Carmustine, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), is a highly effective anti-cancer drug and bone marrow suppressant agent in humans and animals. Pilot studies demonstrated that BCNU induced a time- and dose-dependent supersensitivity to norepinephrine (NE) in rat caudal arteries after a single dose. The studies presented in this thesis were performed to determine the mechanism for this supersensitivity.

Male Sprague-Dawley rats were administered a single dose of BCNU (25mg/kg, i.p.) on day 0. On day 7 a proximal section of caudal artery was doubly cannulated and perfused intraluminally with Krebs bicarbonate physiological buffer. NE, methoxamine, and clonidine were perfused intraluminally. Concentration response (CR) relationships performed with methoxamine and clonidine were not statistically different in caudal arteries from control or BCNU treated rats, however, the number of alpha-1 adrenergic receptor binding sites,
using [$^3$H] prazosin, decreased after treatment with BCNU (p<.05). These studies demonstrated that the supersensitivity induced by BCNU treatment was pre-junctional.

Denervation of caudal arteries with 6-hydroxydopamine led to a significant decrease in the EC50 for NE in caudal arteries from control rats ($7.7 \times 10^{-7}$M to $3.8 \times 10^{-7}$M NE) but not BCNU treated rats ($3.4 \times 10^{-7}$M to $3.2 \times 10^{-7}$M NE). The EC50 for NE in control-denervated arterial segments was not statistically different from BCNU-denervated or BCNU-nondenervated segments.

Metabolism of [$^3$H] NE to its 5 primary metabolites, as determined by thin layer chromatography, and uptake of [$^3$H] NE were significantly lower in caudal arteries taken from BCNU treated rats.

These data demonstrate that a pre-junctional mechanism was responsible for vascular supersensitivity to NE after BCNU treatment in caudal arteries from Sprague-Dawley rats.
Characterization of Dynamic Changes in Vascular Reactivity Following Treatment with Carmustine in Sprague-Dawley Rats

by

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Characterization of Dynamic Changes in Vascular Reactivity Following Treatment with Carmustine in Sprague-Dawley Rats

Chapter 1

Introduction and Overview

Toxicity represents a limiting factor for the application of any chemotherapeutic agent. Carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea) (BCNU) is one of three nitrosourea agents used therapeutically for the treatment of cancer. BCNU was first synthesized by the Southern Research Institute and was selected for development as an anticancer agent in 1962 after it was shown to be extremely effective against implanted L1210 leukemia cells in mice (Schepartz, 1976). Clinical trials with BCNU demonstrated that it was extremely toxic to the hematopoietic system, liver, and kidneys (De Vita et al., 1965). Pulmonary fibrosis has also been recognized as a dose-limiting toxicity of BCNU (Wilson James, 1978).

Intravenous administration of BCNU results in its disappearance from plasma within 5-15 minutes but prolonged levels of radioactivity from labelled BCNU remain in plasma and tissue representing fragments or metabolites of the parent molecule (Oliverio, 1976). The primary route of elimination is through the kidneys with biliary excretion and gut resorption contributing to its disposition (Oliverio, 1976). Seventy-eight percent of a
single dose of BCNU is excreted via the kidneys in 24 hours in the mouse. The fate of the remaining 20% is undetermined but appears to distribute evenly in body water (Oliverio, 1976).

BCNU decomposes spontaneously to yield a chloroethyl carbonium ion and chloroethyl isocyanate. The carbonium ion is responsible for its alkylating properties and the isocyanate can react with nucleophilic groups to yield carbamoylated derivatives (Bono, 1976). Alkylation of nucleic acids, proteins and carbamoylation of proteins have been characterized (Bono, 1976). Liver microsomal enzymes will catalyze the denitrosation of BCNU to 1,3-bis(2-chloroethyl) urea (BCU) which has negligible anti-tumor activity (Levin et al., 1979). Induction of these cytochrome P-450 enzymes with phenobarbital results in a decreased systemic toxicity and anti-tumor activity of BCNU (Reed and May, 1978).

