary collagen content. Chvapil and Peng (1975) exposed male Sprague Dawley rats to 9 or 80% concentrations of oxygen for 41 or 84 days, respectively. Both oxygen concentrations produced similar biochemcial changes in lung tissue including: increased hydroxyproline content, increased lung lactate, increased total lactate dehydrogenase activity, increased prolyl hydroxylase activity and enhanced lipid peroxidation (determined by measuring malondialdehyde content and fatty acid profile changes). They conclude that fibrotic change is the result of nonspecific inflammatory reactions in the lung.

However, the mechanisms for hypoxic and hyperoxic lung toxicity appear to differ. Anoxia, possibly through increased levels of lactic acid, enhances the pool of active prolyl hydroxylase. Hypoxia is known to be a specific stimulus for fibroplasia resulting in enhanced fibroblastic activity. Hyperoxia is known to induce lipid peroxidative changes in the lung tissue leading to lung fibrosis.

## C. Carmustine and Pulmonary Fibrosis

Over one hundred nitrosoureas have been synthesized as potential anticancer agents. About 30% of the compounds that were found active against i.p. L1210 leukemia were further evaluated against intracerebral (i.c.) L1210 leukemia in mice. The N-nitrosoureas were found to be the most active of a number of N-nitroso compounds. The nitroso-

ureas most active against i.c. L1210 leukemia were BCNU, CCNU and MeCCNU. 1,3 bis(2-chloroethyl)-1-nitrosourea (also known as BCNU or carmustine) was judged to be the most active based on its significant and reproducible activity against i.c. L1210 leukemia. Experimentally, this was the first indication of the potential usefulness of this compound in effectively crossing the blood brain barrier.

Carmustine, a bifunctional alkylating agent, is the most common nitrosourea used in the treatment of malignant gliomas, lymphomas, gastrointestinal malignancies, melanomas, and myelomas (Bailey et al., 1978; Aronin et al., 1980). It is especially useful in the treatment of central nervous system tumors because of its high lipid solubility which not only facilitates transport across the blood brain barrier, but permits ready diffusion across cell membranes. Although the uptake of BCNU is by passive diffusion, studies of uptake using L5178Y lymphoblasts showed that the intact drug and its decomposition products are actually transported quite differently (Begleiter et al., 1977).

Carmustine decomposes relatively rapidly, especially when exposed to varying conditions of pH, temperature and media (Laskar and Ayres, 1977; Weinkam and Lin, 1979). The chloroethyl nitrosoureas, of which BCNU is the most widely studied, are relatively stable at pH 4-5 ($t_{1/2}$ approx. 500 minutes) but decompose spontaneously at neutral pH in both aqueous solution and in cell culture medium ($t_{1/2}$ approx. 8 minutes at pH 8.0). These agents, are converted to biologically active intermediates although biological activity of the parent compound cannot be ruled out. The chemical half-life of BCNU in vivo is less than five minutes in plasma while the biological half-life has been estimated at

between 15 and 30 minutes (Oliverio, 1976). The primary route of elimination for BCNU and its metabolites is through the kidney. Biliary excretion and enterohepatic cycling are also important. Fecal excretion accounts for less than 5% and expiration less than 10% of an administered dose (Oliverio, 1976).

On the basis of structure-activity work, it is thought that the therapeutic effects act via an alkylating intermediate while the toxic effects result from a carbamylation species (Bono, 1976). Studies of the breakdown products of BCNU in aqueous solution have shown the generation of a number of products by two competing pathways: (1) a cleavage pathway to 2-chloroethyl azohydroxide and 2-chloroethyl isocyanate, or (2) a denitrosation and cyclization to 2-[(2-chloroethyl)amino]-2-oxazoline (Montgomery, 1976; Weinkam and Lin, 1979). Only 45% of the breakdown products of BCNU have been positively identified. The unidentified products are primarily nonvolatile compounds. The volatile reaction byproducts from the 1-(2-chloroethyl) moiety of BCNU are 2-chloroethanol, acetaldehyde, vinyl chloride and 1,2 dichloroethane, the first two being the major breakdown products. Chloroethylamine and 1,3 bis(2-chloroethyl)urea (BCU) have also been identified. Acetaldehyde, vinyl chloride and 2-chloroethanol are also derived from 2-chloroethyl azohydroxide.

Carmustine has also been shown to be a substrate for the microsomal enzymes of mouse liver and to a lesser extent mouse lung (Hill *et al.*, 1975). The $K_m$ using liver microsomes was 1.7 mM with a $V_{max}$ of 417 nmoles product$\cdot min^{-1} \cdot mg^{-1}$ liver for BCNU. The metabolic product was identified as BCU. It is not clear whether the responsible enzyme is linked to cytochrome P-450, but it is known that metabolism

requires NADPH, has a possible requirement for oxygen, and is inhibited by nicotine ($K_i$ = 0.6 mM). Definitive mass spectral fragments of BCU include 2-chloroethylamine.

Studies on the breakdown products may be very important in understanding BCNU toxicity because all of the major products of the cleavage pathway (2-chloroethanol, acetaldehyde, 2-oxazolidone and 2-chloroethylamine) are toxic substances (Weinkam and Lin, 1979). The toxicity of the primary product of denitrosation and cyclization is not well known but other substituted 2-aminooxazolines are highly toxic.

The therapeutic effects are believed to be mediated by a chloroethylcarbonium ion ($ClCH_2CH_2^+$), a powerful alkylating moiety that results in interstrand cross linking in DNA, although RNA and proteins can apparently be alkylated to about the same extent. Such alkylating species are capable of attacking any number of nucleophilic sites within a cell such as N or O atoms, the phosphate backbone of DNA, or a thiol group of proteins. In DNA, cross linking results from a secondary interaction by a nucleophilic site on an adjacent strand displacing the $Cl^-$ ion leading to interstrand cross linking. The ability to alkylate a number of cellular components, however, cannot be ruled out as being the lethal event.

In vivo evidence indicates the therapeutic action of the nitrosoureas results from their ability to act more effectively against proliferating than nonproliferating cells. It is well established that BCNU stops cell cycle progression in $G_2$ phase. The $G_1$ to S phase progression proceeds normally, S to $G_2$ phase proceeds at a reduced rate, and progression through $G_2$ is arrested. It appears then that

DNA effects are subtle enough to permit the completion of DNA synthesis, but not its functioning in cellular events involved in the $G_2$ phase. Cells could be sensitive to alkylation during $G_1$/S because they have less chance of repairing damage before the next phase of synthesis is initiated. This specificity for arresting cell progression in the $G_2$ phase is substantiated by the evidence that all the nitrosoureas tested with exponentially growing Chinese Hamster Ovarian cells arrested progression in the latter third of the 186 minute $G_2$ phase. All of the above evidence for a therapeutic mechanism must be tempered by the finding that *in vitro*, the nitrosoureas were more active against nonproliferating cells (Bono, 1976). The discrepancy, however, apparently lies in different kinetics associated with each system.

The toxic effects are thought to be mediated through a carbamylating isocyanate product that presumably inhibits the functioning of various repair enzymes (Meyn and Murray, 1984). The isocyanate moiety (R-N=C=O) reacts with electron rich primary amino groups, especially on proteins, yielding stable, inactive urea derivatives. Additionally, any product capable of attacking a nucleophilic site can deplete cellular GSH levels, especially when catalyzed by GSH-S-transferase which has been demonstrated for BCNU (Meyn and Murray, 1984). Such reagents are reportedly capable of inducing lipid peroxidation (Younes and Siegers, 1981).

The first major clinical trials using BCNU were reported in 1965 (DeVita *et al.*, 1965). In this study, 144 patients with various malignancies were treated with BCNU at several dose levels and schedules. On the basis of these trials, 3 intravenous (i.v.) dose

schedules were found to be comparable in toxicity: (1) a single dose of 250 mg/m², (2) 125 mg sq•$m^{-1}$•$day^{-1}$ for three days, or (3) 90 mg sq •$m^{-1}$•$week^{-1}$ for six weeks. A delayed and prolonged hematopoietic toxicity was reported by DeVita et al., (1965) along with minor but reversible hepatic and renal toxicity. The hematopoietic toxicity was the factor that appeared to be dose limiting. This toxicity occurred 3 to 4 weeks after drug administration and was independent of the drug dosage or schedule. The depression was characterized by thrombocytopenia and neutropenia of 2 to 3 weeks duration. Acute toxicity reported with the intravenous administration of BCNU involved intense flushing of the skin and suffusion of the conjunctiva. Nausea and vomiting appeared within 2 hours. Burning sensations at the site of infusion were common. All of the delayed toxicities have also been reported in studies using dogs and monkeys given a daily oral (p.o.) or i.v. administration of BCNU. Reports from other clinical studies (Wasserman, 1976) indicated that gastrointestinal and bone marrow toxicity could be expected in all patients treated with BCNU. Other less frequent toxicities reported included: stomatitis, alopecia, mild anemia, anorexia and possible neuro-opthalmologic effects. The possibility of pulmonary toxicity was mentioned. Reports of ocular and central nervous system toxicity associated with BCNU therapy have appeared in the literature (Schold and Fay, 1980; Greenberg et al., 1981). Extensive studies describing various hepatic effects associated with BCNU administration have been reported (Thompson and Larson, 1969; Lu and Larson, 1972; Wilson and Larson, 1981; Hoyt, 1984; Stolzenbach, 1984).

The increased use of BCNU resulted in a number of reports in the literature of a delayed, irreversible, apparently lethal pulmonary toxicity (Holoye et al., 1976; Crittenden, 1977; Bailey et al., 1978; Bellot and Valdiserri, 1979; Durant, 1979; Weiss et al., 1979; Lieberman, 1980; Melato, 1980; Litam, 1981; Ryan, 1981). The pulmonary toxicity manifested itself as a diffuse interstitial pulmonary fibrosis. The patchy distribution of fibrosis was most marked in the lower lobes. In general histologic findings were consistent with an iatrogenic, drug-induced lung toxicity, alveolar cell dysplasia, interstitial pneumonia and interstitial fibrosis. Tissue preparations from various lobes of both lungs showed the interstitum contained abundant fibrous connective tissue most prominent within the alveolar septa. The fibrosis was patchy, more severe in the lower lobes and occasionally involved bronchioles and small muscular pulmonary arteries. Hyperplasia of the type II alveolar epithelial cells was noted as was a broadening of the visceral pleura resulting from extensive proliferation of the fibrous connective tissue. In most cases there was no consistent evidence of an inflammatory reaction.

This rash of reports involving pulmonary toxicity associated with BCNU was somewhat surprising. Although preclinical studies in animals had produced pulmonary toxicity, it was more of an acute effect manifested as pleural effusion and pulmonary edema (Holoye et al., 1976). There was no postmortem evidence of fibrosis. Though no chronic animal toxicity studies were performed, the available animal data did not predict the human pulmonary toxicity being so widely reported (Weiss et al., 1979).

The prevalence of this problem in 1980 led researchers at the Division of Neurological Surgery at the University of North Carolina to undertake an extensive study in an attempt to predict the onset of pulmonary toxicity during the course of BCNU therapy (Aronin et al., 1980). Before this study, the incidence rate for pulmonary toxicity associated with BCNU had been estimated at from 1.3 (Durant, 1979) to 15% (Aronin et al., 1980). In the University of North Carolina study, 93 patients with malignant gliomas were treated with BCNU under various treatment dosing protocols. Whole brain radiation therapy was also administered as was a random assignment to levamisole immune stimulation. Twenty percent of the patients showed signs of symptomatic pulmonary disease. Multivariate analysis of all the contributing factors showed a positive correlation between pulmonary toxicity and the following: total cumulative BCNU dose, number of cycles over which BCNU was administered, preexisting history of lung disease, the platlet count nadir after the first course of BCNU and the patient's age. The equation shown below, derived from this multivariate analysis, is 80% accurate in predicting pulmonary toxicity during the course of BCNU therapy. A more striking result of the study is that the incidence rate of pulmonary fibrosis can approach 50% at total cumulative BCNU doses of 1500 $mg/m^2$.

Aronin's Predictive Equation*

$$L = -2.95903 + 0.00355(B) - 0.49286(NT) + 1.68236(LD) + 0.00285(PN) - 0.00487(A)$$ where,

L = likelihood that pulmonary toxicity will develop,

B = total cumulative dose BCNU ($mg/m^2$),

NT = number of treatment cycles,

LD = history of lung disease (1 = no, 2 = yes),

PN = platelet count nadir in thousands during first treatment cycle, and

A = age in years at diagnosis.

*Aronin et al. (1980).

From the oncologist's point of view then, good pretreatment analysis of the individual followed by careful monitoring of hematopoietic indices after BCNU treatment is initiated should help prevent most of the serious cases involving pulmonary toxicity. However, the mechanism of BCNU-induced pulmonary fibrosis remains to be elucidated.

The approach of this thesis research is to define some biochemical changes associated with the acute administration of BCNU. The parameters chosen were based on biochemical changes measured using other known fibrotic agents as well as a knowledge of the well described hepatic toxicity associated with BCNU (Hoyt, 1984; Stolzenbach, 1984). The first set of experiments involved measuring the loss of endothelial integrity, an early event preceding fibrosis, but well defined histologically. The endothelial lining of the pulmonary capillary bed is a logical site for exposure to systemically administered

BCNU. It may be possible to identify an early biochemical event that precedes fibrosis by measuring changes in specific enzyme activity primarily associated with these endothelial cells.

The loss of endothelial integrity at best could only be an initiating event and even the appearance of endothelial lesions is preceded by cytoplasmic and subendothelial edema. The second set of experiments were designed to measure the content and activity of the primary protective protein in the lung. This approach has not been taken with other fibrotic agents but has some merit based on the known hepatic toxicity associated with BCNU. Fibrotic agents might directly inhibit this protective mechanism in the lung or may act via liver toxicity to decrease the synthesis of a functional proteinase inhibitor responsible for protecting the lung from a variety of proteolytic enzymes.

Elastase, a specific proteolytic enzyme released by polymorphonuclear cells, was assayed to confirm whether changes in proteinase inhibitor activity were reflected by increased pulmonary proteolytic activity. Malondialdehyde concentration was measured to determine a time course for membrane damage, and collagen content was followed over the same time course to try to determine exactly when, chronologically, the onset of fibrosis occurred.

An oxygen radical mechanism has been described for bleomycin and paraquat (Bus et al., 1976; Phan et al., 1983) toxicity and on the basis of the malondialdehyde concentration results, studies were conducted to measure oxidized and reduced glutathione levels in the lung as well as the enzymes associated with the glutathione shuttle system. This system would be expected to protect against membrane damage from lipid peroxidation. Catalase activity was also measured

because it is involved in an alternate pathway for the inactivation of oxygen radicals.

Lastly, selected biochemical parameters that were felt to be significant in events leading to the development of fibrosis, were evaluated in a second experimental model. These data were crucial in showing strain variation that helped to explain differences in the single i.p. dosing model using the Sprague Dawley rat versus the multiple i.p. dosing model with the Fischer 344 strain used by other investigators (Smith and Boyd, 1983; Smith and Boyd, 1984; Reznik-Schuller et al., 1984).

# Chapter II. The Effects of Fibrotic Agents on Rat Angiotensin Converting Enzyme Activity

## A. Introduction

A variety of agents are known to induce pulmonary fibrosis whether by inhalation or systemic routes of exposure. For both routes, the initial site of injury often appears to be the endothelial lining of pulmonary blood vessels. Histologically, the loss of endothelial integrity has been well described for bleomycin toxicity (Adamson, 1976). Loss of endothelial integrity is preceded by subendothelial edema resulting from increased movement of serum derived fluids through the endothelial barrier. Edema is the result of increased permeability of the vascular endothelial lining in response to chemical mediators.

Pulmonary endothelial cells are rather active metabolically (Ryan and Ryan, 1977). These cells are responsible for selectively removing the biogenic amines 5-hydroxytryptamine, norepinephrine and beta-phenylethylamine (Bend et al., 1985) from the circulation. They form the prostaglandins $PGE_2$ and $PGI_2$, possess alpha-2-macroglobulin, plasminogen activator and factor VIII antigen hemostatic factor (Ryan and Ryan, 1980).

There exists a rather unique enzyme marker associated with the endothelial lining of the pulmonary vasculature, angiotensin converting enzyme (ACE). This enzyme is associated with the endothelial cells of virtually all vascular beds (Ryan and Ryan, 1977) but the enzyme is particularly abundant on the luminal surface of the

pulmonary endothelial cells (Ryan et al., 1975). Here the enzyme is responsible for the bulk conversion of angiotensin I to angiotensin II as well as inactivating bradykinin and perhaps other oligopeptides such as neurotensin and enkephalins (Ryan and Ryan, 1977). It was demonstrated by Ryan et al. (1977) that the endothelial cells were involved in the specific synthesis of ACE and were not merely a receptor site for circulating angiotensin converting enzyme.

Various investigators have proposed measuring ACE activity in pulmonary lavage fluid as a measure of lung toxicity (Kim and Roberts, 1980; Newman, et al, 1980; Tom and Montgomery, 1980; Newman et al., 1981; Smith and Boyd, 1983) because of the specific association of ACE with the pulmonary endothelial cells. The rationale behind the assay is that any cytotoxic effects on the endothelial cells should cause measurable increases in alveolar lavage ACE levels as the enzyme is carried across the barrier passively with fluid movement. It may also be possible to detect increases in serum ACE levels associated with release of ACE into the general circulation as well as changes in total lung tissue ACE levels.

A rational approach to estimation of ACE activity would be to measure variations in enzyme activity by measuring differences in pulmonary arteriovenous gradients of ACE substrates and products such as angiotensin I, angiotensin II or bradykinin. Bioassays based on a differential sensitivity to angiotensin II using the rat aorta strip, rat uterus, or rat colon have been described (Vane, 1964). Angiotensin I, angiotensin II, and bradykinin occur in the blood at concentrations less than $10^{-10}$M and the bioassay suffered some sensitivity problems at these concentrations and was only an indirect measure of

ACE activity. Radioimmunoassays, with sensitivities down to 30 picograms for angiotensin II, have been used but suffered precision and specificity problems because the antibodies cross reacted with bradykinin. Most investigators studying ACE activity have chosen a spectrophotometric assay (Lieberman, 1975) or a radioassay developed by Ryan et al. (1977).

Because of sensitivity and specificity problems associated with the previously mentioned assays and the disposal problem peculiar to working with radiolabelled substrates, the first portion of these experiments involved developing a simple, rapid high performance liquid chromatography procedure for the analysis of angiotensin converting enzyme activity. The method was then used in a comparative study involving the systemic administration of BCNU, paraquat or bleomycin. Carmustine was the primary drug of investigation because the main purpose of this research was to describe pulmonary biochemical changes associated with exposure to this drug. The latter two were employed essentially as positive controls for fibrosis because when these studies were undertaken, a definitive experimental model for BCNU-induced pulmonary fibrosis had not yet been described (Smith and Boyd, 1983; Reznik-Schuller et al., 1984). Using i.p. administration of all three agents, dose and time dependent changes in ACE activity were measured.

## B. Methods

Carmustine (BCNU) was obtained from the NCI, Drug Synthesis and

Chemistry Branch, Bethesda, MD, outdated bleomycin from Bristol Laboratories, Syracuse, NY, and paraquat dichloride from Ortho, Raritan, NJ. The substrate for the hippuric acid determination, hippuryl-L-histidyl-L-leucine, hippuric acid standard (sodium salt) and Hepes buffer (sodium salt) were obtained from Sigma Chemical Company, St. Louis, MO. The internal standard, o-hydroxyhippuric acid, was obtained from Aldrich Chemical Company, Rochester, NY. Porcine angiotension converting enzyme was obtained from Calbiochem, Inc., San Diego, CA. Methanol (HPLC grade), sodium chloride, sodium sulfate, and acetic acid as 'Baker Analyzed' grade or better were obtained from J.T. Baker Chemical Company, Phillipsburg, NY. Water was obtained from a Millipore filtration system.

Male Sprague Dawley rats were obtained from Simonsen Laboratories, Gilroy, CA. Animals were housed, five per cage, in suspended stainless steel wire rodent cages (41 x 24 x 18 cm) at 21°C ± 1°, 60 ± 5% relative humidity and with a 12 hour dark/light cycle. They were provided food and water ad libitum. The animals weighed 200-275 grams and were 8 to 11 weeks old at the time of treatment.

All drugs were prepared fresh prior to administration. Carmustine was dissolved in corn oil at concentrations of either 15 or 20 mg/ml. Paraquat dichloride and bleomycin were dissolved in physiological saline at a concentration of 17 or 20 mg/ml. Animals in each experimental group received a single i.p. dose at 0.1 ml/100g body weight with the appropriate drug or vehicle control. Doses of all three agents were chosen that were in the high sublethal range over the desired time period. The 20 mg/kg BCNU dosage (80 mg/m$^2$) was approximately equivalent to a clinical dose in humans.

At intervals of 1, 3, 7, 14 and 21 days a minimum of 5 animals from each experimental group were lavaged for measurement of alveolar ACE activty. The rats were anesthetized with sodium pentobarbital in physiological saline at 60 mg per kg body weight i.p. and a tracheostomy was performed. The trachea was intubated with a length of polyethylene tubing (O.D. of 0.075 inches, PE 50) cut to rest at mid-trachea and secured with tracheal sutures.

Through the use of a 3 way valve, 3 ml aliquots of warm sterile saline were slowly administered via the trachea tube and the lavage fluid from each wash was collected cumulatively in a 20 ml glass syringe. After each aliquot of saline, the animal was provided 15 seconds of respiratory support by a small animal respirator (Phipps and Bird, Inc., Richmond, VA) set to deliver 80 breaths per minute at 80% of the total inspiratory capacity of the animal. The above procedure was repeated until a total volume of 12 ml of saline had been administered of which 10-11 ml was recovered and pooled. Blood specimens were obtained by cardiac puncture of the right ventricle and blood was collected in 5 ml vacutainer tubes. The rats were then killed by cervical dislocation followed by thoracotomy.

Lavage and blood specimens were immediately centrifuged for 10 minutes at 900 x g and 1400 x g, respectively. Serum samples were removed from the blood clot into a clean test tube and stored at 4°C along with the centrifuged lavage specimen until assayed. Assays were performed within 24 hours of collection.

A second experiment was designed to measure the effects of equimolar dosing of the 3 compounds to determine if there existed any correlation among the relative toxicities described for the 3 agents,

and their effect on serum or lavage ACE activity. Animals were dosed at 93 moles per kg body weight by injecting 0.1 ml/100g body weight of solutions of either BCNU (20 mg/kg), paraquat (17 mg/kg) or bleomycin (113 mg/kg). These animals were sacrificed 7 days after treatment. Lavage and serum specimens were procured and treated as previously described.

The assay for determining angiotensin converting enzyme was a modification of one proposed by Ryan et al.(1977). A 16 mM solution of the hippuryl-L-histidyl-L-leucine substrate was prepared in 0.05 M Hepes buffer, pH 8.0 containing 0.1 M NaCl and 0.6 M $Na_2SO_4$. Reactions were conducted in a 11 x 75 mm test tube using 0.05 ml of the buffered substrate plus 0.05 ml of the neat lavage fluid or 0.05 ml of a 1:10 dilution of the serum. Serum aliquants of 0.05 ml were diluted with 0.5 ml of Hepes buffer. The reaction mixture was incubated for 60 minutes at 37°C in a Dubnoff Metabolic Shaking Incubator under 95% oxygen.

The reaction was terminated by the addition of 0.07 ml of 2.4% methanol in acetic acid containing the internal standard o-hydroxyhippuric acid (o-OH H.A.) at a concentration of 40 g/ml. The mixture was vortexed briefly and then centrifuged for 8 minutes at 1400 x g. A 0.005 to 0.03 ml volume of the resulting supernatant was injected on the HPLC for determination of the hippuric acid (H.A.) concentration. Protein concentrations in both the lavage and serum samples were estimated by the method described by Lowry et al. (1951).

High performance liquid chromatography analysis was performed using a Waters Model 6000A pump and Model 480 variable wavelength detector. Chromatograms were recorded on a linear strip chart

recorder. Separation was effected with a Bondapak $C_{18}$ reverse phase column (30 cm x 3.9 mm) with a 1% acetic acid:methanol (60:40) mobile phase. Operating conditions for the analysis included a flow rate of 1.7 ml/min at 2800 psi, a chart speed of 30 cm/hr and a sensitivity of 0.20 absorbance units full scale (AUFS). The absorbance of the effluent was monitored at 240 nm. The run time for the analysis of each specimen required 8 minutes.

Quantification of the hippuric acid was made by determining the peak height ratio (H.A./o-OH H.A.) of the unknown and extrapolating from a standard curve run with each set of assays. Analysis of variance (ANOVA) was applied to all data and and when a significant F-ratio was calculated a least significant difference test was used to inspect the differences between mean values.