Wheeler et al. (1974) proposed that the therapeutic effects of BCNU were produced via alkylation and the cytotoxic effects were a result of carbamoylation. Alkylating agents are highly reactive electrophiles that can potentially react with a large number of nucleophilic sites in mammalian cells. Their two major effects on cells are: 1) lethality and 2) a delayed progression through the cell cycle (Meyn and Murray, 1984). The mechanism of anti-tumor action has not been established but it is thought to involve the bifunctional crosslinking characteristics of BCNU. Interstrand DNA
crosslinks may result from chloroethylations at guanine-\(\text{O}^6\) positions followed by further reactions to form G-C crosslinks (Erickson et al., 1980). Normal human cells and some tumor cell strains contain a guanine-\(\text{O}^6\)-alkyltransferase which removes monoadducts from the guanine-\(\text{O}^6\) position and prevents the formation of interstrand crosslinks (Lindahl, 1982). The repair activity of the transferase is expended in its reaction with alkylated DNA, "suicide inactivation". Guanine-\(\text{O}^6\)-alkyltransferase was found lacking in human tumor cell lines which were more sensitive to chloroethyl nitrosoureas (Sariban et al., 1984). Cells without this enzyme were more likely to form DNA interstrand crosslinks after exposure to chloroethyl nitrosoureas. BCNU and other nitrosoureas with carbamoylating potential, were shown to carbamoylate the transferase, rendering it inactive (Sariban et al., 1984).

Kann et al. (1980) demonstrated that nucleotide excision repair may be crucial to the ability for normal human cells to recover from the action of the chloroethyl nitrosoureas. Ligase was shown to be inhibited by carbamoylation (Erickson et al., 1980; Fornace et al., 1978; Kann et al., 1980), which inhibited the strand rejoining step in DNA excision repair. Inhibition of ligase by BHCNU, a nitrosourea with only carbamoylating activity and no alkylating potential (Tew et al., 1985), acted synergistically with radiation to induce DNA damage
in mouse leukemia cells, yet it did not possess any lethal activity on its own (Kann et al., 1980). Inhibition of ligase activity with BCNU may amplify its toxicity since an excision repair mechanism is involved in the repair of DNA damaged by alkylation.

Younes and Siegers (1981) showed that depletion of intracellular levels of GSH occurred via the detoxification of carbonium ions from BCNU. Babson and Reed (1978) demonstrated that both BCNU and its isocyanate derivative induced a time-dependent inactivation of purified yeast glutathione reductase. Depressed levels of glutathione reductase activity are found in erythrocytes, leukocytes and platelets in patients exposed to therapeutic doses of BCNU (Frischer and Ahmad, 1977). BHCNU was shown to produce a dose-dependent inhibition of glutathione reductase and a depletion of thiols in vitro in a Walker rat carcinoma cell line (Tew et al., 1985). GSH is the major non-protein thiol found in cells and serves many important biological functions. Crucial among these is the protective function whereby free radicals and xenobiotics are conjugated with the sulfhydryl residue of GSH. Depletion of GSH can potentially induce lipid peroxidation of membrane lipids which is thought to be important to the overall damage or death of a cell.

BCNU was shown to enhance the lysis of cultured vascular endothelial cells by H₂O₂ through its inhibitory action on glutathione reductase (Harlan et al., 1984).
Nicolson and Custead (1985) demonstrated that incubation of vascular endothelial cell monolayers with 40 μg/ml of BCNU for 15-20 minutes resulted in the enhanced attachment of metastatic cells and platelets to the subendothelial matrix. Many of the endothelial cells were shown to be nonviable. The endothelium may frequently be exposed to oxygen products generated by inflammatory cells, toxic agents, or hyperoxia (Harlan et al., 1983). The endogenous antioxidant properties of the glutathione cycle may be critical in preventing or limiting vascular injury in vivo (Younes and Siegers, 1981).

Inhibition of glutathione reductase or depletion of cellular glutathione by 60-85% will eliminate the mitotic apparatus (Tew et al., 1985). Brodie et al. (1980) demonstrated that direct binding of isocyanates to purified tubulin inhibited its polymerization. Tew et al. (1985) recognized chromosomal aberrations typical of spindle damage following sublethal BHCNU treatment.

Recently, Seidenfeld and Komar (1985) showed that depletion of intracellular polyamines induced chemosensitization to BCNU in tumor cell lines which were previously resistant. Higher levels of DNA cross-linking was observed after inhibition of ornithine decarboxylase with DFMO that could be reversed by the addition of putrescine.