## C. Results

Sample chromatograms are shown in Figure 1. Retention times for the compounds of interest are: hippuric acid, 2.7 minutes, o-OH H.A., 3.3 minutes and hippuryl-L-histidyl-L-leucine, 4.4 minutes. Serum and lavage blanks had no endogenous peaks that interfered in the peak height determinations for the compounds of interest. Samples have been run on a fixed wavelength detectors (Waters Model 440) at 254 nm with some loss of sensitivity, but quantification was still possible by loading larger sample volumes on column. Assayed samples were stable with refrigeration for periods up to 96 hours with no apparent change in the quantified values of hippuric acid.

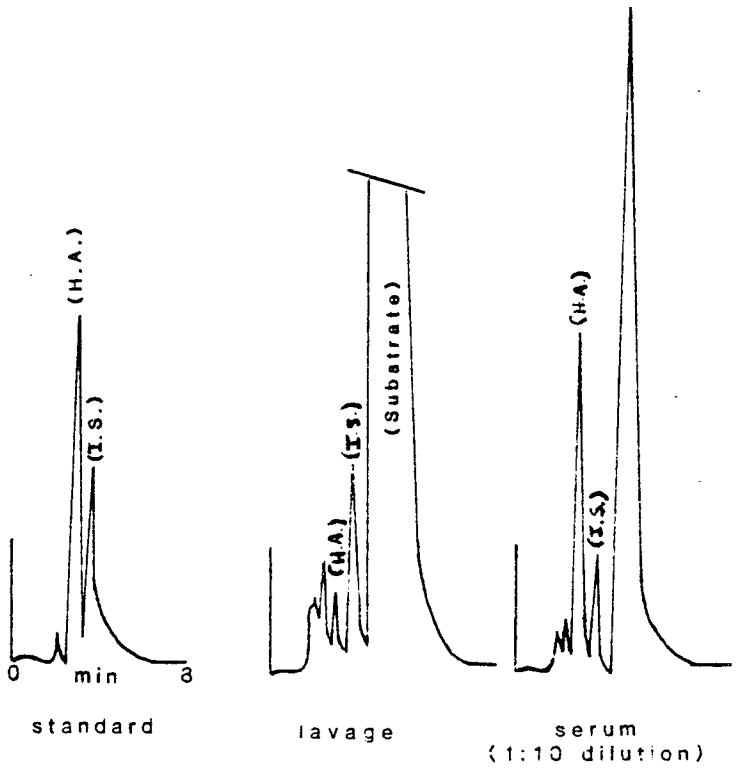


Figure 1. Sample Chromatograms from ACE Assay.

Analytical conditions: μBondapak $C_{18}$ (30 cm x 3.9 mm), 240 nm, 0.20 AUFS, 1% HAc:MeOH (60:40) at 1.7 ml/min and 2800 psi, chart 30 cm/hr. Retention times: hippuric acid (H.A.), 2.7 minutes, o-hydroxyhippuric acid internal standard (I.S.), 3.3 minutes and hippuryl-L-histidyl-L-leucine (substrate), 4.4 minutes. Total run time: 8 minutes.

A computer generated curve for triplicate standards of the free hippuric acid over the range of 1.0 to 100 μg/ml (5.6 to 558 x $10^{-6}$ moles) is shown in Figure 2. This range includes the expected values for the neat lavage specimens and the 1:10 dilutions of the serum samples. The detection limit for this assay is 0.5 μg/ml (2.8 x $10^{-6}$ moles) of the hippuric acid product formed. The correlation coefficient for the standard curve is 0.998. Intraassay reproducibility had a coefficient of variation (CV) of less than 8% over the entire concentration range and the interassay CV for seven runs over a 7 week period was 13%.

The serum and lavage ACE activities following BCNU administration are shown in Figure 3. Control determinations were made at each time point but were constant over the time periods studied and are shown as the pooled values for all control animals because one-way ANOVA revealed no significant differences over the time course $p > 0.25$. Both doses of BCNU significantly ($p < 0.005$) increased serum ACE activity 24 hours after administration. The activity in sera from rats receiving low dose treatment (15 mg/kg) had returned to control levels by 72 hours and in sera from the high dose (20 mg/kg) by 7 days. From 14 to 21 days, serum activity started to increase again although the values were not significantly different from controls (14 day: $p > 0.25$; 21 day: $0.10 > p > 0.05$).

Lavage ACE activity was significantly lower ($p < 0.005$) at 24 and 72 hours postdosing at both dosages. From one to three weeks, lavage activity gradually returned to normal reaching 111% of control levels by 21 days in both treatment groups.

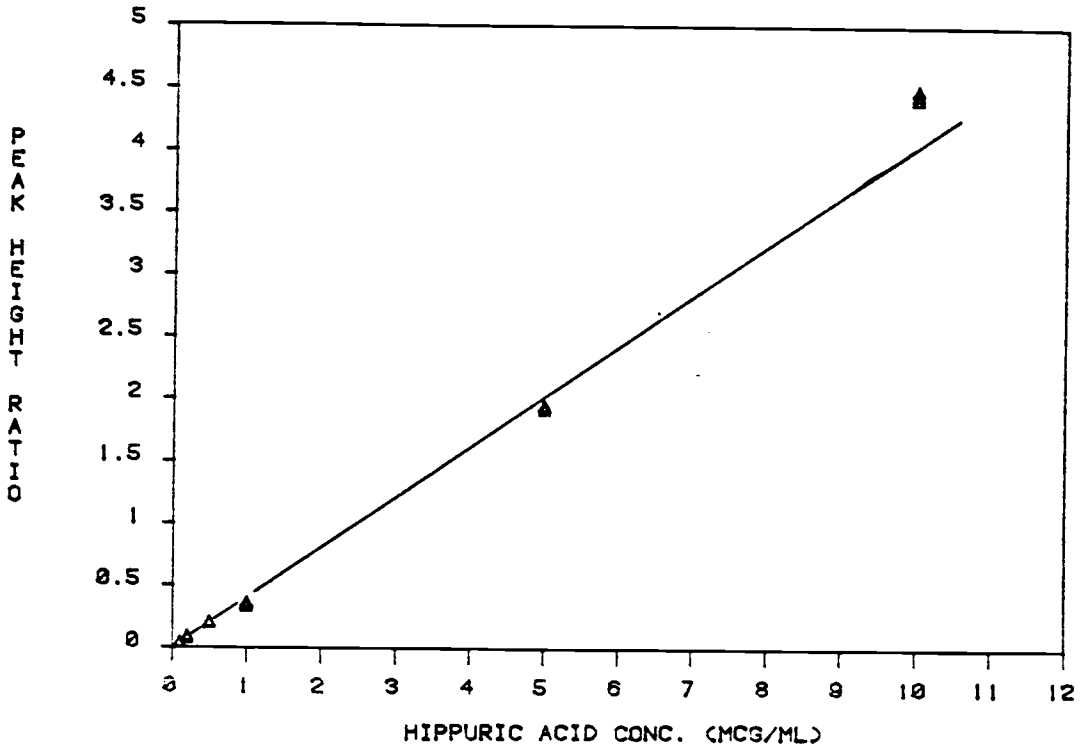


Figure 2. Hippuric Acid Standard Curve.

This curve is generated by treating triplicate spiked standards as described in Methods and injecting 0.01 ml of the resulting supernatant on column and running under the described analytical conditions. The curve is linear over the range of 5.6 to 588 μmoles of hippuric acid with a detection limit of 1.4 μmoles. Intraassay CV was 8%. The linear equation represented by this line is: $y = 0.44x + .05$. The correlation coefficient, r, is 0.9978.

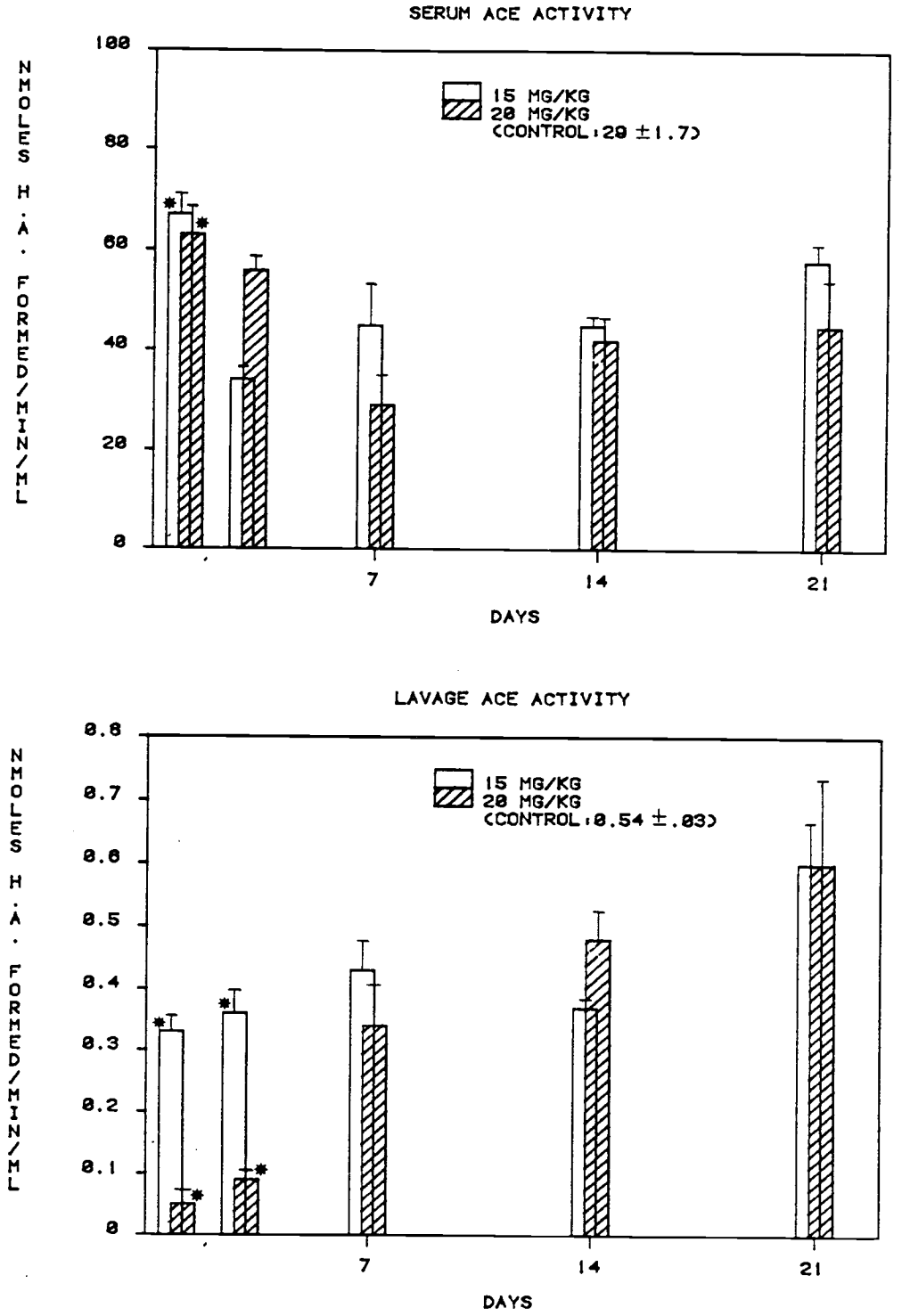


Figure 3. Serum and Lung Lavage ACE Activity in Rats After BCNU Administration.

Values are plotted as the mean ± S.E.M. for 4-11 animals.
*Significantly different from controls by protected LSD, $p < 0.005$.

Bleomycin treated animals exhibited a transient rise in serum ACE activity at 3 and 7 days ($p < 0.005$), returned to normal at 14 days, and then increased again to 297% of control levels by 21 days post-dosing (Figure 4). Lavage ACE activity in these animals was depressed 24 hours after treatment. As with BCNU there was an indication of a possible elevation in lavage activity beginning at 21 days.

High dose treatment with the herbicide paraquat, (20 mg/kg) resulted in significantly elevated ($p < 0.005$) serum ACE activity at all time points except 72 hours (Figure 5). This increase in serum ACE activity was quantitatively much more dramatic than were the effects seen with BCNU. Low dose treatment (17 mg/kg) resulted in significantly increased serum ACE activity only at 21 days. Lavage ACE activity was dramatically depressed from 1 to 14 days at 20 mg/kg ($p < 0.005$). In the low dose groups, however, there was only a transient depression in lavage ACE activity at 14 days.

The data for equimolar dosing of the 3 agents is shown in Table 2. There existed no significant differences in serum ACE activity among any of the treatment groups or control animals ($p > 0.25$). The serum activity in the bleomycin treated group was the only value increased, representing 159% of the control level. Lavage ACE activity, although depressed in the BCNU and bleomycin groups to 63% and 57% of control levels, respectively, was not significantly different from control or paraquat treatment by ANOVA ($0.25 > p > 0.10$).

Based on the early decreases in lavage ACE activity produced by all three compounds, an *in vitro* inhibition study using the three agents was performed. Various concentrations of each agent (including the expected range of exposure) were added in 0.05 ml portions to test

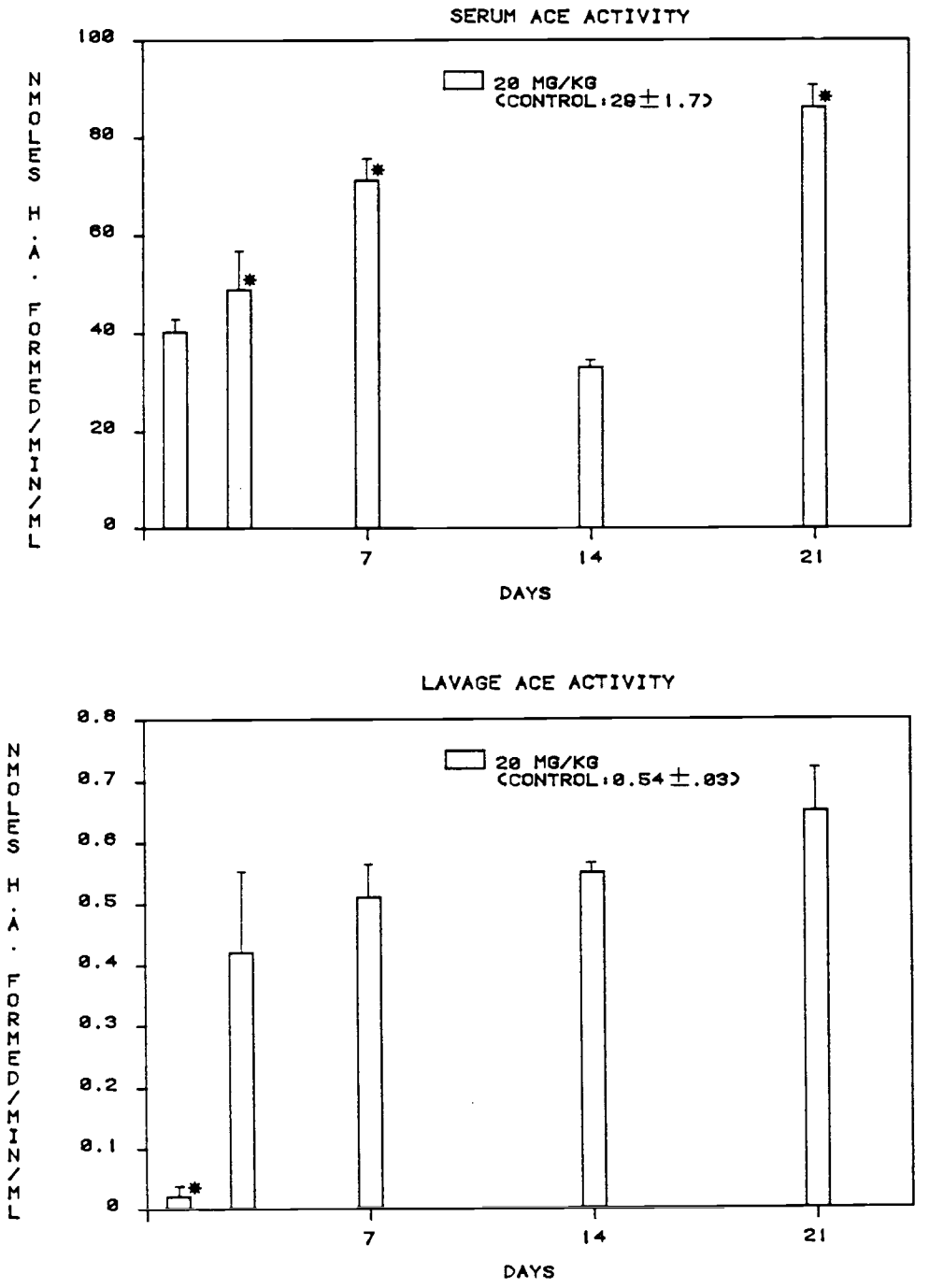


Figure 4. Serum and Lung Lavage ACE Activity in Rats After Bleomycin Administration.

Values are plotted as the mean ± S.E.M. for 4-11 animals.
*Significantly different from controls by protected LSD, $p < 0.005$.

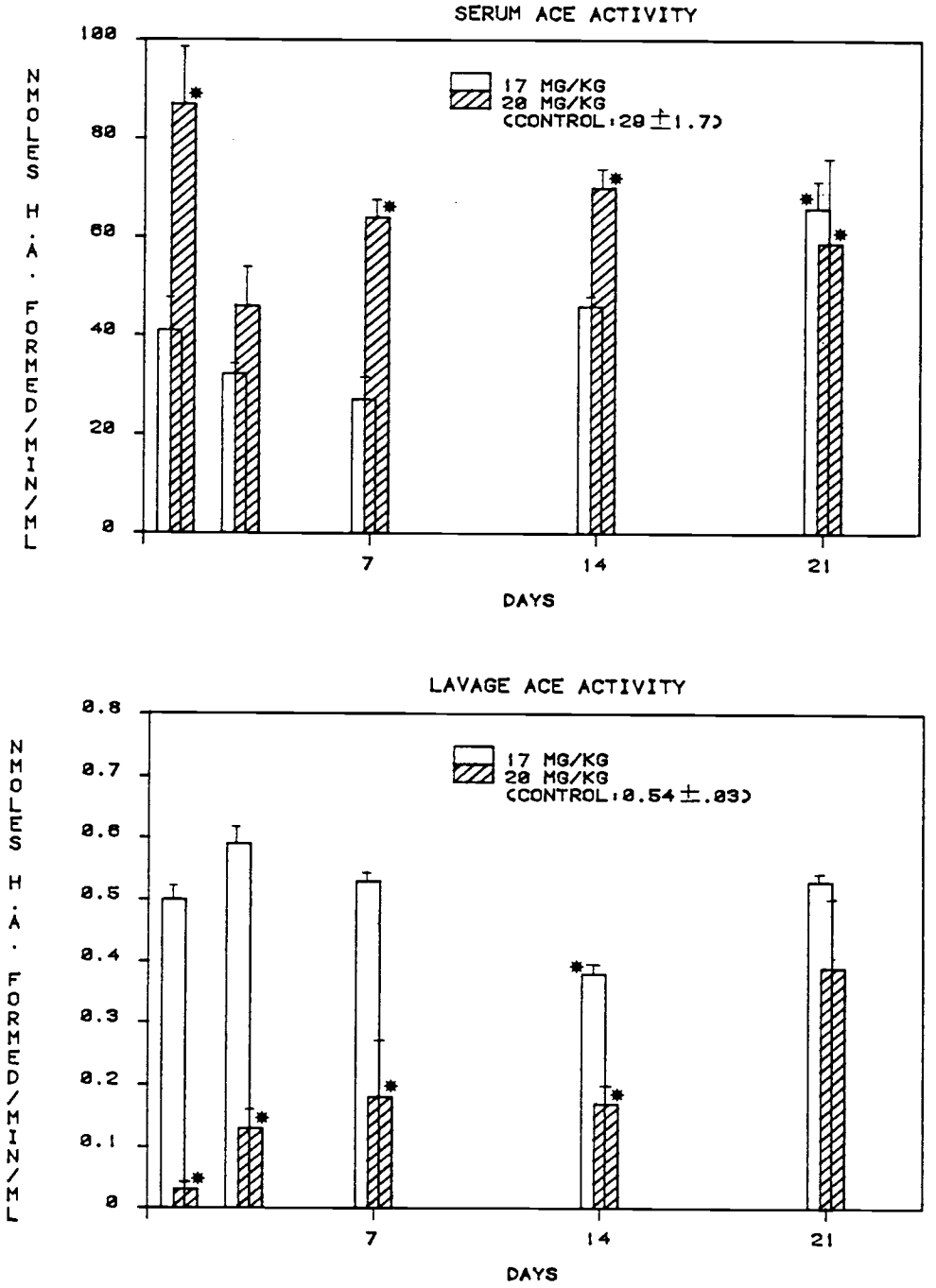


Figure 5. Serum and Lung Lavage ACE Activity in Rats After Paraquat Administration.

Values are plotted as the mean ± S.E.M. for 4-11 animals.
*Significantly different from controls by protected LSD, $p < 0.005$.

Table 2. Comparison of Angiotensin Converting Enzyme Activity in Serum and Lung Lavage Following Equimolar Dosing[a] of Rats with BCNU, Paraquat or Bleomycin

| | Control | BCNU | Paraquat | Bleomycin |
|---|---|---|---|---|
| Serum | 29.0 ± 1.7 [b] | 29.3 ± 5.0 | 27.2 ± 4.1 | 46.1 ± 2.2 |
| Lavage | 0.54 ± 0.03 | 0.34 ± 0.07 | 0.53 ± 0.01 | 0.31 ± 0.03 |

a. Specimens taken seven days after a single i.p. administration of BCNU, paraquat, bleomycin or vehicle control at 93 μmoles/kg body weight.

b. Values are expressed as the mean activity ± S.E.M. in nmoles of hippuric acid formed·$min^{-1}$·$ml^{-1}$ for 3-5 animals.

No significant differences by ANOVA for serum ($p > .25$) or lavage ($.25 > p > .10$).

tubes containing 0.05 ml of buffered substrate and 0.06 ml of porcine ACE (176 mg/ml). The reaction mixtures were then treated as previously described. One unit of activity is defined as the amount of enzyme that results in one nanomole of hippuric acid formed from substrate in one minute at 37°C (Cushman and Cheung, 1971). Even at the highest concentration of 5.4 mM paraquat, 75% of the porcine ACE activity remained (Figure 6). In contrast, BCNU at 2.3 mM decreased porcine ACE activity by 50% to 1.09 ± 0.01 nmoles hippuric acid formed/min (control: 2.12 ± 0.10). At the highest dose of BCNU tested, 4.7 mM, ACE activity was only 38% of control levels. Bleomycin had the most profound inhibitory effects on porcine ACE activity. At concentrations as low as 0.07 mM bleomycin, only 41% of the original activity remained.

Near the completion of these studies, reports of a multiple dosing model using Fischer 344 (F-344) rats for BCNU-induced fibrosis were reported by Smith and Boyd (1983) and Reznik-Schuller et al. (1984). On the basis of their findings, a multiple dosing experiment was performed using Sprague Dawley rats and the results of this study are shown in Table 3. Treated animals were dosed i.p. with BCNU in corn oil 5 $mg \cdot kg^{-1} \cdot wk^{-1}$. Controls received 1 $ml \cdot kg^{-1} \cdot wk^{-1}$ of the corn oil vehicle. At intervals of 28, 35 and 42 days, representing total cumulative doses of 15, 20 and 25 mg BCNU per kg body weight, three animals were sacrificed, samples procured, and analyses performed as previously described for ACE activity. Serum and lavage ACE activity was modestly but significantly ($p < 0.05$) increased only at the 5 week time period representing a total cumulative dose of BCNU of 20 mg/kg.

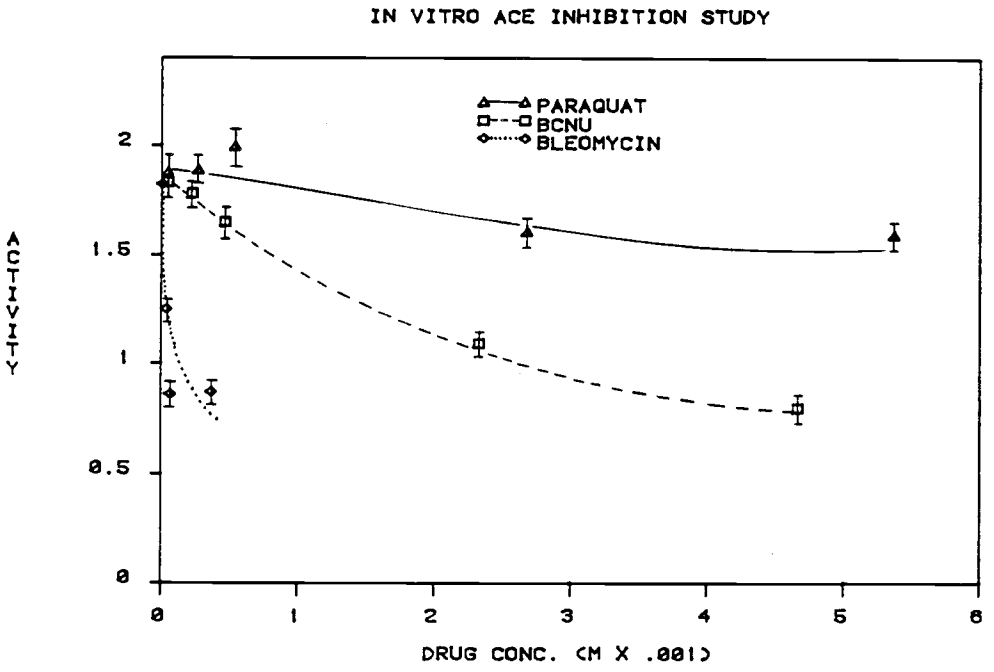


Figure 6. In Vitro ACE Inhibition Study.

Various concentrations of each compound were added in .05 ml portions to .05 ml of buffered substrate and .06 ml of porcine ACE (176 mg/ml). Each concentration was run in triplicate and the reaction was carried out as described under Methods and the supernatants analyzed for hippuric acid product as described under analytical conditions. Control porcine ACE activity formed 2.12 ± .10 nmoles hippuric acid $\cdot min^{-1} \cdot 0.05\ ml^{-1}$ under these conditions.

Table 3. Comparison of Multiple Dosing[a] of BCNU on Serum and Lavage Angiotensin Converting Enzyme Activity[b]

| | | Treated | | |
|---|---|---|---|---|
| | Control | 4 Weeks | 5 Weeks | 6 Weeks |
| Serum | 33.0 ± 0.05 | 31.6 ± 1.3 | 38.1 ± 1.3[c] | 29.1 ± 3.5 |
| Lavage | 0.47 ± 0.01 | 0.52 ± 0.02 | 0.54 ± 0.03[c] | 0.48 ± 0.01 |

a. Animals were dosed i.p. with BCNU at 5 $mg \cdot kg^{-1} \cdot wk^{-1}$ or the corn oil vehicle at 1 $ml \cdot kg^{-1} \cdot wk^{-1}$.

b. Activity is expressed as the mean ± S.E.M. in nmoles hippuric acid $formed \cdot min^{-1} \cdot ml^{-1}$ for 3 animals (pooled controls = 7 animals).

c. Significantly different from controls by protected LSD, $p < 0.05$.

At this time point, serum and lavage ACE activity from treated rats was 115% of the control levels.

D. Discussion

The validity of assessing pulmonary toxicity by measuring ACE activity has been well documented for intraperitoneal and intratracheal administered bleomycin (Tom and Montgomery, 1980; Newman et al., 1980; Newman et al., 1981). The studies of Newman et al. (1980) have largely ruled out the central serum compartment as being the source of the increased lavage angiotensin converting enzyme activity. Rather, it appears that any measurable increase in lavage activity is the result of serum enzyme enriched by angiotensin converting enzyme released upon lysis of the endothelial lining. Newman concluded from his experiments that the simultaneous determination of both lavage and serum ACE activity is more predictive than the measurement of either alone.

The three agents used in this comparative study were chosen based on their reported ability to induce fibrosis in the lung, presumably via different mechanisms. In man BCNU has a plasma half life of 5-15 minutes. The incidence of pulmonary fibrosis can be as high as 50% at cumulative doses of 1500 mg/m$^2$ (Aronin et al., 1980). Bleomycin is a glycopeptide product from Streptomyces verticilus. Plasma half life in man is approximately 115 minutes. In man, cumulative doses of 200 mg/m$^2$ are associated with the development of pulmonary fibrosis (Salmon, 1980). Paraquat is a bipyridinium herbicide with a plasma

half life in man of 150 minutes. Doses of 4 mg/kg (160 mg/m$^2$) in humans produce lethal pulmonary fibrosis (Fairshter and Wilson, 1975).

One purpose of this study was to develop a new methodology for measuring ACE activity in animals treated with agents known to induce pulmonary toxicity. A simple, rapid high performance liquid chromatography method for measuring ACE activity in either serum or pulmonary lavage specimens was developed. The method requires a minimum sample size (0.05 ml), with no extraction step, and has sufficient sensitivity (0.5 µg/ml) to permit analysis of both lavage and serum ACE activity.

Comparing the data for all three agents (Figures 3-5), it would appear that if ACE activity is related to the development of pulmonary toxicity, it is certainly reflected differently by these three compounds. At dosages of 20 mg/kg, paraquat and bleomycin quantitatively had a more dramatic effect on serum ACE activity than did BCNU, though all three agents elevated serum ACE activity significantly. At this dosage, lavage ACE activity was more dramatically effected by paraquat or BCNU treatment, which acted to decrease lavage ACE activity. At no time was increased lavage activity observed although if BCNU and bleomycin-treated groups had been monitored at longer time points, significant increases in lavage ACE activity might have become apparent. Other investigators, (Newman et al., 1980) have advocated measuring lavage ACE activity based on the assumption that endothelial damage would increase lavage ACE activity. No increases in lavage ACE activity were evident in this study. Based on these data, serum ACE determination appears to be more predictive for pulmonary toxicity. Any definitive conclusions in comparing the three agents would depend

on correlating a histological confirmation of acute endothelial injury with increased levels of serum ACE activity.

Equimolar dosing with BCNU, bleomycin or paraquat did not appear to predict the relative toxicities reported for these three agents. On the basis of $mg/m^2$, in man, the relative order of pulmonary toxicity would be paraquat > bleomycin > BCNU. Seven days after equimolar treatment, there were no significant differences in either lavage or serum ACE activity among the three treatment groups. Lavage ACE activity was slightly depressed in the BCNU and bleomycin-treated groups but this depression was not unexpected in view of *in vitro* results demonstrating direct ACE inhibition by these two drugs. *In vitro* inhibition studies clearly demonstrated that bleomycin had the greatest inhibitory capacity on porcine ACE activity and paraquat the least. Based on biological half-life, the body burden of both BCNU and bleomycin would be eliminated by 7 days. The decrease in lavage ACE activity at 7 days is more likely a reflection of ACE activity returning to normal levels.

Multiple treatment with BCNU did not significantly increase serum or lavage ACE activity except at 5 weeks or a total cumulative dose of 20 mg/kg. If multiple BCNU dosing is required to induce pulmonary toxicity, again ACE activity does not reflect consistent changes that could be associated with the onset of toxicity.

These data throw into question the validity of clinically measuring lavage ACE activity as a diagnostic tool in predicting pulmonary toxicity although this argument has been made before (Krieger *et al.*, 1984). It might be argued that increased serum ACE activity in the presence of decreased lavage ACE activity would

indicate a net loss of lung tissue ACE activity although tissue ACE levels in lung homogenates were not measured in these studies. Also, the in vitro inhibition studies could account for the decreased lavage ACE activity if sufficient concentrations of any of these agents could be maintained in the lung for any length of time after administration. Paraquat does actively accumulate in pulmonary tissue (Bend et al., 1985) and the enzyme responsible for inactivating bleomycin is not present in pulmonary tissue (Adamson, 1976; Lazo and Humphreys, 1983; Lazo et al., 1984). Carmustine has known cytotoxic effects on endothelial cells (Harlan et al., 1984; Nicolson and Custead, 1985) although it does not appear to be concentrated in any tissue in particular. It appears that increased serum ACE activity may actually reflect a generalized effect on the vascular endothelium.

A number of assays for measuring ACE activity have been previously reported. Vane (1964) proposed a bioassay system to detect active substances circulating in the blood. Boyd et al. (1976) described a radioimmunoassay procedure to measure angiotensin II. A spectrophotometric assay reported by Cushmann and Cheung (1971) and modified by Lieberman (1975) measured the formation of hippuric acid from hippuryl-L-histidyl-L-leucine at an absorbance of 228 nm. Friedland and Silverstein (1976) measured the rate at which the histidyl-leucine fragment was released by the addition of o-phthaldialdehyde to form a fluorophor. Ryan et al. (1977, 1980) has described a series of labelled substrates that can be used in a radiometric assay to quantitate ACE activity. There have been previous descriptions of HPLC methods for determining hippuric acid concentration in urine (Matsui et al., 1978; Astier and Deutsch, 1980) and a logical extension of

these methods was to use the HPLC to measure the ACE mediated release of hippuric acid from a synthetic substrate. Work by Astier and Deutsch (1980) showed no chromatographic interference among the peaks of interest and creatine, urea, oxalic acid, glycolic acid, glyoxylic acid, benzoic acid, salicylic acid, salicyluric acid, malonic acid or acetaminophen. The surgical and sample collection procedures used in this study were adapted from the methods described by Newman et al. (1980) and the enzymatic reaction is a modification of the radiometric assay reported by Ryan et al. (1977).

Comparison of results obtained in this study with the results obtained by others (Kim and Roberts, 1980; Newman et al., 1980; Tom and Montgomery, 1980; Smith and Boyd, 1983) are only qualitative due to differences in dosages, routes of administration, parameters measured and methodology. Smith and Boyd (1983) reported that single i.p. doses of BCNU, up to 80 mg/kg, did not alter pulmonary ACE nor cause histologically observable acute lung damage in the Fischer 344 rat. At this dose, they reported that serum ACE was decreased by 25% within 1 hour but returned to control values by 8 hours. These investigators only followed serum ACE activity through 48 hours presumably because 80 mg BCNU/kg i.p. is a 3 day $LD_{100}$ dose (Smith and Boyd, 1984).

Based on this lethal toxicity, and because a multiple dosing regimen is commonly given to patients (Reznik-Schuller et al., 1984), the majority of Smith and Boyd's studies (1983, 1984) have utilized a multiple dosing model of 5 mg BCNU$\cdot kg^{-1} \cdot wk^{-1}$ for 6 weeks. Using this dosing schedule they found a significant depression in lung tissue ACE activity after 4 doses (total cumulative BCNU of 20 mg/kg). Lung

homogenate ACE remained low through the end of the dosing period at 6 weeks and then returned to control levels by 8 weeks, 14 days after the last dose. Over this same time frame, serum ACE activity remained at control levels. Unfortunately they did not measure lavage ACE activity. Their pulmonary ACE activity was a measure of whole lung activity determined from the supernatant of a lung homogenate treated to release membrane bound enzymes (Newman et al., 1980). If serum ACE activity is a reflection of endothelial cell ACE content or integrity (Newman et al., 1980, 1981) the fact that Smith and Boyd did not observe some change in serum activity over the same time course is somewhat surprising.

Smith and Boyd (1983) did perform in vitro inhibition studies and found BCNU inhibited both pulmonary and serum ACE. Using 4 mM BCNU, pulmonary activity was decreased 60% and serum activity was decreased 57%. This is in good agreement with the 62% inhibition found in these studies measuring porcine ACE activity in the presence of 4.7 mM BCNU (see Figure 6). Carmustine-induced inhibition of ACE activity is not surprising. Other reports of enzyme inhibition associated with BCNU involve glutathione reductase (Frischer and Ahmad, 1977; Babson and Reed, 1978; Maker et al., 1983; Smith and Boyd, 1984), creatine kinase (Maker et al., 1983), malate dehydrogenase (Maker et al., 1983), lipoamide dehydrogenase (Ahmad and Frischer, 1985) and lactate dehydrogenase (Maker et al., 1983) although this latter enzyme was not inhibited in the studies of Smith and Boyd (1983). The activity of twenty other enzymes tested was unaffected (Frischer and Ahmad, 1977; Maker et al., 1983, Smith and Boyd, 1984; Ahmad and Frischer, 1985).

In view of these inhibition data, it is not surprising that Smith and Boyd (1983) saw a 25% decrease in serum ACE activity from 1 to 8 hours after i.p. administration of 80 mg/kg BCNU. The decrease probably reflects direct inhibition of serum ACE by carmustine. The lack of effects on lung ACE activity which they noted are not easily explained in view of results in Figure 3 showing acute reduction of lavage ACE activity at 24 and 72 hours for both BCNU doses. At 20 mg/kg, in a 250 g rat, BCNU would be present at approximately a 1.2 mM concentration assuming total body distribution. In this study, at 1.2 mM BCNU, in vitro, porcine ACE activity was reduced about 30% (Figure 6) a concentration where Smith and Boyd (1983) saw only minimal reduction in serum ACE activity.

The acute inhibition in lavage ACE activity seen within 24 hours with both doses of BCNU in this study, might be readily explained then on the basis of a direct inhibition by BCNU or its breakdown products. It is interesting to speculate, again based on the inhibition work, that the acute rise in serum ACE activity, seen at 24 hours, might actually be as much as 30% higher. It also appears that there is a time dependent increase in lavage ACE activity and if it had been possible to include time points beyond 21 days, statistically significant increases might have been observed. This may imply that more chronic endothelial damage is actually occurring later.

Much of the work using ACE activity as a measure of pulmonary toxicity was reported by Newman et al. (1980, 1981) and Tom and Montgomery (1980) studying bleomycin-induced pulmonary fibrosis. Newman et al. (1980), using an i.t. route for bleomycin administration, found a time dependent decrease in lung tissue ACE activity that minimized 3

to 7 days postdosing and returned to control values at 21 days. This corresponded with a maximum serum ACE activity noted at 3 days. These results were seen in Fischer 344 animals treated i.t. with 3.5 mg bleomycin/kg. They also noted that lavage activity from excised lungs was greater than the lavage activity from animals maintained on respiratory support.

The i.t. administration of bleomycin to induce pulmonary fibrosis is based on a series of studies performed by Newman et al. (1980) utilizing i.t. instillation of bleomycin in mice, rats, and hamsters. The histopathological lung changes were identical to those seen in studies using more conventional subcutaneous, intraperitoneal, intravenous or intramuscular routes of exposure. Other reported advantages of this i.t. model are the small amounts of drug required, high prevalence of pathologic changes observed and the rapid onset of these histopathologic changes. Intratracheal injection of bleomycin results in initial exposure of the epithelial lining rather than the endothelial cells, which would otherwise be exposed by a systemic route of administration. Indeed, work by Lazo and Pham (1984), showed that pulmonary levels of bleomycin comparable to those attained with a single i.t. dose of 2 mg/kg could not be obtained after a s.c. injection even at severly toxic doses of 100 mg/kg. However, the i.t. bleomycin model remains popular because of its predictability and is especially important in mice where single dosings by more conventional routes did not elicit a fibrotic response (Lazo and Pham, 1984).

This study used the i.p. administration of bleomycin based on the desire to determine endothelial cell effects. Direct comparison with the results of Newman et al. (1980, 1981) are therefore difficult

because of different routes of exposure. Newman et al. (1980) did observe a 125% increase in serum ACE activity at 3 days (3.5 mg/kg, i.t.) but activity returned to control levels by 7 days. The control serum value of 22.4 ± 1.1 units reported by Newman et al. (1980) compares favorably with the control value of 29.0 ± 1.7 in this study. At 3.5 mg/kg i.t. lung tissue ACE activity declined to 40% of control values at 3 days and did not normalize again until 21 days post dosing. In this study using 20 mg/kg i.p., lavage ACE activity returned to control levels by 72 hours substantiating the fact that the i.t. administration of bleomycin probably induces greater endothelial damage.

Tom and Montgomery (1980) used i.p. dosing twice weekly, but only followed lung tissue ACE activity. At high dose treatment (15 units or approximately 15 mg bleomycin i.p. twice weekly) they observed a 32% increase in lung ACE activity at 10 days and a 41% decrease at 21 days. The time course for decreased lung ACE activity seems to be a function of the route of exposure.

The acute serum ACE effects in this study (Figure 4) are similar to those observed by Newman et al. (1980). Activity is significantly increased at 3 days. Except for the unexplained return to control levels at 14 days, serum activities continue to increase with time. Once again it appears that lavage ACE activity is also increasing with time. If multiple i.p. dosing is indeed required to induce fibrosis, increased lavage activity would not be expected.

Tom and Montgomery (1980) did perform an in vitro bleomycin inhibition study using porcine ACE and reported concentration-dependent inhibition of activity at concentrations greater than .010 mM. This

report is in good agreement with the inhibition work reported here (Figure 6) showing 41% inhibition at .040 mM and 60% inhibition at 0.07 mM. Such a dramatic inhibition could possibly account for the decreased activity observed in the lavage samples at 24 hours.

There is only one other study (Kim and Roberts, 1980) that has attempted to correlate paraquat toxicity with changes in ACE activity. Kim and Roberts treated mice with a single i.p. dose of 30 mg paraquat/kg, a 4 day $LD_{70}$ dose. They observed almost a fourfold increase in lung tissue ACE activity in treated animals. Within the treated group, lung tissue ACE activity was higher in mice that died than in those that survived. They performed no inhibition studies, but based on the results of this work (Figure 6) paraquat exhibited the least inhibition of any agent studied. Seventy-five percent of the porcine ACE activity remained even at the highest paraquat dose tested *in vitro* (5.4 mM).

In this study, high dose paraquat (20 mg/kg) produced an acute increase in serum activity at 24 hours. Serum activity briefly decreased and then remained elevated from 7 to 21 days. Once again, one would expect increased lavage activity if there was a loss of endothelial integrity. This assumption, coupled with a minimal inhibitory activity, makes the decreased lavage activity seen from 1 to 14 days with high dose paraquat somewhat difficult to explain. The results might be rationalized by reports that paraquat actively accumulates in Clara and type II cells on the epithelial side of the air-blood barrier by an energy dependent, sodium independent, saturable transport system (Bend *et al.*, 1985). The continuous presence of paraquat, even with minimal inhibitory activity, might be

enough to account for decreased lavage ACE activity seen with high dose paraquat treatment (Figure 5).

The potential clinical use of lavage ACE determinations in assessing pulmonary fibrosis remains to be studied. The use of fiberoptic bronchoscopes to collect lavage specimens would appear to offer a safe and rapid method even in compromised patients although serum ACE activity might be more predictive. Documented increases in serum ACE activity have been described for sarcoidosis, liver cirrhosis, acute hepatitis, Gaucher's disease, leprosy, hyperthyroidism and diabetic retinopathy among others (Lieberman, 1975; Matsuki and Sakata, 1982). The studies performed using this HPLC method, however, were not conclusive with regard to the diagnostic value of ACE determination in predicting pulmonary toxicity. It would be interesting to design studies with any of the known fibrotic agents where lavage ACE determinations and lung histopathological studies could be conducted to confirm if there is a correlation between increased lavage ACE activity and breakdown of the endothelial cells. Even should a positive correlation exist, it is only another biochemical marker of pulmonary toxicity. It tells little about any possible mechanisms. That answer requires other avenues of investigation.

# Chapter III. The Effects of BCNU and BHCNU on Alpha-1-Proteinase Inhibitor Content and Activity.

## A. Introduction

The major alpha-1-globulin of plasma is known as alpha-1-antitrypsin or alpha-1-proteinase inhibitor. The protein was first known as alpha-1-antitrypsin after the trypsin inhibitor activity of the protein was identified. It is now known that alpha-1-antitrypsin actually inhibits the actions of a number of proteolytic enzymes, especially polymorphonuclear (PMN) cell proteinases, and is now more commonly identified as alpha-1-proteinase inhibitor (Travis and Johnson, 1981). Enzymes known to be inactivated to a varying degree by alpha-1-proteinase inhibitor (PI) include: trypsin, chymotrypsin, plasmin, thrombin, pancreatic elastase, skin collagenase, renin, urokinase, Hageman-factor cofactor, the neutral proteases of PMN cells (elastase and collagenase) and the acid protease present in alveolar macrophages (Cohen, 1973; Morse, 1978).

Human PI has been well characterized, but comparatively little is known about the structural and chemical properties of PI from different animal species. Carrell et al. (1982) reviewed the structure of human PI and their data can be used in comparing the published work on rat PI by Fretz and Gan (1980), Roll and Glew (1981) and Verbanac and Heath (1983). Human PI is relatively small and polar permitting rapid movement into tissue fluids. Indeed, PI has been identified in tears, perilymph, lymph, saliva, colostrum, breast milk, duodenal fluid,

bile, synovial fluid, cervical mucus, semen, amniotic fluid, platelets, and megakaryocytes (Morse, 1978) in addition to plasma and lavage (Gadek et al., 1981; Travis and Johnson, 1981; Stockley and Afford, 1983). The human PI molecule is a single chain of 394 amino acid residues and three carbohydrate side chains giving a total molecular weight of 51,000-54,000. A single reactive site is located on the methionine residue at position 358. There exists only one cysteine residue and therefore the strength of the postulated tertiary structure is not augmented by disulfide bridges so characteristic of other inhibitors.

In contrast, the rat molecule was found to have a smaller molecular weight of 47,000-50,000. That rat PI is somewhat smaller is supported by sedimentation equilibrium measurements showing rat PI has a value of 3.29 Svedberg units (human 3.33). There also exist some significant differences in amino acid residues though the carbohydrate composition is qualitatively similar. Double diffusion Ouchterlony experiments have shown distinct immunological identity between rat PI and that of primates. In spite of their structural differences, all available evidence indicates that functionally the proteins act similarly in both species as a general scavenger of proteinases.

The sequence of biochemical events leading to the hepatic synthesis of PI are well described in humans (Yunis et al., 1976) and in rats (Vebanac and Heath, 1983). In general, the biosynthetic pathway is the same as that determined for other secretory glycoproteins. Rat PI is synthesized in the rough endoplasmic reticulum as a 45,000 dalton preprotein. Cleavage of the signal peptide from the preprotein results in the 42,000 dalton polypeptide backbone of mature PI. The

sequential addition of three oligosaccharide side chains at glycosylation sites occurs prior to translocation to the lumen of the endoplasmic reticulum. The addition of terminal N-acetylglucosamine, galactose and sialic acid residues occurs in the Golgi apparatus. Studies by Bhan et al. (1976) indicate the completed PI is then rapidly excreted in a manner similar to albumin, because newly synthesized labeled PI could be detected in hepatic tissue culture medium but not intracellularly. Although inhibitor studies indicate glycosylation is not an essential step for transport of PI out of the hepatocyte (Verbanac and Heath, 1983), unglycosylated PI accumulations do occur in the hepatic smooth endoplasmic reticulum of phenotypic variant individuals deficient in circulating PI (Morse, 1978).

The regulation of plasma levels of PI is unknown (Laurell et al., 1977) although serum PI levels are known to double or even triple when stimulated by acute inflammation (Verbanac and Heath, 1983) neoplastic disease, pregnancy, or estrogen therapy (Morse, 1978). Phenobarbital induction has no effect in raising plasma PI levels or in clearing hepatocyte precursor materials (Morse, 1978). The normal half-life for circulating PI is 3 to 7 days with most of the loss resulting from hepatic catabolization of complexed PI (Carrell et al., 1982).

In serum alpha-1-PI accounts for 90% of the total trypsin inhibitor capacity (Yunis et al., 1976). Of the other serum proteinase inhibitors [alpha-2-macroglobulin, alpha-1-antichymotrypsin, inter-alpha-trypsin inhibitor, $C_1$ esterase inhibitor and antithrombin III (Stockley and Afford, 1983)], only alpha-2-macroglobulin appears to have appreciable inhibitory activity (Morse, 1978). It appears that PI is not concentrated in the normal lung but rather represents a

filtrate of plasma (Gadek et al., 1981). In humans alpha-2-macroglobulin represents less than 0.5% of the molar content of the lower respiratory tract antielastases; PI being present at a minimum 200-fold higher concentration.

Determining lavage PI content and activity appeared to be an attractive avenue of investigation based on the protease-antiprotease theory of emphysema (Morse, 1978; Gadek et al., 1981), the reported potentiation of cadmium induced pulmonary injury in PI suppressed rats (Chowdhury et al., 1983), the possible association of byssinosis and tuberculosis with PI deficiencies (Evans and Bognaki, 1979), and findings of oxidative inactivation of lavage PI in patients with adult respiratory distress syndrome (Cochrane et al., 1983).

Two possible mechanisms of action, both affecting pulmonary PI, might be attributed to acute BCNU exposure. First, based on the known hepatic toxicity (Hoyt, 1984; Stolzenbach, 1984), it might be possible that the livers of BCNU treated rats are not capable of synthesizing a functional alpha-1-PI. Second, based on the known alkylation and carbamylation properties of BCNU (Bono, 1976), perhaps BCNU, or a breakdown product, directly inactivates the proteinase inhibitor.

Studies were undertaken to investigate these two possibilities by first measuring dose-response relationships between PI content and activity after acute BCNU administration. Secondly, a nitrosourea acting through a purely carbamylation mechanism, 1,3-bis(hydroxycyclohexyl)-1-nitrosourea or BHCNU (Tew et al., 1982; Sariban et al., 1984;) was used in an attempt to differentiate between an alkylation and carbamylation mechanism. Similar responses with BCNU and BHCNU might imply a mechanism dependent on the carbamylating intermediate.

Different responses might imply BCNU is actually acting via an alkylating intermediate, especially if the response to BHCNU does not differ from control values.

B. Methods

Human alpha-1-proteinase inhibitor, trypsin (EC 3.4.21.4) and N-benzoyl-DL-arginine-p-nitroanilide-HCL (BAPNA) were obtained from Sigma Chemical Company, St. Louis, MO. All other chemicals were obtained from J.T. Baker Chemical Company, Phillipsburg, NJ, as 'Baker Analyzed' grade or better. Carmustine was obtained from the NCI and BHCNU was obtained from the NCI Developmental Therapeutics Program, Bethesda, MD. Male Sprague Dawley rats were obtained and maintained as previously described.

Carmustine and BHCNU were prepared fresh prior to administration. Carmustine was dissolved in corn oil at concentrations of 15, 20 and 30 mg/ml. 1,3-bis(hydroxycyclohexyl)-1-nitrosourea was dissolved in ethanol:propylene glycol (20:80) at concentrations of 20 or 25 mg/ml (doses approximately equimolar to 15 and 20 mg/kg BCNU, respectively). Animals in each experimental group were dosed i.p. as previously described with the appropriate drug or vehicle control.

At intervals of 1, 3, 5, 7, 14, 21 or 28 days specimens were obtained from a minimum of five animals. Animals were anesthetized, a tracheostomy was performed, and pulmonary lavage samples collected as described in the preceeding chapter. Blood samples were obtained by cardiac puncture of the right ventricle into a 5 ml vacutainer tube. Rats were then killed by cervical dislocation followed by thoracotomy.

All samples were immediately centrifuged and stored as previously described for subsequent analysis.

Alpha-1-PI quantification was performed using a Waters I-250 size exclusion protein column (30 cm x 7.8 mm, size exclusion range 10,000-500,000 MW) in a procedure described by Feste and Gan (1982). All samples were filtered through a 0.45 micron filter prior to HPLC analysis. High performance liquid chromatography analysis was performed using a Waters Model 6000A pump and Model 480 variable wavelength detector. Chromatograms were recorded on a linear strip chart recorder. Separation was effected using a mobile phase of 0.05 M $NaH_2PO_4$-$H_2O$ (pH 6.5). Operating conditions for the analysis included a flow rate of 1.5 ml/min at 1100 psi, a chart speed of 15 cm/hour, a sensitivity of 0.02 AUFS, and an injection volume of 0.015 ml of the neat lavage or diluted serum sample. Sera were diluted 1:5000 in 0.05M sodium phosphate buffer. The absorbance of the effluent was monitored at 230 nm. Run time for analysis of each specimen required 13 minutes. Quantification of alpha-1-PI from each sample was made by measuring the peak height of the PI peak and extrapolating from a standard curve of human alpha-1-PI run with each set of assays. Under these analytical conditions, there was no measurable difference in the retention volumes of rat PI versus human PI.

Total serum and lavage trypsin inhibitory capacity was assayed spectrophotometrically by monitoring the tryptic hydrolysis product from a BAPNA substrate (Erlanger *et al.*, 1961) as described by Briscoe *et al.* (1966). Serum samples required dilution to 0.25% (V/V) using 0.1 M Tris. Trypsin was made up to a concentration of 100 µg/ml in

0.1M Tris (pH 7.6) containing 0.01M $CaCl_2$. The BAPNA substrate was prepared at a concentration of 0.001M in the same buffer as trypsin.

The assay was performed by adding 1.0 ml of the neat lavage sample or the 0.25% diluted serum sample to a 14 x 175 mm test tube followed by 0.1 ml of trypsin (100 µg/ml). The reaction mixture was briefly vortexed and incubated for 60 minutes at 37°C under ambient atmosphere in a Dubnoff Metabolic Shaking Incubator. After the initial incubation, 3.0 ml of 0.001M BAPNA substrate was added to each sample. The tubes were again vortexed and incubated for an additional 15 minutes at 37°C at which time the reaction was terminated by the addition of 1.0 ml of 0.2N HCl. Sample absorbance was read at 400 nm versus a reagent blank (containing no trypsin) on a Bausch and Lomb Spectronic 600 UV/VIS spectrophotometer. Quantification of residual trypsin activity in each sample was made by extrapolating the absorbance of each sample against a standard curve prepared by running various concentrations of trypsin with each assay. Data were reported as micromoles of trypsin inactivated per micromole of alpha-1-PI for each sample.

Analysis of variance (ANOVA) was applied to all data and when a significant F-ratio was calculated, a least significant difference test was used to inspect the difference between mean values.

## C. Results

At selected time points, serum PI concentration was measured to determine if BCNU treatment caused any changes in circulating PI content. At 3 and 7 days postdosing neither BCNU treatment resulted

in significant changes in serum PI concentration ($p < 0.005$) (Table 4). At 28 days postdosing, however, the 20 mg/kg treatment resulted in a 336% increase in serum PI content (control: 506 ± 20 µg/ml). 1,3-bis(hydroxycyclohexyl)-1-nitrosourea, the agent reported to act purely via a carbamylation byproduct, did not significantly affect serum PI concentration at either of the doses tested which were approximately equimolar with the BCNU doses employed. Twenty-eight days after high dose BHCNU treatment (25 mg/kg) sera PI concentrations were only 55% of the control values.

BCNU treated lavage PI content measured as a percentage of control values is shown in Table 5. At 30 mg/kg, animals showed severe signs of acute toxicity and these animals were only followed for 14 days. The 20 and 30 mg/kg doses acted to acutely depress lavage PI concentration between 3 and 5 days after treatment. At these time points lavage PI concentration reached a nadir of approximately 50% of control levels. Lavage PI content in treated animals had returned to control levels by 7 days postdosing and then began to exhibit dose dependent increases from 14-21 days (Figure 7). Maximum lavage PI content was 683 g/ml 28 days after a 20 mg/kg dose of BCNU (control: 141 ± 18 µg/ml). Low dose recipient animals (15 mg/kg) did not exhibit the acute depression in lavage PI content but significant elevations did occur by 14 days post-treatment and they appeared to return to control levels by 28 days.

Treatment with BHCNU did result in earlier, more prolonged elevations in lavage PI content (Figure 8). There was also an acute depression of lavage PI concentration 5 days after treatment with 25 mg/kg BHCNU, a response comparable to that seen with 20 mg/kg BCNU.

Table 4. Serum Alpha-1-Proteinase Inhibitor Concentration [a] in Rats After Treatment with BCNU or BHCNU

| Drug | Dosage[b] | Post-dosing Interval (days) 3 | 7 | 28 |
|---|---|---|---|---|
| BCNU | 15 mg/kg | 526 ± 13 | 507 ± 27 | 460 ± 23 |
| | 20 mg/kg | 529 ± 15 | 504 ± 7 | 1701 ± 690[c] |
| BHCNU | 20 mg/kg | 486 ± 20 | 511 ± 16 | 444 ± 42 |
| | 25 mg/kg | 511 ± 33 | 470 ± 17 | 279 ± 13[c] |
| Control | | 506 ± 20 | | |

a. Values are expressed as the mean ± S.E.M. in μg PI/ml serum ($n \geq 5$).
b. Single i.p. administration of drug or vehicle control.
c. Significantly different from controls by protected LSD, $p < 0.005$.

Table 5. Lung Lavage Alpha-1-Proteinase Inhibitor Concentration[a] in Sprague Dawley Rats After BCNU Treatment

| | Post-dosing interval (days) | | | | | | |
|---|---|---|---|---|---|---|---|
| Dosage[b] | 1 | 3 | 5 | 7 | 14 | 21 | 28 |
| 15 mg/kg | 117 ± 16 | 143 ± 30 | 129 ± 19 | 110 ± 24 | 215 ± 53 [c] | 220 ± 69 [c] | 153 ± 27 |
| 20 mg/kg | 96 ± 17 | 94 ± 15 | 52 ± 2 [c] | 136 ± 21 | 125 ± 10 | 484 ± 132 [c] | 260 ± 50 [c] |
| 30 mg/kg | 120 ± 20 | 53 ± 7 [c] | 65 ± 4 [c] | 89 ± 8 | 111 ± 7 | --- | --- |

a. Values are expressed as the mean ± S.E.M. as percentage of controls (141 ± 18 μg PI/ml lavage) for 4-10 animals.

b. Single i.p. administration of drug or vehicle control.

c. Significantly different from controls by protected LSD, $p < 0.005$.

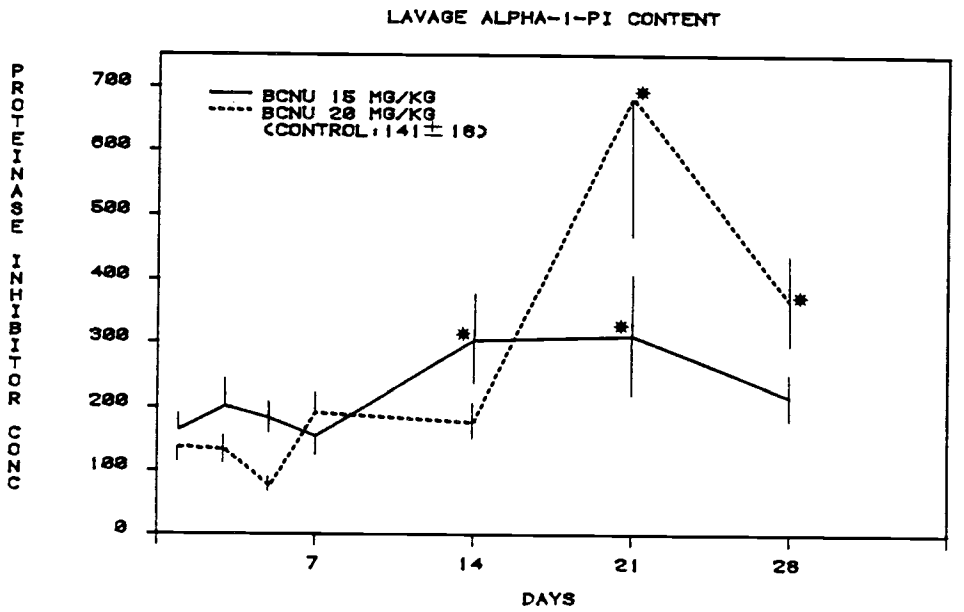


Figure 7. Lung Lavage Alpha-1-PI Concentration in Sprague Dawley Rats After BCNU Administration

Values are plotted as the mean ± S.E.M. in μg/ml for five animals.
*Significantly different from controls by protected LSD, $p < 0.005$.

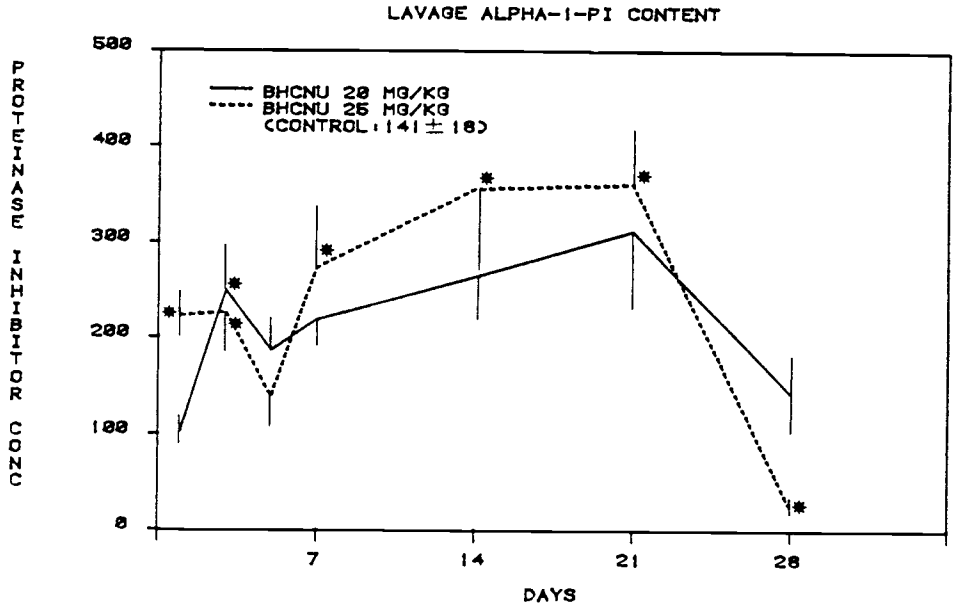


Figure 8. Lung Lavage Alpha-1-PI Concentration in Sprague Dawley Rats After BHCNU Administration

Values are plotted as the mean ± S.E.M. in μg/ml for five animals.
*Significantly different from controls by protected LSD, $p < 0.005$.

Lavage PI content increased over the acute time course (1-3 days) in animals doses with 20 mg/kg BHCNU, decreased slightly at 5 days, and then remained elevated through 21 days postdosing. Maximum lavage PI content was 360 ± 57 µg/ml 21 days after administration of 25 mg/kg BHCNU compared to 683 ± 187 µg/ml 21 days after an equimolar dose of 20 mg/kg BCNU (Figure 7). There was an 83% decrease in lavage PI content compared to control levels 28 days after high dose BHCNU treatment in parallel with the decreased serum PI concentration seen at this same dose and time.

Determination of alpha-1-PI activity actually is a measure of the ability of PI to inactivate trypsin. The experiment more correctly measures total trypsin inhibitory capacity of the physiological fluid in question. Because PI accounts for the largest portion of trypsin inhibitor activity, the data in Table 6 are a measure of the ability of serum PI to react with and inactivate a fixed concentration of trypsin. Seven days after the administration of 15 mg/kg BCNU, there was a 35% decrease in serum PI activity. After 20 mg/kg BCNU, total serum trypsin inhibitory capacity was decreased 72 hours postdosing representing 68% of the control activity of 6.08 ± .46 µmoles trypsin inactivated/µmole PI. The most striking observation was the marked inhibition of serum PI activity at 28 days in the high dose BCNU group. At this time point only 19% of the control inhibitor activity was present. This was in the face of a 2.5-fold increase in PI concentration of the serum (Table 5; Figure 7). Low dose BHCNU treatment decreased serum PI activity 3 and 28 days after treatment while high dose BHCNU treatment decreased serum PI activity at 7 and 28 days. Quantitatively the decrease in serum PI

Table 6. Serum Alpha-1-Proteinase Inhibitor Activity [a] in Sprague Dawley Rats After Treatment with BCNU or BHCNU

| Drug | Dosage[b] | Post-dosing Interval (days) 3 | 7 | 28 |
|---|---|---|---|---|
| BCNU | 15 mg/kg | 5.48 ± .33 | 3.93 ± .51[c] | 7.72 ± .55 |
| | 20 mg/kg | 4.14 ± .60[c] | 7.03 ± .48 | 1.16 ± .56[c] |
| BHCNU | 20 mg/kg | 3.95 ± .35[c] | 6.64 ± .23 | 2.09 ± .23[c] |
| | 25 mg/kg | 7.57 ± .80 | 4.85 ± .43[c] | 4.43 ± .51[c] |
| Control | | 6.08 ± .46 | | |

a. Values are expressed as the mean ± S.E.M. in μmoles trypsin inactivated/μmole PI ($n \geqslant 5$).
b. Single i.p. administration of drug or vehicle control.
c. Significantly different from controls by protected LSD, $p < 0.005$.

activity was actually greater at the low dose.

The data in Figure 9 depicts lavage PI activity after BCNU treatment. High dose BCNU treatment resulted in decreased total lavage trypsin inhibitory capacity at 24 and 72 hours postdosing, representing 46 and 40%, respectively, of the control activity of 0.070 ± .009 μmoles trypsin inactivated/μmole PI. Three days after the administration of 15 mg/kg BCNU, there was a 59% decrease in lavage PI activity. At all other time points measured in treated animals lavage PI activity was within control values although by 28 days postdosing there is a definite trend towards decreased activity again. In contrast to the acute inhibition after BCNU treatment, high dose BHCNU treatment increased lavage PI activity 7 and 28 days after dosing to 180 and 246% of the lavage control value of .070 ± .009 μmoles trypsin inactivated/μmole PI (Table 7).

## D. Discussion

The apparent association between decreased levels of alpha-1-PI and pulmonary diseases such as emphysema (Gadek et al., 1981), cadmium induced pulmonary injury (Chowdhury et al, 1983), and byssinosis (a pneumoconiosis reported in cotton, flax, and hemp workers) and tuberculosis (Evans and Bognacki, 1979) could not be overlooked in considering BCNU-induced pulmonary toxicity. Though emphysema, tuberculosis and byssinosis are pathologically different disease processes, it is not entirely unrealistic to expect that yet another disease process, fibrosis, might be causally related to some aberration in PI content or activity.

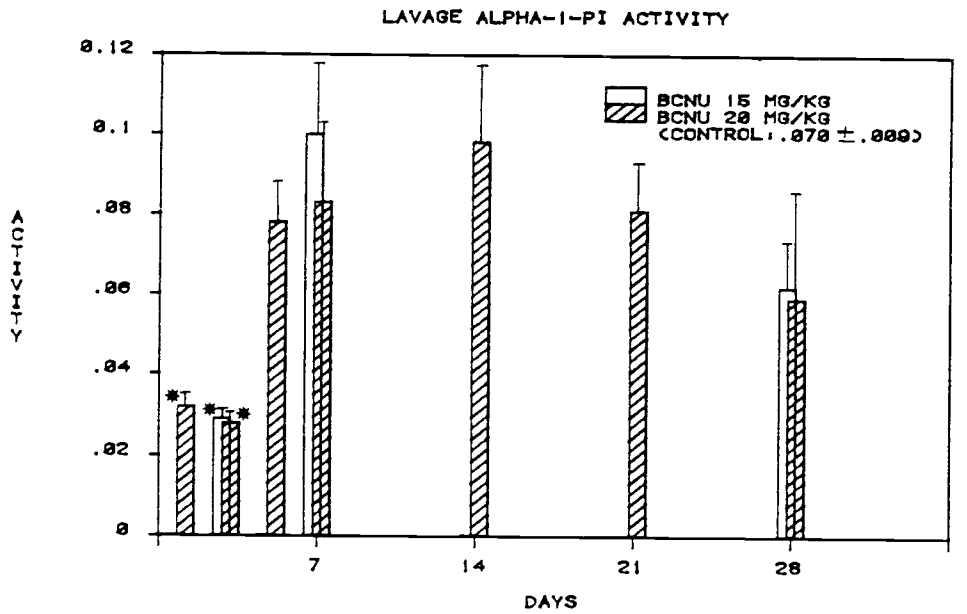


Figure 9. Lung Lavage Alpha-1-PI Activity in Sprague Dawley Rats After BCNU Administration

Values are plotted as the mean ± S.E.M. in μmoles trypsin inactivated/μmole PI for five animals.
*Significantly different from controls by protected LSD, $p < 0.005$.

Table 7. Lung Lavage Alpha-1-Proteinase Inhibitor Activity [a] in Sprague Dawley Rats After Treatment with BCNU or BHCNU

| Drug | Dosage[b] | Post-dosing Interval (days) 3 | 7 | 28 |
|---|---|---|---|---|
| BCNU | 15 mg/kg | .029 ± .001[c] | .100 ± .012[c] | .062 ± .006 |
| | 20 mg/kg | .028 ± .003[c] | .083 ± .010 | .059 ± .015 |
| BHCNU | 20 mg/kg | .081 ± .004 | .060 ± .004 | .068 ± .011 |
| | 25 mg/kg | .056 ± .012 | .126 ± .014[c] | .172 ± .025[c] |
| Control | | .070 ± .009 | | |

a. Values are expressed as the mean ± S.E.M. in μmoles trypsin inactivated/μmole PI ($n \geq 5$).
b. Single i.p. administration of drug or vehicle control.
c. Significantly different from controls by protected LSD, $p < 0.005$.

Carmustine treatment elicited a marked increase in both serum and lavage PI concentrations. Because lavage levels appear to result from the movement of PI from the circulation into the lung (Gadek et al., 1981) the fact that both physiological fluids qualitatively responded in a similar manner is not surprising. However, it is noteworthy that this is diametrically opposite to what was seen in the ACE studies. If decreased PI levels are to be associated with fibrosis, especially in the lung, it would appear that these data showing increased lavage PI content would contradict that theory.

One other possibility exists. It may be possible that the liver is no longer capable of synthesizing a functional alpha-1-PI based on the known liver toxicity associated with acute BCNU administration. Lu and Larson (1972) demonstrated decreased side chain oxidation of pentobarbital by liver microsomes 7 days after BCNU treatment, with maximal affects at 21 days. Carmustine caused marked cholestasis and inhibition of organic ion transport in rats 48 hours after a single i.p. injection of 20 mg/kg (Hoyt, 1984). Low dose treatment (15 mg/kg) was somewhat less effective at inducing cholestasis, but more significantly, maximum low dose cholestasis occurred 13 days after treatment. Carmustine also results in decreased microsomal P-450 content by 21 days, decreased ethylmorphine N-demethylase activity by two weeks, and changes in hepatic heme metabolism by 14 days (Stolzenbach, 1984). Based on this known hepatic toxicity, it seemed possible that there could be some definitive effects on PI. The ability to release PI into general circulation does not appear to be impaired based on the dramatic dose dependent increases in PI content seen from 21 to 28 days. However, the ability of the liver to

synthesize a functional PI does appear to be compromised based on data from these studies. Total lavage trypsin inhibitory activity was acutely depressed from 1 to 3 days after BCNU administration though this may be a direct inactivation of the PI since BCNU inhibited ACE activity. Over the more prolonged time course, 21 to 28 days, lavage trypsin inhibitor activity remained at control levels though lavage PI content increased from two- to fourfold. The fact that PI activity can be inhibited is even further demonstrated by the almost sixfold reduction in serum trypsin inhibitor capacity 28 days after 20 mg/kg BCNU administration. Based on these data, there is an apparent induction of alpha-1-PI synthesis in response to an as yet undetermined stimulus. The circulating PI is apparently not as functionally active, however, based on decreased serum trypsin inhibitory capacity.

The discrepancies in PI activity between lavage and serum is at first difficult to explain because all data has been normalized on a per μmole PI basis. Control lavage PI activity is .070 ± .009 μmoles trypsin inactivated/μmole PI compared with a serum value of 6.08 ± .46. It is possible that the other proteinase inhibitors present in serum (Stockley and Afford, 1983), especially alpha-2-macroglobulin are responsible for the increased trypsin inhibitor capacity seen in serum. Other serum proteinase inhibitors are effectively excluded from access to the alveolar space, so the majority of the lavage trypsin ihibitor capacity will be due to available active PI.

The preferential inactivation of serum PI activity compared to lung lavage PI activity by BCNU has at least two possible explanations. First, if the PI inactivation was a simple result of oxidation of the methionine reactive site, Met(O) reductase present in

the lung should be capable of reactivating the PI once it enters the lung (Abrams et al., 1981; Brot et al., 1981). Another possibility is that BCNU treatment is actually inactivating the other serum proteinase inhibitors to such an extent that the total trypsin inhibitor capacity of the serum is dramatically decreased. Since alpha-1-PI accounts for the greatest portion of serum inhibitor capacity, the extent of serum inhibition implies that alpha-1-PI must be inhibited at least in part. The BCNU-induced inactivation of ACE has been demonstrated earlier in this thesis, and it appears that PI might also be inactivated in vivo. These observations, coupled with the reported inactivation of glutathione reductase (Frischer and Ahmed, 1977; Babson and Reed, 1978; Maker et al., 1983; Smith and Boyd, 1984), malate dehydrogenase and lactate dehydrogenase (Maker et al., 1983), and lipoamide dehydrogenase (Ahmad and Frischer, 1985) would seem to indicate that BCNU has a much greater capacity to inactivate enzymes than previously established (Frischer and Ahmad, 1977). The problem is that the most severe PI inactivation occurs long after the body burden of BCNU is eliminated (Oliverio, 1976). Generalized proteinase inhibitor inactivation at these later time points may therefore be the result of a BCNU-induced modification of protein synthesis.

The interaction of the primary proteinase inhibitors, alpha-1-PI and alpha-2-macroglobulin (MG) in protecting against neutrophil generated elastase activity has been well described by Morse (1978). Alpha-1-PI combines rapidly ($K_{assoc}$ = 6.5 x $10^7$) with elastase to inactivate its elastinolytic actions (Weiss and Regiani, 1984). The half-life of the PI-elastase complex is approximately 90 minutes. Under normal circumstances elastase would rapidly combine with the

primary inhibitor and then be passed slowly to alpha-2-MG for complexation and transport to the reticuloendothelial system for metabolism. The elastase:alpha-2-MG complex is almost entirely cleared from circulation in as little as 12 minutes. It is known that in individuals having lower PI concentrations resulting from a genetic variation, the alpha-2-MG scavenger pathway is overloaded resulting in excess elastase-MG complexes in the alveoli. These complexes are phagocytized by macrophages and concentrated centrilobularly. It is now known that elastase-PI complexes can also be taken up by macrophages (Morse, 1978). The key feature is that elastase is still active when complexed to MG and therefore the complex released from dead macrophages could cause localized destructive breakdown of elastin. A similar mechanisms might be postulated for collagenase as well. It appears that when the system is overloaded, either by excessive elastase or by lack of alpha-1-PI, the primary protective mechanism for the lung is compromised. Defects inducing a MG deficiency in content or function have not yet been reported (Morse, 1978) leaving the PI defects as a primary area of investigation.

The actual source of PI inactivation in response to BCNU treatment is only speculative at this point. Based on the known liver toxicities, and the ability of BCNU moieties to alkylate DNA, RNA and various proteins, it is possible that some translational event in the synthesis of PI is altered, producing a functionally impaired PI. It has also been well established that PI can readily be inactivated (Abrams et al., 1981; Cochrane et al., 1983) by oxidation of the methionyl residue in the reaction site of the molecule. Evidence of oxidant activity in bronchoalveolar lavage fluid (BAL) has been

reported in patients with respiratory distress syndrome (Cochrane et al., 1983; Merritt et al., 1983). Weiss and Regiani (1984) demonstrated that PMN cells are capable of counteracting the effects of PI and thus are able to attack the subendothelial matrix. Zaslow et al. (1983, 1985) found that the myeloperoxidase system is effective in oxidizing PI in vivo via the release of elastase and an oxidant with characteristics similar to hypochlorous acid. They reported that $1.25 \times 10^7$ neutrophils can potentially inactivate 162.5-325 µg of PI.

Oxidation cannot reactivate PI bound elastase. However, the enzymatic reduction of oxidized PI to restore its biological activity has been reported (Abrams et al., 1981; Brot et al., 1981). Methionine sulfoxide [Met(O)] peptide reductase was found to restore the biological inhibitory capacity of oxidized canine alpha-1-PI. High levels of Met(O) reductase would not be expected in tissues but low levels might be present to reduce the methionine sulfoxide that accumulates in proteins in vivo under the influence of various oxidizing agents, i.e. peroxides, hydroxyl radicals and superoxides. The harmful effects of these oxidants would normally be neutralized by cellular enzymes such as catalase, peroxidases and superoxide dismutase. Brot et al. (1981) demonstrated progressively decreasing Met(O) reductase activity in rat liver, kidney, heart, lung and brain implying that the well described cellular antioxidant systems might not be entirely sufficient.

This study has demonstrated significant increases in PI in response to a presently unknown stimulus. The serum PI is functionally less active and is therefore presumably less capable of filling its primary protective role though in the lung it retains its activity and

is more likely to be overwhelmed by localized increases in elastase concentration. The demonstration of a functionally compromised serum PI opens several avenues for further investigation. If the PI is inactivated via an oxidative process prior to or after its release from the liver, Met(O) reductase experiments should be able to restore PI activity. Perhaps liver Met(O) reductase is inactivated by BCNU or a BCNU breakdown product as has been described for other enzyme systems (Smith and Boyd, 1984; Ahmad and Frischer, 1985). An inflammatory response capable of increasing PMN cells to a level sufficient to inactivate PI, has not been well described for BCNU or other fibrotic agents. Alternatively, histological examination of hepatocytes might demonstrate abnormalities in PI synthesis and release in a manner similar to that reported in PI deficient children (Morse, 1978).

While the demonstration of reduced PI activity was a significant finding, the next phase in the research required the demonstration of elastase in concentrations sufficient to induce pulmonary damage. Also, assuming tissue damage was occurring, it should be possible to obtain biochemical evidence of this damage.

# Chapter IV. The Effects of BCNU on Elastase Activity, Lung Lipid Peroxidation and Collagen Synthesis in the Lung

## A. Introduction

The marked induction of alpha-1-proteinase inhibitor (PI) content after BCNU administration was an important observation. As previously mentioned, two possible explanations might account for these results. Induction of the hepatic synthesis of PI in response to BCNU treatment might well force more PI into general circulation than would occur under normal physiological sequences. Although it appears BCNU has no inhibitory effects on general protein synthesis, in view of other manifestations of hepatic toxicity it does not seem reasonable to assume that increased basal synthesis of PI in response to BCNU would be occurring.

More probable is the second possibility, that increased PI concentrations in plasma and lung lavage are the result of some damaging mechanism in the lung initiating the release of PI in an attempt to counteract the insulting agent. Even in the presence of very high concentrations of PI, it might be possible to generate localized concentrations of proteolytic activity that cannot be effectively neutralized by PI (Morse, 1978). The enzymes actually capable of initiating proteolytic activity in the lung are rather limited. Trypsin is not capable of acting on native collagen or elastin and in fact is rarely found in general circulation (Morse, 1978). Trypsin is, however, known to produce pulmonary hemorrhage and edema but not emphysema (Osman et al., 1985). Collagenase also apparently has

little effect on the elastic properties of the lung. However, the ability of elastase to induce emphysema has been described by several investigators (Morse, 1978; Gadek et al., 1981; Valentine et al., 1983; Osman et al., 1985). Though emphysema and fibrosis are two different clinical and pathological processes, it has been proposed that they may actually be divergent responses to the same initiating event (Niewoehner and Hoidal, 1982). Niewoehner and Hoidal (1982) demonstrated that $CdCl_2$-induced lung injury can be selectively modified by simultaneous exposure to beta-aminoproprionitrile fumarate, resulting in emphysematous lesions rather than the fibrotic lesions usually observed when the modifying agent was not present.

The primary sources of elastase are polymorphonuclear (PMN) cells and macrophages. Neutrophil generated elastase apparently accounts for the greatest portion of enzyme activity (Janoff and Scherer, 1968; Merritt et al., 1983). Increased elastase activity appears to be the result of an initial acute inflammatory response (Chvapil and Peng, 1975). Increased elastase activity might occur in the apparent absence of an inflammatory cell population if the neutrophils and macrophages were tissue bound, secreting lysosomal proteases that are measurable though the cells themselves may have no access to airway lavage fluids (Merritt et al., 1983). Such a scenario does suggest a relationship between some form of inflammatory response and elastase activity. Therefore the measurement of elastase activity may provide a biochemical marker for potential lung parenchymal injury. For these reasons this study was designed to measure activity in response to acute BCNU exposure.

As a possible primary cause of the inflammatory response and in view of the inhibitory actions of BCNU on glutathione reductase, studies were conducted looking for evidence of involvement of lipid peroxidation in lung tissue taken from BCNU-treated rats. Lipid peroxidation is the oxidative deterioration of polyunsaturated lipids (Plaa and Witschi, 1976). Lipid peroxidation *in vivo* is thought to play a major role in the aging process, cellular damage from air pollution, some types of atherosclerosis, toxic liver injury from several chlorinated hydrocarbons and oxygen toxicity to the lung. The possible role of lipid peroxidation in paraquat (Bus *et al.*, 1976) and oxygen (Chvapil and Peng, 1975) induced pulmonary toxicity has already been outlined. Dahle *et al.* (1962) described the process responsible for the formation of malondialdehyde (MDA), a secondary product derived from lipid peroxides of polyunsaturated fatty acids with a minimum of three double bonds. The formation of MDA has been associated with the functional loss of selected microsomal enzymes in the liver including glucose-6-phosphatase, oxidative demelthylation of aminopyrine, hydroxylation of aniline, NADPH oxidation and menadione-dependent NADPH oxidation (Plaa and Witschi, 1976). Decreased cytochrome P-450 concentration in hepatic microsomes has also been reported (Plaa and Witschi, 1976). Under appropriate *in vitro* conditions, lipid peroxidation has induced a loss of functional membrane integrity in microsomes, mitochondria and lysosomes.

Based on this apparent association of lipid peroxidation and membrane damage taken together with reports that agents known to deplete cellular GSH could possibly induce acute lipid peroxidation (Younes and Siegers, 1981), the measurement of pulmonary MDA content

appeared to be a reasonable course of investigation. A number of assays have been used in measuring lipid peroxidation including the thiobarbituric acid (TBA) reaction with MDA (Dahle, et al, 1962; Ohkawa et al., 1979) the detection of conjugated dienes by measuring $^{14}C$ incorporation from tetracyanoethylene-$^{14}C$ (Waller and Recknagel, 1977), the fluorescent measurement of lipid peroxidation products (Fletcher et al., 1973) and ethane evolution (Riely et al., 1974). The TBA methodology described by Ohkawa et al. (1979) was chosen based on the popularity of the method, as well as its sensitivity and freedom from bilirubin interference. Bilirubin interference could be a significant problem since many animals were jaundiced in appearance by 14 days after BCNU treatment.

The association of collagen and fibrosis has already been described. While the fibrotic process after BCNU exposure is well defined in humans, the experimental development of the disease in an animal model is less certain (Kehrer, 1984; Kehrer and Paraidathathu, 1984; Reznik-Schuller et al., 1984). These previous studies had used BALB/C mice and Fischer 344 rats, respectively. If, indeed, the toxic effects of BCNU lead to pulmonary fibrosis, demonstrable changes in collagen content or composition should be possible. The inability of other investigators to demonstrate increased collagen synthesis (Reznik-Schuller et al., 1984; Kehrer, 1984) might be due to the apparent resistance of the model chosen, or the fact that they analyzed time points prior to the actual deposition of collagen. Intuitively it seems that the biochemical demonstration of increased collagen content might be possible at a time point before the material can be histologically demonstrated. While BCNU-induced biochemical

changes in hepatic metabolic processes have been demonstrated in mice, experiments with these animals have not yet demonstrated increased collagen. Fischer 344 rats also appear to be more resistant in view of the multiple dosing requirement reported by other investigators (Smith and Boyd, 1984; Reznik-Schuller et al., 1984).

Collagen content as determined by hydroxyproline measurements (Woessner, 1961; Stegemann and Stalder, 1967) were performed using the Sprague Dawley rats to ascertain whether they respond to BCNU treatment with increased collagen deposition. Because the collagen process is normally a balance between synthesis and degradation processes, if increased lung collagen deposition is detected, then serum and lavage samples will be measured for collagen synthesis and breakdown products. This should help determine whether the fibrotic response is actually a result of an increased synthesis rate, a decreased degradation rate, or some combination of both processes.

B. Methods

N-succinyl-L-(alanyl)$_3$-p-nitroanilide (SAPNA) and 1,1,3,3-tetramethoxypropane were obtained from Sigma Chemical Company, St. Louis, MO. Sodium-N-chloro-p-toluene sulfonamide (chloramine-T) was obtained from Eastman Kodak Company, Rochester, NY. Para-dimethylaminobenzaldehyde was obtained from Calbiochem, San Diego, CA. Hydroxy-L-proline was from Nutritional Biochemicals Corp., Cleveland, OH. Porcine pancreas elastase was procured from Calbiochem-Behring, La Jolla, CA. Amberlite IRA 410 (100-200 mesh) was obtained from Mallinckrodt Chemical Works, St. Louis, MO. All other chemicals were 'Baker

Analyzed' grade or better and were obtained from J.T. Baker Chemical Company, Phillipsburg, NJ. Carmustine was obtained from NCI. Male Sprague Dawley rats were obtained and maintained as previously described.

Carmustine solutions were prepared fresh prior to administration. Carmustine was dissolved in corn oil at concentrations of 15 or 20 mg/ml. Animals in each experimental group were dosed i.p. as previously described with the appropriate BCNU dose or vehicle control.

At intervals of 7, 14, 21, 28 and 35 days post treatment, specimens were obtained from a minimum of five animals from each experimental group. For the lavage and serum elastase and collagen determinations animals were anesthetized, a tracheostomy performed, and pulmonary lavage samples collected as previously described. Blood samples were obtained by cardiac puncture also as previously described. Rats were then killed by cervical dislocation followed by thoracotomy. All samples were immediately centrifuged and stored as previously described for subsequent analysis.

For lung lipid peroxidation measurements, animals were fasted for 12 hours prior to the experiment. Five animals from each experimental group were killed by cervical dislocation. The lungs were removed and trimmed free from excessive tissue, rinsed with saline, blotted dry and weighed. The entire lung was then placed in 5.0 ml of 0.05M $NaH_2PO_4$-$H_2O$ (pH 7.8) containing 0.9% NaCl and 0.001M $Na_2EDTA$. The lung was finely minced and washed a second time with fresh buffer. After washing, fresh buffer was added (1 g tissue: 9 ml buffer) and the tissue homogenized for 30 seconds at full speed on a Janke and

Kunkel polytron. Aliquants of the fresh homogenate were used for lipid peroxidation determination.

For lung collagen determination, the lungs were excised as described above. After trimming excess tissue the lungs were weighed in ice cold saline. The lungs were then finely minced and rinsed with iced saline before being homogenized as described above in ice cold 1.15% KCl (1 g tissue: 9 ml 1.15% KCl). Aliquants of the homogenate were used for lung collagen determination.

The elastase procedure is one described by Merritt et al. (1983) utilizing a p-nitroanilide substrate described by Yasutake and Powers (1981). A 0.3 mM solution of SAPNA substrate was prepared in 0.2M Tris containing 1 mg/ml of bovine serum albumin (pH 8.0). Acetic acid (1.0 N) was prepared in deionized water. Elastase standard was prepared similarly to SAPNA in Tris buffer. The reactions were carried out in 11 x 75 mm test tubes using 0.5 ml of the neat lavage or 0.5 ml of a 1:5 dilution of serum in 0.9% NaCl solution. Various concentrations of elastase standard were also added in 0.5 ml portions and run with each set of assays. All samples received 0.5 ml of 0.3 mM SAPNA. The tubes were briefly mixed and incubated 15 minutes at 25°C in a Dubnoff Metabolic Shaking Incubator under ambient atmosphere. At the end of the incubation period, 0.5 ml of 1N acetic acid was added to stop the reaction and the tubes were briefly vortexed. Sample absorbance was read at 410 nm against a reagent blank containing no elastase using a Bausch and Lomb Spectronic 600 UV/VIS spectrophotometer. Quantification of elastase activity in each sample was made by extrapolating the absorbance of the unknown from a standard curve prepared using various concentrations of elastase.

Activity was reported as elastase units per milliliter of physiologic fluid. One unit of elastase activity is defined as the amount of enzyme that will hydrolyze 1.0 micromole of N-acetyl-tri-L-alanine methyl ester per minute at 25°C, pH 8.0.

Lipid peroxidation was determined using a malondialdehyde assay described by Ohkawa et al. (1979). Sodium dodecyl sulfate (SDS) and 2-thiobarbituric acid (TBA) were prepared in distilled, deionized water at concentrations of 8.1% and 0.8%, respectively. A 20% acetic acid solution in 0.27M HCl was adjusted to pH 3.5 using 1.0N NaOH. N-butanol and pyridine were combined in a ratio of 15:1. 1,1,3,3-tetramethoxy propane (TMP) as a standard was used as supplied by the manufacturer. The reaction was performed in a 14 x 175 mm test tube to which was added 0.2 ml of lung homogenate, 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid, 1.5 ml of 0.8% TBA and 0.6 ml of distilled deionized water. The contents were briefly vortexed and incubated for 60 minutes at 95°C in a water bath. At the end of the incubation period 1.0 ml of water was added to each tube followed by 5.0 ml of n-butanol:pyridine (15:1). The entire contents were vortexed and centrifuged for 10 minutes at 2000 x g to separate the organic and aqueous layers. An aliquant from the upper organic layer was diluted 1:21 with additional n-butanol:pyridine and the fluorescence read against a reagent blank on a Turner (Model 12) fluorometer at an excitation wavelength of 515 nm and an emission wavelength of 553 nm. Values for unknowns were extrapolated from a standard curve of 1,1,3,3-tetramethoxypropane run under identical conditions with each assay group. Malondialdehyde for each sample was reported as TMP equivalents.

Lung collagen was determined using an assay described by Huszar et al. (1980). The working buffer was prepared by adding 50 g citric acid $H_2O$ + 120 g Na-acetate-$3H_2O$ + 34g NaOH + 12 ml (96%) acetic acid + 700 ml $H_2O$. The buffer was adjusted to pH 6.0 and then additional water added to a final volume of 1.0 liter. Ten drops of toluene were added as a preservative. A 0.017M chloramine-T solution was prepared by the addition of 1.41 g chloramine-T + 10 ml n-propanol + 10 ml $H_2O$ and then adjusting to a final volume of 100 ml with the buffer solution. The chloramine-T solution was stable for approximately 4 weeks if stored in a dark bottle at 4°C. Fresh aldehyde/perchloric acid solution for each assay was prepared by the addition of 15 g of p-dimethylaminobenzaldehyde + 60 ml n-propanol + 26 ml (60%) perchloric acid and adjusting to a final volume of 100 ml by the addition of n-propanol. A 0.1 mg/ml hydroxyproline standard was made using the working buffer. Sodium hydroxide (4N), 1.4N citric acid, and 1N HCl were prepared in distilled water.

The collagen assay was performed by the addition of 0.2 ml of tissue homogenate to a 14 x 175 mm test tube. The homogenate was then dried in an oven at 100°C. After drying, 0.05 ml of 4N NaOH was added to each tube, vortexed and then autoclaved for 10 minutes at 120°C. After autoclaving, 0.05 ml of 1.4N citric acid was added to each tube and mixed. Each tube was checked and adjusted if required to approximately pH 6 with 1.4N citric acit or 4N NaOH. One milliliter of the 0.017M chloramine-T solution was then added to each tube. Contents were mixed and incubated for 20 minutes at room temperature. After incubation each tube received 1.0 ml of aldehyde/perchloric acid solution. Tubes were again vortexed and incubated for 15 minutes at

65°C in a water bath. At the end of this last incubation step, the reaction was terminated by placing the tubes in an ice bath. The absorbance of each tube was measured at 550 nm versus a reagent blank (no tissue homogenate) using a Bausch and Lomb Spectronic 600 UV/VIS spectrophotometer. The collagen content for each sample, as hydroxyproline concentration, was determined by extrapolating from a standard curve of hydroxyproline run with each set of assays.

Lavage and serum were analyzed for hydroxyproline content after a chromatographic separation into water soluble and acid soluble fractions. Sera were autoclaved at 120°C and 30 psi for three hours. Both the autoclaved serum and the neat lavage were filtered and the aqueous fraction collected. All precipitates were washed twice with 3.0 ml of water and the washing combined with their respective aqueous fraction. The pooled samples were then applied to a 0.8 cm x 10 ml Amberlite IRA 410 column previously equilibrated with working buffer consisting of 50 ml of collagen buffer plus 450 ml of water. The high molecular weight products of collagen breakdown were eluted from the column with 35 ml of water. Low molecular weight hydroxyproline peptides and free hydroxyproline were eluted from the column using 50 ml of 1N HCl. The column was reactivated before the next sample was applied using a 200 ml wash of the working buffer. One milliliter of each ion exchange column eluent was assayed for hydroxyproline content as described above.

White blood cell (WBC) counts were performed on whole blood to establish the time course of hematopoietic depression associated with BCNU administration. The procedure for counting WBCs is described by Fox et al. (1982). A 1:20 dilution of whole blood was made in a

standard WBC pipette with 3% acetic acid as the diluting fluid. The pipette was placed on a Yankee Pipette Shaker, Parsippany, NJ, and mixed for 3 minutes to lyse the red blood cells. After discharging a small volume from the pipette, both sides of a Neubauer counting chamber (hemocytometer) were charged. The four large corner squares of each chamber were counted using a 10x objective and the counts from both chambers averaged. The WBC count per $mm^3$ was obtained by multiplying the average count by the dilution factor.

Analysis of variance was applied to all data and when a significant F-ratio was calculated, a least significant difference test was used to inspect the difference between mean values.

C. Results

Lavage elastase activity began to increase 21 days after administration of 20 mg/kg BCNU reaching a maximum of .138 ± .016 elastase units/ml (control: 0.070 ± .004) at four weeks (Figure 10). Low dose BCNU treatment (15 mg/kg) did not significantly elevate lavage elastase activity at any time point tested through 28 days ($p > .25$). Serum elastase activities were 185 and 212% of the control level of 1.04 ± .16 elastase units/ml 28 days after the administration of 15 or 20 mg/kg BCNU, respectively.

Lung lipid peroxidation, as measured by malondialdehyde concentration was significantly elevated ($p < 0.005$) by seven days after the administration of either dose of BCNU (Figure 11). Over the next 21 days the MDA concentration of the lung homogenates decreased toward control values with the 15 mg/kg BCNU group returning to 0.11

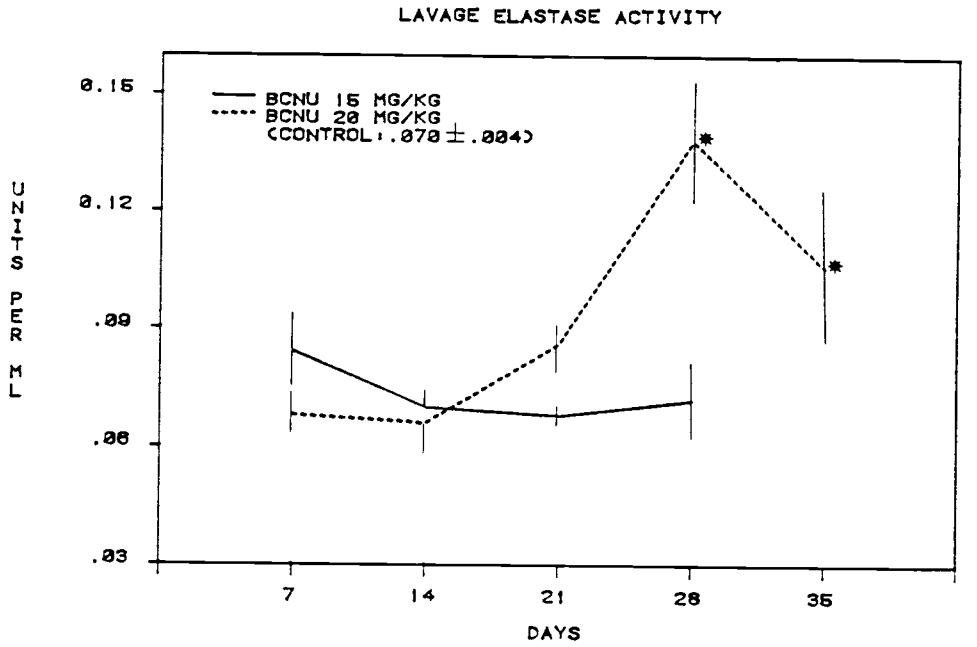


Figure 10. Lung Lavage Elastase Activity in Sprague Dawley Rats After BCNU Administration

Values are plotted as the mean ± S.E.M. in elastase units/ml for five animals.
*Significantly different from controls by protected LSD, $p < 0.005$.

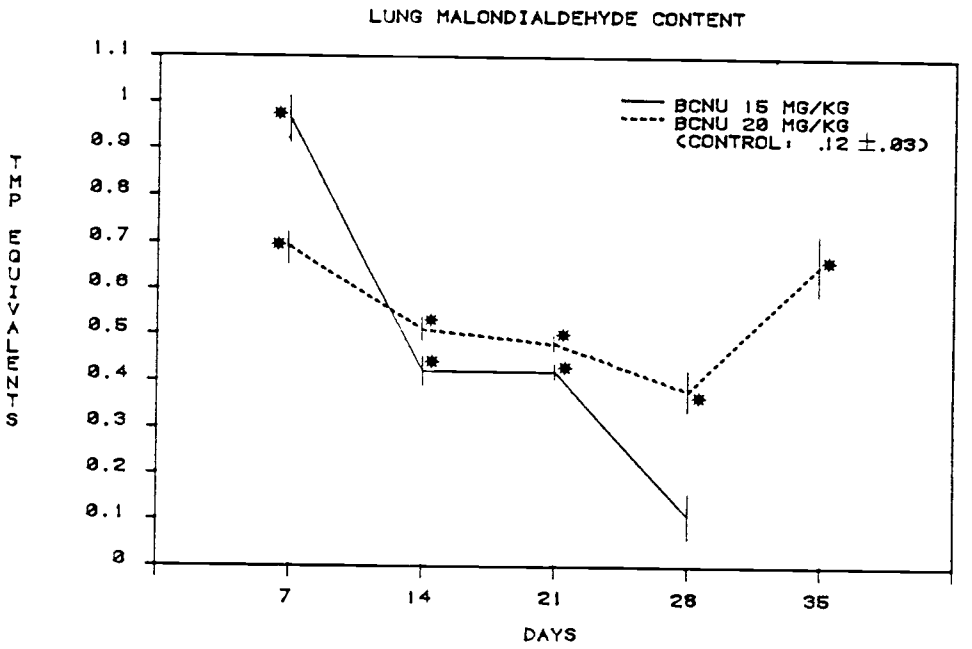


Figure 11. Lung Malondialdehyde Concentration in Sprague Dawley Rats After BCNU Administration

Values are plotted as the mean ± S.E.M. in TMP equivalents/ml for five animals.
*Significantly different from controls by protected LSD, $p < 0.005$.

± .05 TMP equivalents (control: 0.12 ± .03) at 28 days postdosing. The high dose treatment group reached a nadir at 28 days of 0.38 ± .04 TMP equivalents (317% of control levels) and then the MDA concentration began to increase again as evidenced by a rise to 0.66 ± .07 TMP equivalents at 35 days.

From 7 to 14 days, lung collagen content dropped significantly ($p < 0.005$) from 105 to 59% of control levels (Figure 12). Lung collagen content increased after 14 days reaching 159% of controls by 28 days. The source of increased collagen concentration would appear to be increased levels of the collagen precursors, i.e., acid soluble low molecular weight hydroxyproline peptides plus free hydroxyproline (Table 8). From 14-28 days there was approximately a 50% increase in collagen precursors in the serum with no significant change in the high molecular weight products of collagen breakdown ($p > .25$). Additionally, while lavage showed a transient increase in precursors at 21 days, lavage breakdown products significantly decreased from 14-28 days ($p < 0.005$).

White blood cell counts in whole blood decreased at three days to 67% of control levels of 6076 ± 467 WBC/mm$^3$ (Figure 13). By 14 days the treated groups were 84% of controls and by 28 days postdosing they were 117% of control.

## D. Discussion

The dose- and time-dependent increases in lavage elastase concentration roughly paralleled the time course observed for increased lavage proteinase inhibitor content. Under normal physiological

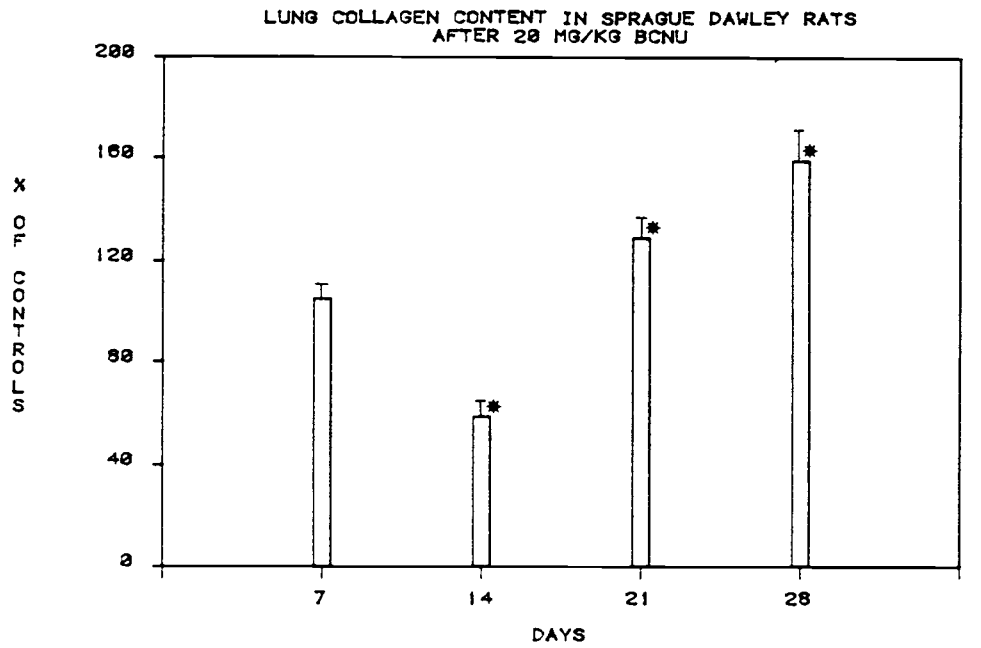


Figure 12. Lung Collagen Concentration in Sprague Dawley Rats After BCNU Administration

Values are plotted as mean ± S.E.M. as percentage of age matched controls (n = 5), control: 1.44 ± .032 µg hydroxyproline/mg protein.
*Significantly different from controls by Student's t-test ($p < 0.05$).

Table 8. Serum and Lung Lavage Collagen Fractions in Sprague Dawley Rats After BCNU Administration [a]

| Tissue | Fraction | Post-dosing Interval (days) Control | 21 | 28 |
|---|---|---|---|---|
| Serum | $H_2O$ soluble [b] | .059 ± .002 | .061 ± .006 | .058 ± .005 |
| | $H^+$ soluble [c] | .122 ± .019 | .118 ± .012 | .177 ± .007 [d] |
| Lavage | $H_2O$ soluble | .072 ± .004 | .051 ± .004 [d] | .047 ± .005 [d] |
| | $H^+$ soluble | .079 ± .008 | .119 ± .020 [d] | .063 ± .007 |

a. Single i.p. administration of 20 mg/kg BCNU or vehicle control. Values expressed as mean ± S.E.M. in µg hydroxyproline/mg protein (n=5).
b. High molecular weight products of collagen breakdown.
c. Low molecular weight hydroxyproline peptides plus free hydroxy proline for collagen synthesis.
d. Different by Student's t-test ($p < 0.05$).

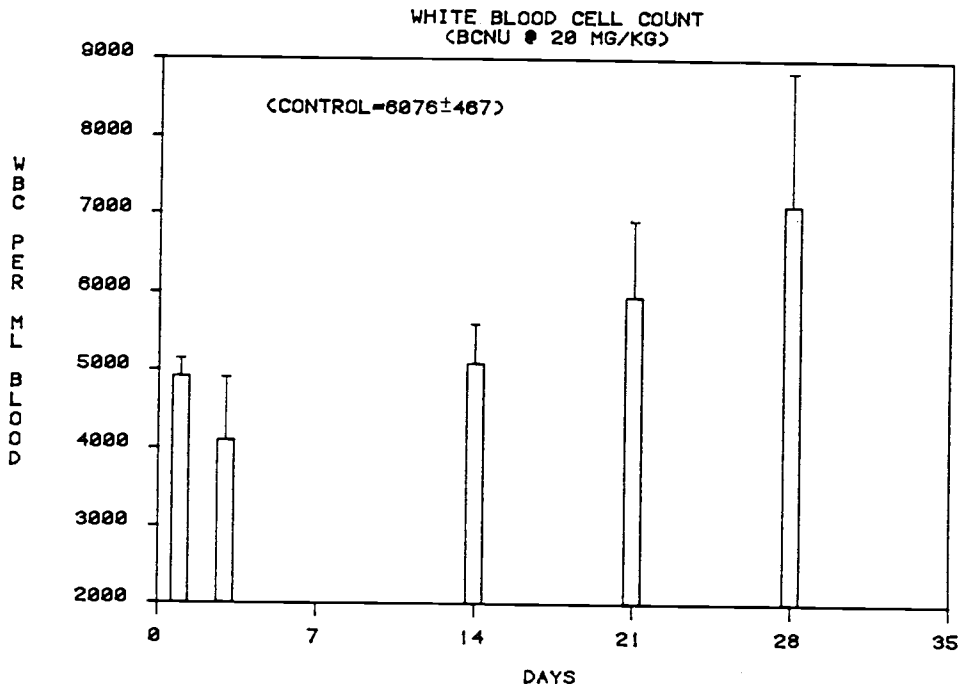


Figure 13. Whole Blood White Blood Cell Count in Rats After BCNU Administration

Values are plotted as mean ± S.E.M. in WBC/mm$^3$ (n=5). No significant differences by protected LSD, $p > 0.25$.

conditions this pattern might be expected. As elastase concentrations rise, the liver synthesizes more PI for eventual transport to the lung where it can inactivate the elastase. The actual relationship between increased lung elastase and PI concentration was a little peculiar however, in that lung lavage PI content in treated animals decreased from 21 to 28 days (Figure 7), a time over which elastase activity increased to its maximal values (Figure 10). Over this same time course lavage PI activity, as measured by total trypsin inhibitor capacity, remained essentially unchanged from control levels (Figure 9). Thus, even in the presence of increased amounts of functionally active proteinase inhibitor, BCNU-treated rats had significant increases in lavage elastase concentration. This possibility has been reported previously by Morse (1978) who postulated that high localized concentrations of proteolytic activity might not be effectively neutralized by PI. Nevertheless, because the lavage procedure samples virtually the entire pulmonary air compartment it would also sample any localized areas of increased elastase content.

From 21 through 35 days increased pulmonary elastase content might be present in sufficient quantities to initiate localized areas of tissue damage. It would also appear that elastinolytic damage was occurring because from 21 to 28 days treated lung collagen content increased from 129% of control to 159% of control. These observed increases in treated lung collagen concentration in Sprague Dawley rats appear to be the first experimental biochemical demonstration of BCNU-induced fibrosis since neither Reznik-Schuller *et al.* (1984) using the Fischer 344 rat, nor Kehrer (1984) using mice, were able to demonstrate increased collagen content in BCNU-treated groups. The

lack of demonstrable collagen in the Fischer-344 is peculiar because these same investigators claim all treated animals developed pulmonary fibrosis (Reznik-Schuller et al., 1984).

Accompanying the changes in total lung collagen content where some significant changes in the water and acid soluble collagen fractions from treated lavage and serum samples. In treated sera, the hydroxyproline concentration in the water soluble fractions, representing the high molecular weight products of collagen breakdown, was not significantly increased in the concentration of hydroxyproline/mg protein from 2 to 4 weeks after BCNU treatment. Over this same time course, there was a significant increase in the low molecular weight, acid soluble precursors of collagen synthesis. Taken together, increased precursor material in the absence of any change in collagen destruction could account for the increased lung collagen content observed. During this same period of time, lavage precursor material from treated animals showed a transient increase at 21 days and then a slight decrease at 28 days. There was a significant reduction in the lavage concentration of the high molecular weight collagen breakdown products in treated animals. The final result would be similar to that seen in serum, increased net collagen content resulting from unchanged or increased synthesis and decreased degradation.

The primary enzyme responsible for collagen degradation is collagenase although there are two additional processes, intracellular fibroblastic degradation and a phagocytic process (Bienkowski et al., 1978). Neutrophils are a prime source of collagenase as well as elastase and therefore collagenase content would probably show localized increases in concentration paralleling increases in elastase.

The primary difference is that collagenase exists in both a latent and an active form (Lindblad and Fuller, 1983); the latent form is probably activated by trypsin. Decreased collagenase activity would be expected to lower the concentration of collagen breakdown products explaining the lavage results from BCNU-treated rats seen in these experiments. In the early stages of $CCl_4$-induced hepatic fibrosis, hepatic collagenase activity increased (Lindblad and Fuller, 1983) and the same is true for bleomycin-induced IMR-90 fibroblasts *in vitro* (Sterling *et al.*, 1982). In the liver at least, long term fibrosis is associated with decreased collagenase activity (Lindblad and Fuller, 1983). The observed increases in lung collagen content following BCNU treatment might be explained by an inactivated collagenase (e.g., via increased proteinase inhibitor activity) resulting in less degradation accompanied by fibroblastic activation and therefore net collagen deposition.

Finally, there is considerable support for BCNU-induced peroxidative membrane damage based on the time- and dose-dependent changes in thiobarbituric acid (TBA) reactive products seen in these experiments. The acute increase in MDA concentration seen at seven days with both BCNU doses would tend to support the argument that compounds which can deplete cellular GSH levels are capable of inducing lipid peroxidation (Younes and Siegers, 1981). Subsequently, the MDA content of lungs of rats treated with 15 mg/kg BCNU returned to basal control levels by 2 weeks after treatment. A bimodal response was observed, however, in rats receiving 20 mg/kg. Lungs of these animals never contained less than twice the control MDA concentration and after 28 days again began

to show increased levels of TBA reactive products implying increased membrane damage over this delayed time frame.

Peroxidative membrane effects over the longer time periods (greater than 7 days) cannot be the result of a direct BCNU action because of its relatively short plasma half-life. Although at present there is only indirect evidence for an increased inflammatory cellular response (i.e., because of increased lavage elastase concentration), the presence of neutrophils could account for increased lipid peroxidation based on experimental evidence that stimulated neutrophils are capable of generating oxidants (Cohen, 1979; Schraufstatter et al., 1984) and because of reports of oxidants derived from endothelial cells (Martin, 1984).

# Chapter V. The Effects of BCNU on Catalase Activity, Glutathione Content and Glutathione Shuttle Enzyme Activity in the Lung

## A. Introduction

In light of results suggesting BCNU-induced increases in lipid peroxidation and collagen deposition using the Sprague Dawley rat and the known effects of BCNU on glutathione reductase activity, the next question involved investigating the possibility of BCNU-induced interference with the primary pulmonary mechanisms for protecting against such damaging processes. Two separate enzymatic systems have been described for protecting against oxygen radical-induced lipid peroxidation: the glutathione redox cycle (Harlan et al., 1984) and the dismutation of superoxide radicals to hydrogen peroxide catalyzed by superoxide dismutase (McCord and Fridovich, 1969) followed by a reduction to oxygen and water under the influence of catalase (Minchin and Boyd, 1983). Additionally, glutathione (GSH) is involved in the enzymatic or spontaneous inactivation of reactive electrophiles or activated neutrophils.

The toxic intermediates of oxygen reduction are superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^{\cdot}$). The dismutation of the superoxide radical, $O_2^{\cdot-}$, can be a spontaneous process by a second order reaction dependent on the superoxide radical concentration. In the absence of scavengers, the anion half-life increases from 0.05 sec at $10^{-4}$M to 14 hr at $10^{-10}$M (Fridovich, 1983). In the presence of superoxide dismutase (SOD), the half-life is independent

of the $O_2^{\cdot-}$ concentration and enzymatic dismutation may be as much as $10^{10}$ faster than the spontaneous process. The dismutation forms another toxic by-product, $H_2O_2$. Catalases and peroxidases are present to reduce $H_2O_2$ to oxygen and water. These enzymes are also mutually protective since $O_2^{\cdot-}$ is known to inactivate catalase and heme containing peroxidases while $H_2O_2$ inactivates two of the three known subtypes of SOD (Fridovich, 1983).

There are a wide variety of sources for superoxide radicals. Autoxidations known to produce $O_2^{\cdot-}$ include hemoglobin, myoglobin and catecholamines to name just a few. Some $O_2^{\cdot-}$ is produced in the catalytic cycles of xanthine oxidase, aldehyde oxidase, dihydro-orotic dehydrogenase and several flavin dehydrogenases. Mitochondria, microsomes and nuclei are known to generate $O_2^{\cdot-}$ (Turrens et al., 1982).

The potential sources of $O_2^{\cdot-}$ of special interest are the neutrophils and macrophages. The phagocytic process in neutrophils activates membrane bound NADPH oxidase to generate $O_2^{\cdot-}$ (Cohen, 1979) and consequently $H_2O_2$. These products of the 'respiratory burst' (Babior, 1984) then act to generate microbiocidal oxidants such as hypochlorite ($OCl^-$) or hydroxyl radicals ($OH^{\cdot}$). It is also possible that a small portion of the superoxide anion is released into the extracellular environment where it can cause tissue damage. Though macrophages release low levels of $H_2O_2$ at rest, there is no increase in peroxide production in response to the phagocytic process, an event that results in a large release of $H_2O_2$ from neutrophils (Biggar and Sturgess, 1978).

Glutathione and the glutathione redox cycle is an important alternate pathway to protect cells from lipid peroxidation. The

glutathione shuttle system involves glucose-6-phosphate dehydrogenase (G-6-POH) as a source of NADPH, glutathione peroxidase (GPx) and glutathione reductase (GR). Though this cycle can act directly to reduce oxidized glutathione (GSSG), reduced glutathione may also enter the cycle from glutathione biosynthesis (Harlan et al., 1984). Glutathione peroxidase can also catalyze the conversion of $H_2O_2$ to $2H_2O$ by the oxidation of GSH. In the absence of a functional catalase, the peroxidase pathway would serve as an alternate mechanism for reducing $H_2O_2$. Additionally this glutathione shuttle system may utilize GSH to reduce lipid peroxides to lipid alcohols inactivating the lipid free radical mechanism of membrane damage (Minchin and Boyd, 1983).

Studies have shown that agents known to deplete hepatic GSH levels result in enhanced lipid peroxidation even in the presence of oxygen radical scavengers (Younes and Siegers, 1981). In studies of long term exposure to oxidative stress, the protective mechanisms showed a decreased ability to protect against increases in lipid peroxidation (Ichinose and Sagai, 1982). It has also been demonstrated that the induction of the glutathione shuttle system can protect against ozone induced lipid peroxidation (Chow and Tappel, 1972). A number of agents are known to selectively inhibit different portions of the glutathione redox cycle. Carmustine-induced inhibition of GR has already been mentioned (Frischer and Ahmad, 1977; Babson and Reed, 1978). The formation of thioether conjugates from electrophilic agents such as 1-chloro-2,4-dinitrobenzene and diethylmaleate acts to deplete cellular stores of GSH (Harlan et al., 1984). Glutathione

biosynthesis can be blocked by buthionine sulfoximine inhibition of gamma-glutamyl-cysteine synthetase (Meister, 1983).

In view of the importance of these protective mechanisms to the lung, experiments were conducted to measure the effect of BCNU on catalase activity, glutathione content, and the enzyme activity of the glutathione shuttle pathway in the lungs of Sprague Dawley rats. Though BCNU is known to directly inhibit GR, the increases in TBA reactive products seen 3 weeks after treatment imply that the protective mechanisms might be compromised in some other fashion to permit increased lipid peroxidation.

## B. Methods

Catalase (EC.1.11.1.6), glutathione reductase (GR) (EC 1.6.4.2), N-ethylmaleimide (NEM), 5,5'-dithiobis(2-nitrobenzoic acid)(DTNB), hydrogen peroxide ($H_2O_2$), and cumene hydroperoxide were obtained from Sigma Chemical Company, St. Louis, MO. Reduced nicotinamide adenine dinucleotide phosphate (NADPH), nicotinamide adenine dinucleotide phosphate (NADP), and glucose-6-phosphate (G-6-P) were obtained from Calbiochem-Behring, La Jolla, CA. Reduced glutathione (GSH) was obtained from Boehringer-Mannheim, Indianapolis, IN. Oxidized glutathione (GSSG) was obtained from Nutritional Biochemical Corporation, Cleveland, OH. Triethanolamine was obtained from Mallinckrodt Chemical Works, St. Louis, MO. Bovine serum albumin (BSA) was obtained from Calbiochem, San Diego, CA. Carmustine was obtained from NCI, Bethesda, MD. All other reagents were 'Baker Analyzed' grade or better and were procured from J.T. Baker Chemical Company,

Phillipsburg, NJ. Male Sprague Dawley rats were obtained and maintained as previously described.

Carmustine solutions in corn oil were prepared fresh prior to administration at a concentration of 20 mg/ml. Animals received a single i.p. dose of BCNU or the vehicle control at 20 mg/kg (0.1 ml/100g). At intervals of 1, 3, 7, 14 and 21 days animals were sacrificed by cervical dislocation. The lungs were perfused *in situ* with cold heparinized saline via the right ventricle and clipping the pulmonary vein. Lungs were then removed, rinsed, blotted dry, weighed and homogenized in 5.0 ml of 0.05M $NaH_2PO_4$-$H_2O$ (pH 7.8) containing 0.9% NaCl and 0.001M $Na_2$-EDTA. The homogenate was centrifuged at 4°C and 102,000 x g for 60 minutes using a Beckman Model L3-50 ultracentrifuge. The resulting supernatant was used for the analysis of catalase, GPx, GR and G-6-PDH activities as well as protein content.

Animals used for GSSG and total glutathione determinations were monitored for food intake to permit age-matched control animals to be pair fed. Twenty-four and 72 hours after BCNU or vehicle control treatment the animals were sacrificed by an i.p. overdose of sodium pentobarbital (120 mg/kg). Lungs were perfused *in situ* as previously described, removed, rinsed, blotted dry and weighed. Lung tissue was homogenized in two volumes of 0.1M phosphate buffer (pH 6.2) containing 5mM EDTA. One milliliter aliquants of this homogenate were used to determine GSSG and total glutathione concentration.

Catalase determinations were performed using a procedure described by Chance and Maehly (1955). A 0.05M $Na_2HPO_4$ buffer (pH 7.0) was prepared for diluting the $H_2O_2$ substrate and the catalase

standard. A 0.6% solution of $H_2O_2$ was prepared fresh daily by diluting 0.1 ml of 30% $H_2O_2$ with 50 ml of phosphate buffer. A catalase stock standard was prepared fresh daily at a concentration of 280 units/ml in phosphate buffer and various concentrations of working catalase standard diluted from stock. The initial absorbance of the 0.6% $H_2O_2$ standard was read against a phosphate buffer blank at 240 nm (25°C) on a Bausch and Lomb Spectronic 600 UV/VIS spectrophotometer. Additional buffer or 30% $H_2O_2$ was added to the stock substrate until an absorbance of 0.55 to 0.52 was obtained. A 0.1 ml portion of the catalase standards or lung homogenate supernatant was added to a 1 cm light path length cuvette containing 2.9 ml of the optically adjusted $H_2O_2$ substrate. The cuvette was briefly inverted to mix the contents and then the absorbance was measured at 240 nm against the buffer blank. The time required for the optical density to decrease from 0.45 to 0.40 was measured and recorded for each sample. Data were calculated in Sigma units where one Sigma unit decomposes one micromole of $H_2O_2$ per minute at pH 7.0 and 25°C. A decrease of 0.05 absorbance units corresponds to 3.45 µmoles of $H_2O_2$ in the 3ml solution and the Sigma units for each sample equaled 3.45 divided by the time required for the absorbance to decrease from 0.45 to 0.40 absorbance units. Various concentrations of catalase standard were run with each assay to ensure linearity of the enzyme reaction.

Glutathione peroxidase activity (GPx) was determined using a procedure described by Chow and Tappel (1972). A 0.2M Tris-KCl buffer (pH 7.0) was prepared for diluting all assay reagents. The following reagents were prepared prior to each assay: 1.2mM NADPH, 100 units/ml GR, 30mM solution of ethylenedinitrilotetraacetic acid (EDTA), 2.6mM

GSH and 3.9mM cumene hydroperoxide. The reactions were conducted in a cuvette of 1 cm light path length at 25°C. Each sample cuvette was preloaded with the following reagents: 0.05 ml lung homogenate supernatant (or a dilution if required), 0.1 ml (1.2mM) NADPH, 0.5 ml (0.2M) Tris-KCl, 0.1 ml GR, 0.1 ml (30mM) EDTA and 0.1 ml (2.6mM) GSH. The enzyme reaction was initiated by the addition of 0.05 ml of 3.9mM cumene hydroperoxide and the contents briefly mixed by inversion. The change in optical density was monitored at 340 nm versus a reagent blank using a Bausch and Lomb Spectronic 600 UV/VIS spectrophotometer. The change in absorbance was recorded from 2 to 4 minutes after the reaction was initiated. A nonenzymatic reaction was run with each assay group by substituting buffer for enzyme protein and the GPx activity of each sample was normalized by deducting the absorbance change of the nonenzymatic reaction from the absorbance change of the protein reaction. One enzyme unit results in a change of 0.001 absorbance units/minute and the specific activity is recorded as enzyme units/mg protein.

Glutathione reductase (GR) was determined using a procedure described by Racker (1955). A 1.0 M $KH_2PO_4$ buffer (pH 7.6) was prepared for diluting assay reagents. A 1.2 mM NADPH solution was prepared in phosphate buffer. A 1% BSA solution was made using 0.1M phosphate buffer. A 2% GSSG solution was prepared fresh daily using phosphate buffer. The assays were performed in cuvettes of 1 cm light path length at 25°C. Each sample cuvette was preloaded with the following reagents: 0.05 ml (1.0M) phosphate buffer, 0.55 ml distilled $H_2O$, 0.10 ml (1.2mM) NADPH, 0.10 ml (1%) BSA and 0.10 ml (2%) GSSG. The enzyme reaction was initiated by the addition of 0.10 ml of the

lung homogenate supernatant (or a dilution if required), mixing and reading at 340 nm versus a reagent blank on a Bausch and Lomb Spectronic 600 UV/VIS spectrophotometer. The change in optical density at 30 sec intervals was recorded for 2 minutes for each sample. One enzyme unit results in a change of 0.001 absorbance units/minute and the specific activity is recorded as the enzyme units/mg protein.

The determination of glucose-6-phosphate dehydrogenase (G-6-PDH) activity was performed using a method reported by Bergmeyer (1965). The following reagents were prepared prior to the assay: 50mM triethanolamine buffer (pH 7.5) containing 0.2% EDTA, 40mM G-6-P, 30mM NADP and 0.66mM EDTA (prepared in 0.9% NaCl). All assays were run in a cuvette of 1 cm light path length at 25°C. A blank was prepared by mixing 2.9ml of (50mM) triethanolamine buffer + 0.05 ml lung homogenate supernatant. Each sample contained 2.85 ml (50mM) triethanolamine buffer, 0.05 ml lung homogenate supernatant and 0.05 ml (30mM) NADP. The contents were mixed gently and preincubated for five minutes at 25°C. The reaction was initiated by the addition of 0.05ml (40mM) G-6-P, the contents briefly mixed by inversion and the absorbance change read using a Bausch and Lomb Spectronic 600 UV/VIS spectrophotometer. After the sample showed an initial increase of 0.02 absorbance units, the change in optical density was recorded at 2 minute intervals for the next 10 minutes. One enzyme unit results in a change of 0.001 absorbance units/minute and the specific activity is recorded as the enzyme units/mg protein.

Glutathione disulfide or oxidized glutathione (GSSG) and total glutathione (GSH + GSSG) was measured using an assay originally

described by Akerboom and Sies (1981) and modified by Smith and Boyd (1984). For GSSG determination a 1.0 ml aliquant of the tissue homogenate was added to 0.25 ml of 25% perchloric acid which acted to precipitate the proteins. Next a 0.25 ml volume of 0.25M NEM was added to bind GSH. The entire mixture was carefully neutralized to pH 6.2 using 2N KOH which acted to precipitate the perchloric acid. Diethyl ether was used in 3 x 20 ml washes to remove excess NEM. Reagents required for the actual GSSG measurement included: 0.1M $KH_2PO_4$ (pH 7.0) prepared fresh daily, 100mM EDTA, 1mM NADPH in 0.5% $NaHCO_3$ and GR (20 units/ml) diluted in phosphate buffer containing .010mM NADPH. All assays were performed in a cuvette with a 1 cm light path length at 25°C. A 1.0 ml sample of the ether washed GSSG homogenate was placed in the cuvette along with 0.01 ml of 100mM EDTA and 0.01 ml of 1mM NADPH. The contents were mixed and the baseline absorbance of NADPH measured at 340 nm versus a reagent blank on a Aminco DW-2a UV/VIS spectrophotometer. The enzyme reaction was then initiated by the addition of 0.005 ml of (20 units/ml) GR and the change in absorbance monitored for 2 minutes. Quantification of each sample was performed by comparing the change in absorbance for each sample against a standard curve of GSSG run with the assay. The GSSG standard was measured using 1.0 ml of buffer containing various concentrations of GSSG in place of the 1.0 ml of sample.

For total glutathione determination a separate 1.0 ml aliquant of the tissue homogenate was added to perchloric acid as described above. An 0.1ml aliquant of the supernatant was diluted with 1.0 ml of 0.1 M phosphate buffer (pH 6.2) containing 5mM EDTA. Reagents for the total GSH assay were prepared fresh daily and included 0.1M $KH_2PO_4$ (pH 7.0)

containing 1mM EDTA, DTNB (1.5 mg/ml) in 0.5% $NaHCO_3$, NADPH (4 mg/ml) in 0.5% $NaHCO_3$, GR (6 units/ml) in phosphate buffer and .010 mM GSSG. All assays were performed in a 1 cm light path length cuvette at 25°C. One milliliter of phosphate buffer was placed in the cuvette along with 0.1 ml of diluted supernatant (approximately 0.5-2 nmol GSH), 0.05 ml NADPH, 0.02 ml DTNB and 0.02 ml GR. The contents were briefly mixed and the absorbance change monitored for 2 minutes at 412 nm versus a reagent blank using an Aminco DW-2a UV/VIS spectrophotometer. Quantification of each sample was performed by comparing the change in absorbance versus a GSH standard curve run with the assay group.

Protein concentrations were measured using the method reported by Lowry et al. (1951).

Data for catalase GPx, GR, and G-6-PDH were analyzed by a Student's t-test versus aged-matched controls utilized on the same day. For GSSG and total GSH data a paired Student's t-test was used to analyze the data.

## C. Results

Lungs from BCNU treated rats contained significantly less catalase activity than did those from control rats. Catalase activity was approximately 60% of control levels in animals treated with 20 mg/kg BCNU (Figure 14) from 7 through 21 days. Control levels of catalase fluctuated from 0.61 ± .11 to 0.88 ± .03 Sigma units/ml of lung homogenate so treated values are expressed as a percentage of the catalase activity for aged matched control animals run on the same day as the treated groups.

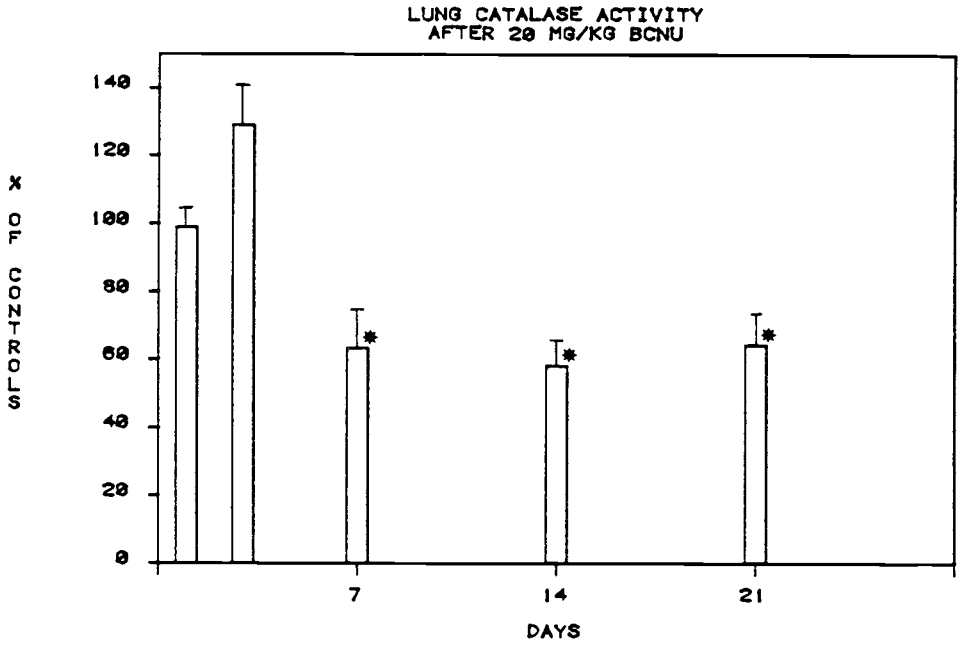


Figure 14. Lung Homogenate Catalase Activity in Rats After BCNU Administration

Values are plotted as mean ± S.E.M. as percentage of controls (n = 5), control: 0.73 ± .07 Sigma units/0.1 ml.
*Significantly different from controls by Student's t-test, $p < 0.05$.

Glutathione shuttle enzyme activity is shown in Figure 15. Glutathione peroxidase activity showed a transient elevation one week after BCNU administration to 153% of the control level (control: 19.3 ± 6.0 enzyme units/mg protein). From 14 to 21 days GPx activity returned to control levels. Glutathione reductase (GR) activity was significantly depressed 24 and 72 hours after BCNU administration. At 24 hours treated GR activity was 34.5% of the control value of 187.0 ± 16.6 enzyme units/mg protein. At 72 hours treated GR activity was 58.9% of the control level of 175.2 ± 14.3 enzyme units/mg protein. From 7 through 21 days, however, GR activity was at control levels. Glucose-6-phosphate dehydrogenase activity was not measured at 24 or 72 hours but treated animals ranged from 99 to 121% of controls from 7 through 21 days after BCNU administration.

Oxidized glutathione and total glutathione quantitation for treated animals versus age matched and pair fed controls is shown in Table 9. The only treated value significantly elevated was the GSSG concentration at 72 hours which was .043 ± .012 umoles/g tissue versus a paired control value of .024 ± .004 umoles/g tissue. This was in correspondence with the observed decrease in GR at this time. At 24 hours treated animals had GSSG concentrations that were within the control range.

## D. Discussion

The uniform reduction in catalase activity from 7 through 21 days was a surprising finding. Giri *et al.* (1983) studying bleomycin pulmonary toxicity in hamsters saw only an acute reduction in catalase

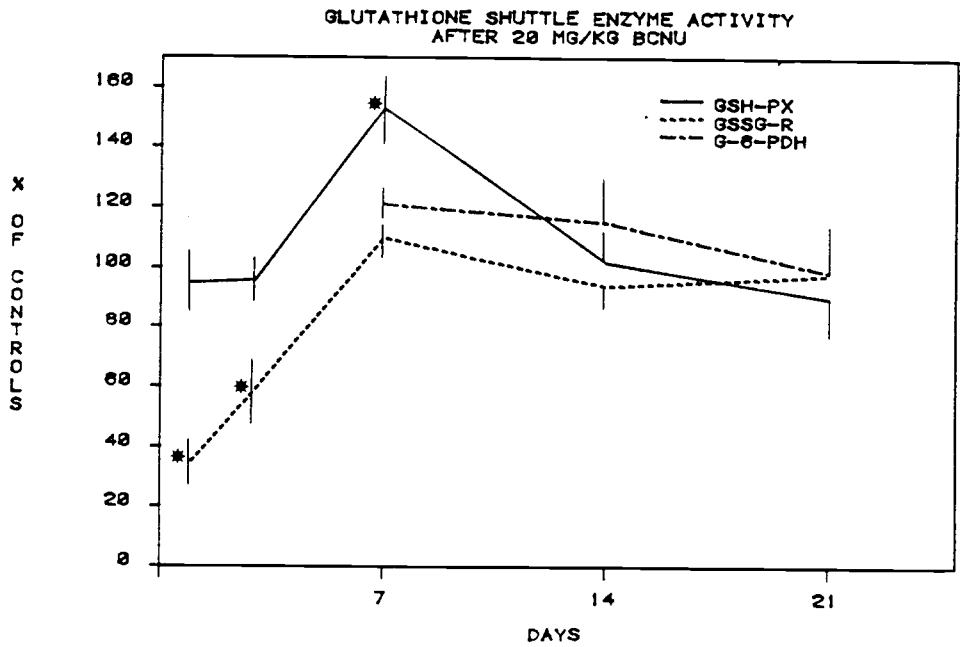


Figure 15. Lung Glutathione Shuttle Enzyme Activity in Rats After BCNU Administration.

Values are plotted as mean ± S.E.M. as percentage of controls (n = 5), control: glutathione peroxidase (GSH-Px) = 9.1 ± 2.9 units/mg protein; glutathione reductase (GSSG-R) = 105 ± 22 units/mg protein; glucose-6-phosphate dehydrogenase (G-6-PDH) = 18.2 ± 2.5 units/mg protein.

*Significantly different from controls by paired Student's t-test, $p < 0.05$.

Table 9. Lung Homogenate Oxidized Glutathione and Total Glutathione Levels in Rats After BCNU Administration

| | Post-Dosing Interval | | | |
|---|---|---|---|---|
| Treatment[a] | 24 hours | | 72 Hours | |
| | [GSH + GSSG][b] | [GSSG][b] | [GSH + GSSG] | [GSSG] |
| BCNU[b] | 1.70 ± .47 (121%)[c] | .025 ± .005 (119%) | 1.52 ± .25 (92%) | .043 ± .012[d] (179%) |
| Control | 1.41 ± .07 | .021 ± .005 | 1.65 ± .11 | .024 ± .004 |

a. Single i.p. administration of 20 mg/kg BCNU or vehicle control.
b. Values are expressed as mean ± S.E.M. in μmol/g tissue (n = 5).
c. Percent of control value.
d. Significantly different from controls by Student's t-test, $p < 0.05$.

activity from 24-48 hours and then statistically significant increases in catalase activity at 7-21 days postdosing. The physiological importance of catalase has been reemphasized in recent work showing that catalase could reduce the endothelial cell injury caused by a neutrophil generated $H_2O_2$ dependent pathway (Martin, 1984). Additionally catalase acts to reduce the cytotoxic effects of nitrofurantoin-induced production of toxic oxygen species by endothelial cells (Martin et al., 1985). Catalase appears to be a readily inducible enzyme that responds to various sources of oxidative damage.

It appears that at seven days, treated animals responded to decreased catalase activity by increasing the alternate enzymatic pathway for reduction of $H_2O_2$ to water, i.e., through glutathione peroxidase. At 7 days treated GPx activity was 153% of control levels. By 14 and 21 days treated GPx activity had returned to control levels implying that the compensatory mechanisms for the catalase deficiency was either no longer required, or not able to respond. A protective mechanism against oxidative damage might not be required over this time course because from 14 to 21 days elastase activity was only marginally increased and lipid peroxidation levels as indicated by MDA concentration were still declining.

The acute depression of lung GR activity 24 and 72 hours after BCNU treatment was to be expected on the basis of the known reductase inhibition demonstrated by other researchers (Frischer and Ahmad, 1977; Babson and Reed, 1978; Maker et al., 1983; Smith and Boyd, 1984; Ahmad and Frischer, 1985). GR activity had recovered by 7 days after treatment and remained at control levels through 21 days. Glucose-6-phosphate dehydrogenase activity in treated animals also remained at

control levels from 7 through 21 days, thus assuring the availability of adequate amounts of the NADPH cofactor.

Taken cumulatively, the results of glutathione shuttle enzyme activity would indicate the pathway is probably responding to increased lipid peroxidation in an attempt to minimize membrane damage. From 7 through 21 days, the mechanism responsible for causing the peroxidative membrane damage must no longer be active, because redox enzyme activity levels return to normal levels.

The studies of oxidized and total glutathione levels coupled with the observed inhibition in GR activity might actually help explain the acute peroxidative damage seen as increased MDA concentration at 7 days (Figure 11). Results of lung GSSG and total glutathione concentration using the Sprague Dawley rat compare favorably with results obtained in the F-344 strain by Smith and Boyd (1984). Younes and Siegers (1981) proposed that agents that could deplete GSH levels could induce lipid peroxidation even in the presence of oxygen radical scavengers. If the GR is less active over this time course, the reduction of GSSG to GSH would be compromised, depriving the cell of reducing equivalents. One would expect GSSG levels to accumulate, a hypothesis supported by increased GSSG content 72 hours after BCNU treatment. Since total glutathione remains unchanged, the GSH concentration (representing the difference between total glutathione and GSSG) should be reduced. In the face of decreased reductase activity, de novo synthesis might not be sufficient to maintain the increased levels of GSH necessary to block lipid peroxidation. Alternately, perhaps from 3 to 7 days, total glutathione levels would actually drop as synthesis of new GSH is limited by the availability

of precursor materials such as glutamate, cysteine or glycine. This latter mechanism might actually be the more correct argument because BCNU is anorexigenic and animals do not regain their appetite until 5 to 7 days after treatment.

The following sequence of events might explain the results of these experiments. The acute inhibition of GR activity and the known anorexiant effects of BCNU could act together to deplete cellular GSH levels sometime between 3 and 7 days after treatment. Thus, in the absence of reducing equivalents, lipid peroxidation would occur over this time as evidenced by elevated MDA concentrations. The lung attempts to counteract this peroxidative process by increasing the enzyme activity associated with the glutathione shuttle pathway. This activity is maximized at 7 days, a time point coinciding with maximal acute lipid peroxidation. Glutathione activity is induced to a greater extent than the other redox enzymes, apparently representing a compensatory response to decreased catalase activity at 7 days. These two observations taken together imply that the actual mechanism for the peroxidative damages might be through an oxidative pathway. Over the next 2 weeks, shuttle enzyme activity normalizes as lung lipid peroxidation decreases, implying that the mechanism responsible for the acute effects is no longer active. By some mechanism(s) that needs to be explored, catalase activity remains depressed. Thus, the initial damaging process in the lung after BCNU treatment may be enhanced lipid peroxidation.

# Chapter VI. The Strain Variation in Response to BCNU Exhibited by Fischer 344 Rats

## A. Introduction

The final portion of this thesis involved a study of the variation in response to BCNU exhibited by the Fischer 344 (F-344) rat. Previous studies of BCNU-induced toxicity have utilized mice or F-344 rats. Studies of BCNU-induced pulmonary fibrosis have specifically utilized the F-344 animal and multiple dosing regimens (Reznik-Schuller et al., 1984; Smith and Boyd, 1984). In the studies reported herein utilizing the Sprague Dawley (S.D.) rat, and a single i.p. dosing regimen, there were significant changes in several pulmonary biochemical activities that are consonant with lung damage that may be ultimately fibrogenic. Notably, alpha-1-PI content, elastase activity, MDA content, collagen content, catalase activity, GSSG content and glutathione redutase activity were all affected in a correlatable fashion after BCNU treatment.

Genetic variation in biochemical responses is certainly not a novel observation. Studies have been conducted on differences in response between species (Gregus et al., 1983). A genetic component in human pulmonary diseases has been reported for familial fibrocystic pulmonary dysplasia (Adelman et al., 1966) and idiopathic pulmonary fibrosis (Javaheri et al., 1980). More recently the role of strain variation in bleomycin-induced murine pulmonary fibrosis has been investigated (Schrier et al., 1983).

The basis for strain variation work by Schrier et al. (1983) was the observation that the effects of intratracheal bleomycin instillation could be partially reversed by suppressing immune or inflammatory function. T-cell depletion or treatment with antilymphocyte globulin and steroids diminished the fibrotic response caused by bleomycin. Using collagen deposition as a measure of fibrotic response, Schrier's group found the C57BL/6 mice were high responders, DBA/2 and Swiss were intermediate responders, and BALB/c were the least responsive. They concluded that the intensity of the fibrotic response is controlled by the major histocompatibility complex (H-2) with a more permissive role played by nonH-2 genes. Because of distinct levels in fibrotic response, they also proposed that bleomycin induced pulmonary fibrosis is under polygenic control.

That an inflammatory component plays some role in the pulmonary response to BCNU in Sprague Dawley rats was suggested by findings in this study with regard to elastase activity. This observation, coupled with the increased collagen synthesis exhibited by the Sprague Dawley after a single i.p. administration of BCNU, suggested that this experimental model might be as valid as the multiple dosing model of F-344 rats proposed by Smith and Boyd (1984). The key would be to demonstrate differences in response between the two strains under a similar dosing protocol. With this objective in mind, F-344 animals were obtained and treated with a single i.p. injection of BCNU at a concentration known to be effective in the Sprague Dawley animals. Selected biochemical parameters were then monitored at various time points to permit a comparison of some of the results previously measured in the Sprague Dawley animals.

B. Methods

Male Fischer 344 rats were obtained from Simonsen Laboratories, Gilroy, CA. Animals were housed and maintained as previously described for the Sprague Dawley rats. The animals weighed 200-225 grams and were 7 weeks old at the time of treatment.

Solutions of BCNU in corn oil were prepared fresh prior to administration at a concentration of 20 mg/ml. Animals in each experimental group received a single i.p. dose at 20 mg/kg (0.1 ml/100g) body weight with the drug or vehicle control.

At intervals of 7, 21 and 28 days a minimum of 5 animals from each experimental group were sacrificed as previously described for determination of alpha-1-PI concentration, alpha-1-PI activity, MDA concentration, elastase activity and collagen content. All samples were obtained and treated as previously described and assays were performed as described in the appropriate chapter. All data were analyzed using a Student's t-test.

C. Results

While control lavage and sera PI were not different in these two strains, Fischer 344 animals exhibited a 58% decrease in lavage PI concentration (control 2.6 ± 0.6 μmoles/liter) 7 days after BCNU treatment (Table 10). By 28 days postdosing, lavage PI content in the treated F-344 group had increased to 154% of the control value. By

Table 10. Variation in Alpha-1-PI Concentration in Sprague Dawley and Fischer 344 Rats After BCNU Treatment[a]

| | Lavage (μmoles/l) | | | Serum (mmoles/l) | | |
|---|---|---|---|---|---|---|
| | Control | 7-day | 28-day | Control | 7-day | 28-day |
| S.D.[b] | 3.0 ± 0.4 | 4.1 ± 0.6[d] | 7.8 ± 1.5[d] | 10.8 ± 0.4 | 10.7 ± 0.2 | 36.2 ± 14.7[d] |
| F-344[c] | 2.6 ± 0.6 | 1.1 ± 0.1[d] | 4.0 ± 0.6[d] | 10.3 ± 0.5 | 9.4 ± 0.5 | 10.8 ± 0.3 |

a. Single i.p. administration of 20 mg/kg BCNU or vehicle control. Values are reported as mean ± S.E.M., ($n \geqslant 5$).
b. Sprague Dawley.
c. Fischer 344.
d. Significantly different from control by Student's t-test, $p < 0.05$.

way of contrast, Sprague Dawley rats at this same time point had a lavage PI concentration which was 260% of the control level of 3.0 ± .04 μmoles/liter. Treated F-344 animals showed no change in serum PI concentration over this 4 week interval whereas treated Sprague Dawley animals showed about a threefold increase in serum PI concentration by 28 days.

The pulmonary lavage fluid from control F-344 rats contained almost twice the PI activity as did that from S.D. controls (Table 11) even though there was no difference in the lavage PI concentration (Table 10). Treated rats of both strains exhibited qualitatively similar response patterns in lavage PI activity at 7 and 28 days (Table 11). At 7 days both strains showed insignificant transient increases in lavage PI activity and by 28 days postdosing, lavage PI activity had decreased. The most striking difference between the two treated strains was in serum PI activity. Both strains had equivalent control activity and neither strain showed significant effects in serum PI activity at 7 days. However, the S.D. had an approximate sixfold decrease in trypsin inhibitor activity at 28 days while at this time point the treated F-344 animals still were within 86% of the control activity.

Lavage elastase concentration also was much higher in F-344 controls than in S.D. controls (Table 12) and in the F-344 rats it was unchanged after BCNU treatment. The BCNU treated S.D. animals had an almost twofold increase in lavage elastase concentration by 28 days. Control F-344 animals had only about one-third of the serum elastase concentration of control Sprague Dawley. Because of this lower control serum elastase concentration (0.36 ± .02 units/ml), the

Table 11. Variation in Alpha-1-PI Activity in Sprague Dawley and Fischer 344 Rats After BCNU Treatment [a]

| | Lavage activity[b] | | | Serum activity | | |
|---|---|---|---|---|---|---|
| | Control | 7-day | 28-day | Control | 7-day | 28-day |
| S.D.[c] | .070 ± .009 | .083 ± .010 | .059 ± .015 | 6.08 ± .46 | 7.03 ± .48 | 1.16 ± .56[e] |
| F-344[d] | .129 ± .017 | .156 ± .009 | .081 ± .012 | 7.59 ± .62 | 8.73 ± .60 | 6.54 ± .53 |

a. Single i.p. administration of 20 mg/kg BCNU or vehicle control.
b. Activity reported as mean ± S.E.M. in μmoles trypsin inactivated/μmole PI.
c. Sprague Dawley.
d. Fischer 344.
e. Different from control by Student's t-test, $p < 0.05$.

Table 12. Variation in Elastase Concentration in Sprague Dawley and Fischer 344 Rats After BCNU Treatment [a]

| | Lavage (units/ml)[b] | | | Serum (units/ml) | | |
|---|---|---|---|---|---|---|
| | Control | 7-day | 28-day | Control | 7-day | 28-day |
| S.D.[c] | .070 ± .004 | .068 ± .006 | .138 ± .016[e] | 1.04 ± .16 | .48 ± .10[e] | 2.20 ± .10[e] |
| F-344[d] | .134 ± .008 | .116 ± .004 | .110 ± .008 | 0.36 ± .02 | 1.92 ± .38[e] | 1.34 ± .10[e] |

a. Single i.p. administration of 20 mg/kg BCNU or vehicle control.
b. Values are mean ± S.E.M. (n = 5) in elastase units/ml where one unit will solubilize 1 mg of elastin in 20 minutes at pH 8.8 at 37°C.
c. Sprague-Dawley.
d. Fischer 344.
e. Significantly different from control by Student's t-test, $p < 0.05$.

treated F-344 animals showed a larger percentage increase in serum elastase content at 28 days after BCNU treatment than did treated Sprague Dawley animals (372% versus 212%, respectively). Treated F-344 animals also did not show the transient reduction in serum elastase content exhibited by treated S.D. animals at 7 days.

Table 13 shows strain comparisons for lung lipid peroxidation and collagen content 28 days after BCNU administration. Though control F-344 animals showed a threefold greater basal lung lipid MDA content compared to control S.D. animals, there was no increased lipid peroxidation in the 28 day treated Fischer group. The treated S.D. group had a 317% increase in by 28 days. Over the 21-28 day treated time course, the S.D. animals showed a time dependent increase in lung collagen content to 156% ± 13% of controls by 28 days. Over this same time course, treated F-344 animals exhibited no statistical change in lung collagen content.

## D. Discussion

The two strains of rats responded quite differently to the acute administration of BCNU. Treated S.D. animals show a much greater increase in lavage and serum PI content than do treated F-344 animals. Perhaps F-344 animals cannot respond because of an acute hepatic sensitivity to BCNU. Such sensitivity might not permit the hepatic synthesis of additional PI. It might also be possible that the feedback mechanism responsible for controlling PI synthesis is disrupted in some manner. The more plausible explanation is that F-344 rats are more resistant to the toxic effects of the drug.

Table 13. Variation in Lung Malondialdehyde (MDA) Concentration and Collagen Concentration in Sprague Dawley and Fischer 344 Rats After BCNU Treatment[a]

| | MDA Concentration[b] | | Collagen Content[c] | | |
|---|---|---|---|---|---|
| | Control | 28-day | Control | 21-day | 28-day |
| S.D.[d] | .12 ± .03 | 0.38 ± .04[f] | 1.44 ± .32 | 1.86 ± .02 | 2.26 ± .44[f] |
| F-344[e] | .38 ± .12 | 0.34 ± .03 | 1.09 ± .22 | 1.05 ± .07 | 0.96 ± .01 |

a. Single i.p. administration of 20 mg/kg BCNU or vehicle control.
b. Values are mean ± S.E.M. in TMP equivalents/ml, n = 5.
c. Values are mean ± S.E.M. in µg hydroxyproline/mg protein, n = 5.
d. Sprague Dawley.
e. Fischer 344.
f. Significantly different from controls by Student's t-test, $p < 0.05$.

Evidence to support F-344 resistance is provided by the remaining data. Treated F-344 animals responded in a parallel manner to S.D. groups as far as lavage PI activity. Lavage trypsin inhibitory capacity, on the other hand, was marginally increased at 1 week but depressed by 4 weeks postdosing in both strains. Serum trypsin inhibitor capacity was only slightly reduced at 28 days in the treated F-344, a time point when treated S.D. serum activity had declined by 81%.

In Chapter III it was postulated that the increases in PI inhibitor content, especially in the lung, may be a response to increased elastase content in the lung. There was no evidence for increased lavage elastase activity in these treated F-344 animals and by extension perhaps no inflammatory response either following a single dose of BCNU. There was a significant increase in serum elastase content but apparently this circulating activity had no pulmonary effects.

The two biggest indications of pulmonary damage from the previous experiments using the S.D. strain were the biphasic increase in lung MDA content and the increase in lung collagen content at 21 and 28 days. Treated F-344 animals showed no evidence of increased peroxidative damage (i.e., MDA content) at 4 weeks nor was any increase in lung collagen content measureable. All of these data, but especially the lack of demonstrable evidence of lung damage or repair, indicates that the Fischer 344 rat strain is more resistant to the pulmonary toxicity of BCNU than is the Sprague Dawley strain, at least following a single i.p. dose of BCNU.

These data tend to support the findings by Reznik-Schuller et al. (1984) and Smith and Boyd (1984) that the F-344 strain requires multiple dosing with BCNU to induce pulmonary toxicity. Multiple dosing does not appear to be a prerequisite for pulmonary toxicity if the right animal model is chosen. In this case, the Sprague Dawley strain seems more sensitive to the effects of a single BCNU treatment.

More importantly, the difference in strain response might actually help to more clearly delineate the mechanism of BCNU-induced lung fibrosis. If there is a genetic component to BCNU-induced fibrosis, as there appears to be for other forms of pulmonary disease, then a detailed knowledge of genetic differences between the S.D. and F-344 strains might provide a useful clue to the possible component and mechanism. A more detailed investigation of strain variation in other biochemical parameters certainly seems warranted.

# Chapter VII. Summary and Conclusion

The experiments described in this study were designed to measure biochemical changes associated with BCNU-induced pulmonary toxicity. The absolute validity of the experimental model awaits histological demonstration of the fibrotic disease process. Though pulmonary fibrosis has been conclusively demonstrated using paraquat and bleomycin in several different animal species and strains (Kim and Roberts, 1980; Newman et al., 1980, 1981; Trush et al., 1981; Kaelin et al., 1983; Schrier et al., 1983) the demonstration of BCNU-induced fibrosis in rats and mice (Kehrer, 1984; Reznik-Schuller et al., 1984; Smith and Boyd, 1984) has not been proven satisfactorily in the absence of complementary histological and biochemical data. Several of the biochemical parameters measured in these studies, however, tend to support the Sprague Dawley rat as a definitive animal model of BCNU-induced fibrosis.

The validity of measuring pulmonary lavage angiotensin converting enzyme (ACE) activity is thrown into question by these studies. All 3 fibrotic agents tested actually decreased lavage ACE activity from 1 to 3 days. Through 21 days, none of the treatment groups showed significant increases in lavage ACE activity. If measuring ACE activity is useful as a diagnostic tool, it would seem to better predict generalized endothelial cell damage. Carmustine and paraquat both showed significant increases in serum ACE activity as early as 24 hours after treatment. All 3 agents caused increased serum ACE activity 21 days after treatment.

This study has provided the first evidence of BCNU-induced effects on alpha-1-proteinase inhibitor (PI) content and activity. The sharp increase in lavage PI content, coupled with the increase in lavage elastase activity implicates an inflammatory component might be involved in a definitive mechanism of BCNU-induced pulmonary fibrosis. The clearest biochemical indication that a fibrotic process was occurring in the lung, was the demonstration of significantly increased lung hydroxyproline concentration. Significant increases in collagen precursor materials were also measured in BCNU treated animals.

The malondialdehyde (MDA) concentration in the lung also increased in a biphasic manner implying possible short term peroxidative membrane damage caused by BCNU and more long term membrane damage probably resulting from neutrophil generated oxidants. Lung catalase activity and glutathione peroxidase activity was increased at 3 and 7 day, respectively, presumably in response to the peroxidative process that resulted in measured increases in MDA concentration. This study has also shown increased lung oxidized glutathione concentrations 72 hours after BCNU treatment. Since total glutathione content remains essentially unchanged, by implication reduced glutathione levels are depleted by BCNU. The BCNU-induced inhibition of glutathione reductase activity described by other investigators (Frischer and Ahmad, 1977; Babson and Reed, 1978; Maker et al. 1983; Smith and Boyd, 1984; Ahmad and Frischer, 1985) was also demonstrated in these studies.

Finally, significant differences in response to BCNU treatment were measured in the Fischer 344 rat. These differences help to explain the independent development of a multiple i.p. dosing model for BCNU proposed by Smith and Boyd (1983, 1984) and Reznik-Schuller

et al. (1984). The variation in strain response might actually prove to be a useful tool in teasing out the mechanism responsible for BCNU-induced pulmonary fibrosis.

Taking all of the experimental BCNU data together, a chronological sequence of biochemical changes can be summarized. Over the first 3 days, BCNU appears to exert direct effects on lung glutathione content and glutathione reductase activity. These experimental observations have also been reported by other investigators (Maker et al., 1983; Smith and Boyd, 1984). A compromised glutathione redox cycle could definitely account for the early increase in lung lipid peroxidation levels. Enzymes associated with the glutathione redox cycle are induced by 7 days postdosing in an apparent physiological attempt to compensate for increased membrane peroxidation. Indeed, the lung would appear to be recovering from this acute toxicity based on continual declines in lipid peroxidation through 28 days postdosing.

Between 21 and 28 days, the biochemical events responsible for more prolonged toxic effects are evident. Increased lavage protein (partially due to ACE but mainly due to serum derived proteins, especially alpha-1-PI) indicates a loss of endothelial integrity. This loss of integrity would be expected to result in interstitial edema that appears to be supported by increases in lung wet weight over this same time period (Appendix C). Loss of endothelial integrity is also supported by a trend towards higher lavage and serum ACE levels from 14 to 21 days. Edema formation could be the pathological state responsible for the recruitment of neutrophils and macrophages leading to an inflammatory response. Evidence for some form of neutrophil recruitment is provided by the very significant increases

in lavage elastase activity from 21 to 28 days after BCNU treatment.

In an attempt to compensate for increased elastase activity, treated animals are able to synthesize greater quantities of the primary proteinase inhibitor, alpha-1-PI. Though this inhibitor is active in the lung, localized concentrations of the elastase are probably high enough to effectively overwhelm the inhibitory capacity of alpha-1-PI. These induced neutrophils are also capable of initiating peroxidative membrane damage by the release of various oxidants, some of which have a half-life approaching 18 hours (Weiss et al., 1983). The localized release of elastase is accompanied by a release of collagenase and both the enzymes are capable of breaking down the tissue matrix structure of the lung.

Malondialdehyde content again increased in the lungs of treated animals after 21 days. Over a similar time course, lung collagen content increased. These results support membrane peroxidative damage occurring as well as a collagen repair process. A net increase in collagen deposition appears to be the result of increased collagen precursors available in general circulation and decreased collagen degradation in the lung. The final result would be interstitial pulmonary fibrosis.

Several investigators have proposed that fibrosis is the result of the lungs inability to undergo reepithelialization (Haschek and Witschi, 1979; Last, 1985). This process requires exposure to an initial agent causing lung damage followed by exposure to a second agent that compromises reepithelialization. Osman et al. (1985) reported impaired elastin resynthesis in the lungs of hamsters receiving sequential treatment with elastase and trypsin. The work by

Niewoehner and Hoidal (1982) indicates that fibrosis and emphysema might actually be divergent responses to the same initiating event, the pathology actually expressed being a function of exposure to a second toxic agent. Whatever the actual cause of fibrosis, it definitely appears to result from a failure of the normal collagen maturation and remodeling process. The normal fibroblastic process appears to be disrupted by a failure to regenerate elastin in the extracellular matrix or an inability to restore an epithelial cell layer (Last, 1985). Perhaps continuous exposure to a localized, elastinolytic process might account for the fibrotic response.

This study has obtained some interesting observations as far as BCNU effects that were not intentionally part of the experimental design, but nevertheless help support some of the experimental evidence. The adverse effects of BCNU administration were grossly evident in the Sprague Dawley animals from 14 to 21 days postdosing. The animals appeared jaundiced and in general no longer maintained the grooming habits exhibited by the experimental controls. Weight loss appeared to be a problem in all treated groups and is substantiated by the dose dependent loss in weight shown in Appendix A. Weight losses from 1 through 5 days are probably due to the apparent anorexic effect of BCNU. After 7 days, appetites of treated animals seemed to return and in general food and water consumption closely approached that of control groups through 35 days postdosing. Because of these early anorexic effects, age-matched and pair fed controls were used in the experiments designed to measure glutathione content and shuttle enzyme activity. In the absence of an anorexic effect, pair-feeding was not deemed necessary for the longer time points.

Another significant observation was that animals dosed on paraquat or bleomycin (20 mg/kg) exhibited no weight loss. In general the growth curves for these treatment groups paralleled the control curve. Animals dosed at 20 or 25 mg/kg BHCNU also increased in weight through 21 days. Over the next week, however, rats treated with both doses exhibited dramatic weight losses. Control F-344 animals also showed consistent weight gain though their growth curve was shallower than that exhibited by control S.D. animals. Carmustine treated F-344 animals also did not exhibit the dramatic weight loss seen in treated S.D. animals.

At necropsy there were also opportunities to get some crude estimates of gross lung pathology. Lung specific gravity determinations (Appendix B) indicated that in treated animals, portions of the lung tended to be more dense than in control or treated day-1 or day-7 animals. There also appeared to be a dose and time dependent increase in wet lung weight (Appendix C). This could be explained by edema formation. Both these measurements, taken together tend to support the claim that BCNU induces some organ specific changes in the lung.

Another indication of gross lung pathology was a simple lavage protein assay. The lavage protein content in treated animals remained relatively close to control values through 21 days post-dosing (Appendix D). At 28 and 35 days, however, there were significant increases in lavage protein. This observation should not be surprising because other experiments demonstrated dramatic increases in lavage PI content during this same period. Some portion of the increased lavage protein content might be explained by a loss of endothelial integrity permitting more ready diffusion of serum

proteins that might otherwise be excluded or limited in their access to the alveolar compartment.

Serum protein concentration increased in a parallel manner to lavage protein (Appendix E) while lung homogenate protein did not change significantly at any of the time points measured. Again, some portion of the increase in serum protein can be explained by the observed increases in serum PI concentration.

The serum and lavage protein data, and the wet lung weight data point out the particular problems in trying to express lung data addressed by Witschi (1975). Wet lung weight is much less stable than wet liver weight and perfusion techniques might drastically alter actual lung weights. It would also have been useful to have determined an absolute measure of edema formation by measuring the differences between wet and dry lung weights over time. Such a determination would not have been cost effective, because drying the lungs rendered them useless for any additional biochemical measurements.

Several parameters that might help define the fibrotic process warrant further investigation. It would be important to determine if the glutathione redox cycle is still functionally capable of responding to the increased peroxidative damage at 28 and 35 days. This study has demonstrated a redox cycle response due to acute lipid peroxidation at 1 week but if the system were compromised at the later time periods it might only compound the problem.

The cause of the decreased serum trypsin inhibitor capacity should be investigated. The newly synthesized PI might be released from the liver as an inactive form. Because PI provides the major portion of the inhibitory capacity in serum, an inactive PI may result

in lower total inhibitory capacity. The other problem is the apparent activation of PI once it enters the lung. Studying pulmonary Met[O]-reductase activity should determine whether there is sufficient capacity remaining after BCNU treatment to restore PI activity.

Another area that warrants special attention is the relationship of surfactant production and fibrosis. Surfactant is synthesized by Type II alveolar cells (Mason et al., 1977; Hollingsworth and Gilfillan, 1984). Destruction of the Type I cells comprising the majority of the normal epithelial lining results in Type II cells maturing much faster than normal which in turn results in decreased surfactant production. Lung surfactant abnormalities have been identified in adult respiratory distress syndrome (Hallman et al., 1982) and the failure of the surfactant system can result in irritation, inflammation and edema formation. This might prove to be the second event responsible for the more long term effects of BCNU.

The high concentration of pulmonary elastase activity observed after BCNU treatment also merits further study. If a continuous source of elastase prevents normal elastin formation and therefore initiates an abnormal fibroblastic response, elastin levels should be measured to determine if their concentration increases or decreases. Urinary valyl-proline levels could be monitored as an indication of increased elastin degradation (Soskel and Sandberg, 1983).

1,3-bis(hydroxycyclohexyl)-1-nitrosourea also provides a unique investigational tool for perhaps teasing out the differences in alkylation versus carbamylation effects of BCNU. 1,3-bis(hydroxycyclohexyl)-1-nitrosourea, a pure carbamylating drug did not decrease

proteinase inhibitor activity as did BCNU. However, BHCNU-treated animals responded with increased PI synthesis. This leaves alkylation as the most likely mechanism for decreased PI activity. This should be verified, but additional biochemical parameters should be measured to ascertain any other differential effects between BHCNU and BCNU treatments.

Perhaps some more fruitful results might come from continuing the research into the apparent differential response to BCNU treatment exhibited by Sprague Dawley and Fischer 344 rats. If there is an important genetic component to the fibrotic process caused by BCNU, the differences in biochemical response coupled with a detailed knowledge of the genetic make-up of each strain should lead to key biochemical mechanisms responsible for fibrosis.

Other potential areas of investigation include BCNU-induced changes in lung metabolism and changes in membrane diffusion capacity and capillary volume. The association of a specific biochemical change with a defined cell population is important. The histological or cytological demonstration of cells associated with the inflammatory response should be undertaken. Carmustine effects on other proteinase inhibitors should also be studied.

There is a very definite and urgent need to correlate the biochemical demonstration of fibrosis with the histological confirmation of the disease process in an experimental model. Until a positive correlation can be established, the statement that BCNU causes pulmonary fibrosis in experimental animals is only an assumption and the extrapolation of biochemical changes in the animal to events going on in human patients would be even more difficult.

This thesis work has made some positive contributions to understanding the biochemical changes associated with BCNU-induced pulmonary toxicity. The research has provided a fast and efficient method for analyzing angiotensin converting enzyme activity. These data have demonstrated a biphasic pulmonary response to BCNU treatment; an acute effect at 1 week probably the direct result of BCNU and a more delayed effect starting at about 21 days, possibly related to some inflammatory response. Finally, this work has again demonstrated the unique nature of BCNU as an investigational tool by adding to the list of toxic effects associated with the use of this drug.

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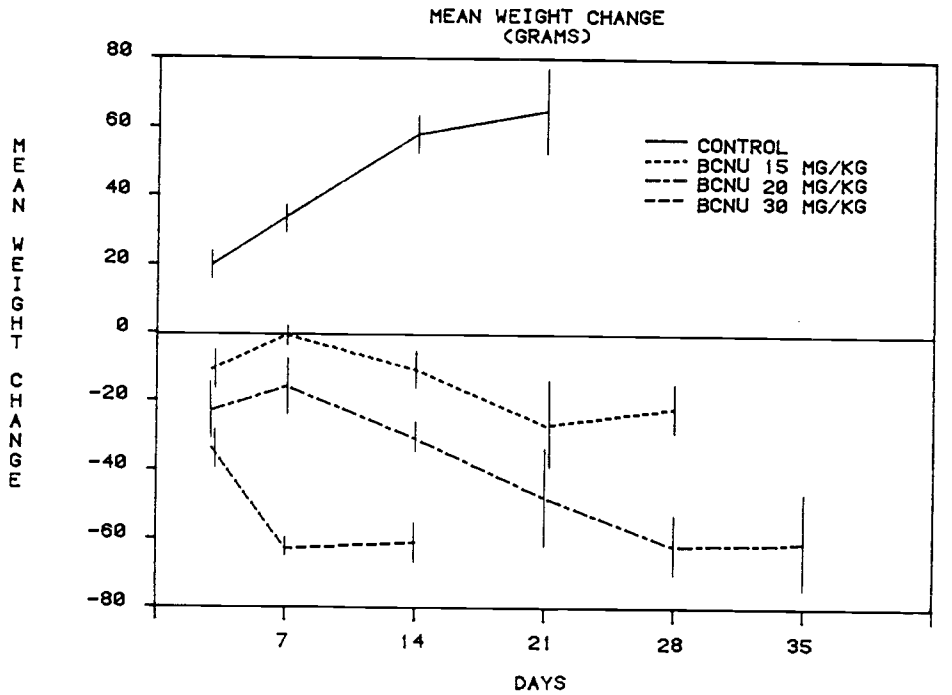
# Appendices




Appendix A. Mean Animal Weight Change of Sprague Dawley Rats After BCNU Administration

Animals received a single i.p. administration of BCNU (one of three doses) or vehicle control. Gross weight changes from time of dosing till sacrificed were recorded and averaged for each experimental group. Values are recorded as mean ± S.E.M.(n = 5).

Appendix B Lung Specific Gravity Determinations [a]

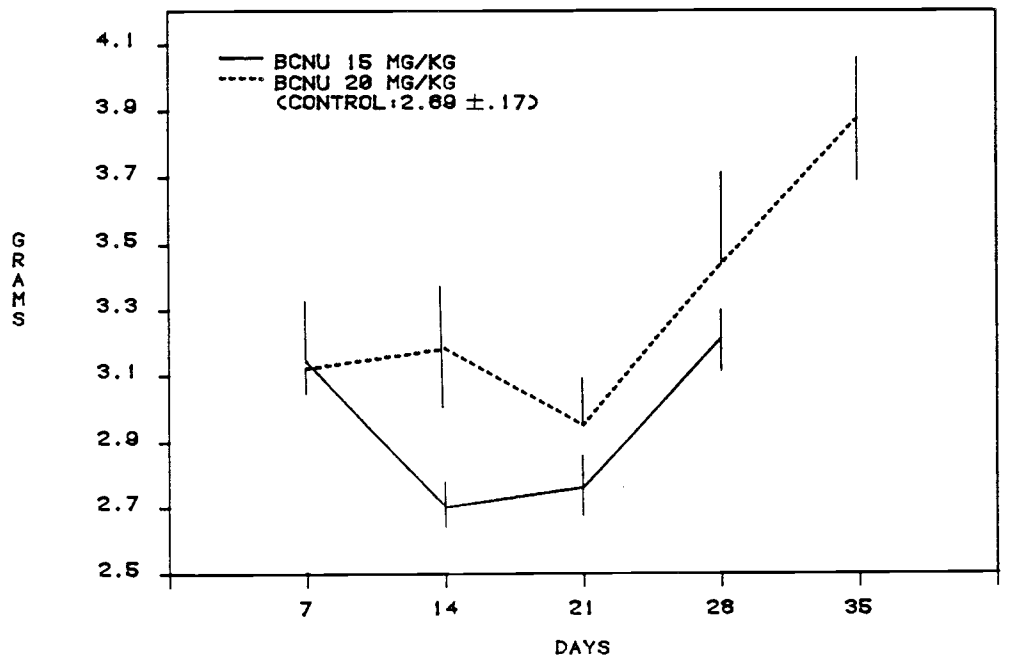
| Sample | | Specific Gravity [b] |
|---|---|---|
| Control | | 1.036 |
| BCNU[c] | day- 1[d] | 1.036 |
| | day- 7 | 1.036 |
| | day-14 | 1.036 - 1.0958 |
| | day-21 | 1.036 - 1.0958 |
| | day-28 | 1.036 - 1.0958 |

a. Three density gradients were prepared using kerosene (sp. gr. = 0.80) and bromobenzene (sp. gr. = 1.4952) in the following proportions: 3:1, 1:1 and 1:3. Small portions (approx. 0.1g) of lung tissue from areas of gross pathology from one lung lobe were placed in each gradient and permitted to come to equilibrium. The final position was marked and recorded.

b. Specific gravity range for a minimum of two animals at each time point was determined by placing drops of known density in each column, establishing their final position, and comparing with lung samples from each of the experimental groups.

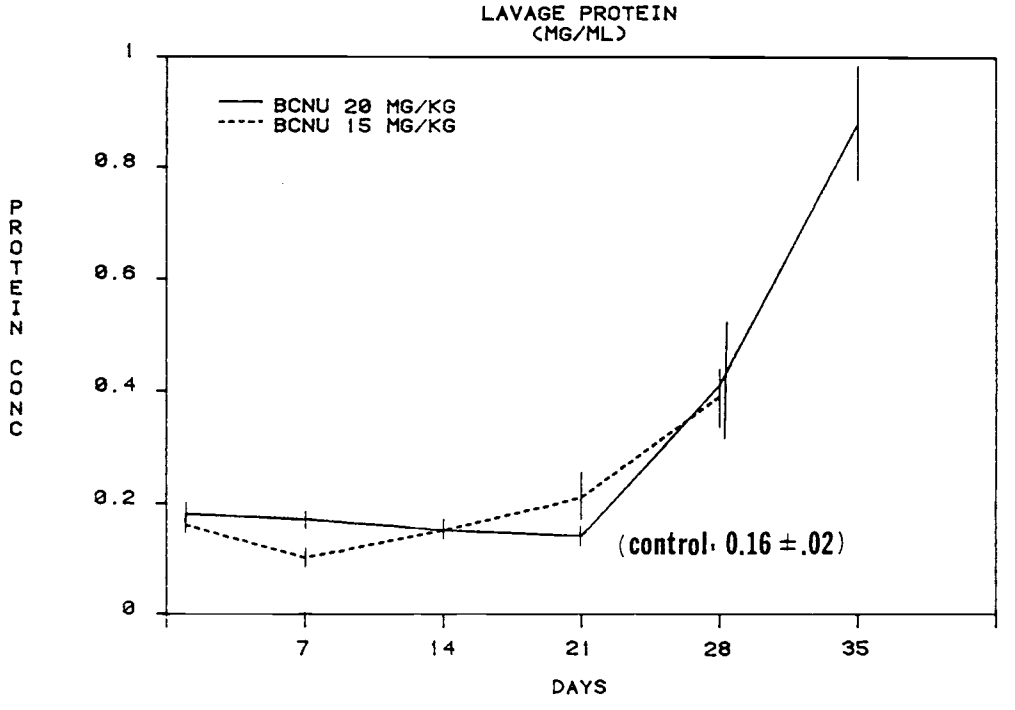
c. Single i.p. administration of 20 mg/kg BCNU

d. Post-dosing interval in days.



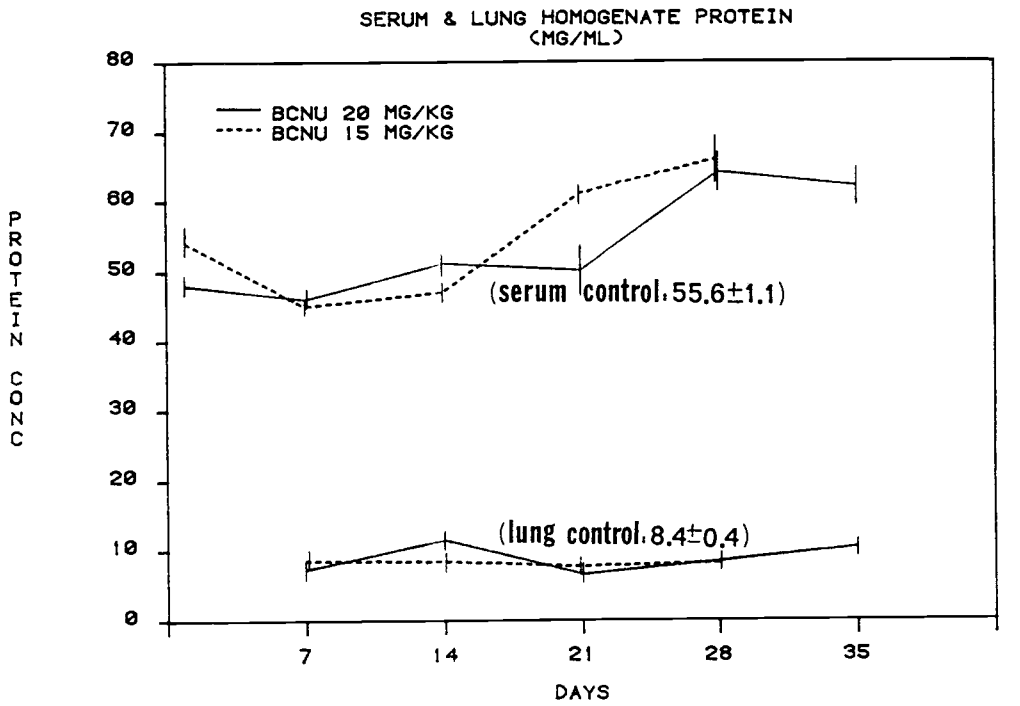

Appendix C. Lung Wet Weights of Sprague Dawley Rats After BCNU Administration

Data is taken from experimental groups where lungs were removed in mass without lavaging or rinsing in situ. Excess tissue was removed, the lungs rinsed with saline, blotted dry and weighed. Values are plotted as mean ± S.E.M. in grams (n = 5).




Appendix D. Lung Lavage Protein Concentration in Sprague Dawley Rats After BCNU Administration.

Data is taken from experimental groups where lavage protein analysis was performed. Values are plotted as mean ± S.E.M. in mg protein/ml lavage.




Appendix E. Serum and Lung Homogenate Protein in Sprague Dawley Rats After BCNU Administration

Data is taken from experimental groups where serum or lung homogenate protein analysis was performed. Values are plotted as mean ± S.E.M. in mg protein/ml serum or homogenate.