The anti-tumor activity of BCNU is not fully understood. It appears to be related mainly to BCNU's
alkylating properties, but carbamoylation of DNA repair proteins via the chloroethyl isocyanate ion may potentiate the anti-tumor effectiveness of BCNU. Therapy with BCNU is complicated and limited by several dose-dependent toxicities. The studies presented in this thesis examine the potential cardiovascular effects of BCNU and its impact on vascular reactivity in the Sprague-Dawley rat. These were undertaken in view of the reported effects on vascular endothelial cells in vitro and because of my own pilot experiments which suggested an increased sensitivity of isolated arterial segments to exogenous norepinephrine.
Chapter 2
Effects of Carmustine (BCNU)
on Cardiovascular Performance

Introduction

Incidental observations during previous studies with BCNU had suggested that there was a cardiovascular component to the toxicity of this carcinostatic drug in the Sprague-Dawley rat. Often the hearts of BCNU treated rats appeared smaller and it was difficult to obtain blood by trans-thoracic cardiac puncture. In many regions the gross appearance of blood vessel walls was changed from smooth and consistent to more irregular. Regions of the vessel walls appeared to have collapsed between segments of normal-looking vasculature giving the appearance of weakened or aneurysmal regions. These changes took place over several weeks post-dosing with a single dose of BCNU administered i.p.. However, except for reported fatal hepatic venoocclusive disease following treatment of cancer with BCNU alone or in combination with other chemotherapeutic agents (McIntyre et al., 1981), no other reports demonstrating compromised cardiovascular performance with BCNU could be found either in the clinical or experimental literature.

These overt characteristics of BCNU pretreatment on the heart and blood vessels of Sprague Dawley rats led me to look into possible changes in cardiovascular performance as well as to examine some of the structural compo-
nants of the vasculature in these rats. Over a protracted time course, rats treated with BCNU became jaundiced as a result of the hyperbilirubinemia associated with the development of cholestasis (Thompson and Larson, 1969; Hoyt and Larson, 1985). The cholestatic properties of BCNU have also been investigated in this laboratory (Hoyt and Larson, 1985). BCNU decreased bile flow by 48 hours after its administration and a progressive course of this defect is followed through at least two weeks (Hoyt and Larson, 1985). Thompson and Larson (1969) demonstrated that total serum bilirubin levels in rats dosed with a single ip administration of 30 mg/kg BCNU reached maximum levels by 28 days post treatment.

These jaundiced, cholestatic rats might also be expected to have elevated serum levels of bile salts. Wakim et al. (1940) reported that i.v. infusions of bile led to decreases in blood pressure and heart rate in dogs. The hearts from these dogs appeared flabby and dilated at necropsy. Small blood vessels had ruptured and interstitial hemorrhages into the myocardium were present. Bile duct ligation has been shown to lead to the accumulation of serum bile salts and bilirubin in rats (Bogin et al., 1983). Levels of deoxycholic acid (DOC) after 6 days of ligation were 30 times the level seen in control serum. This same high level of DOC (40 μmoles/L) caused a marked inhibition in the cell beating rate of
cultured rat heart cells by 8 hours. Within 48 hours after its addition there was complete cessation of beating (Bogin et al., 1983). Decreases in pH and increases in lactic acid formation were observed within 24 hours after the addition of DOC or of bile duct ligated rat serum to cultured rat heart cells. DOC has been shown to cause swelling of mitochondria and sarcoplasmic reticulum and to affect oxidative phosphorylation leading to decreased levels of cellular ATP (Bogin et al., 1983).

Other studies have shown that BCNU leads to lethal effects in cultured arterial endothelium within two hours of incubation, in vitro (Harlan et al., 1984). This type of vascular endothelial damage was also suggested from studies with Sprague-Dawley rats in vivo by Jarvi (1986). Serum from these rats had elevated levels of angiotensin converting enzyme activity, indicative of endothelial damage (Nicolson and Custead, 1985), within 24 hours following a single ip dose of BCNU (20 mg/kg). This initial type of endothelial injury by BCNU could lead to alterations in vascular responsiveness to many drugs and endogenous compounds as well as to other long term effects (Fuster, 1982; Gryglewski and Szczeklik, 1982; Wight, 1985). The confluent monolayer of endothelial cells lining blood vessels has recently been discovered to play a significant role in vascular reactivity by the secretion of several endothelium-dependent factors (Furchgott and Zawadzki, 1980; Demay et al., 1982; Singer
and Peach, 1983; Furchgott, 1984). The release of endothelium-dependent relaxing factor (EDRF) by such compounds as acetylcholine and bradykinin could be impaired in damaged endothelial cells. Also, impairing the release of prostacyclin (PGI₂), a vasodilator, from vascular endothelium by norepinephrine (NE) (Armstrong, 1982) may alter vascular smooth muscle responsiveness to NE. Edema of the vascular smooth muscle could also result from endothelial damage and produce mechanical interference with responsiveness of the blood vessels. Other structural changes could also play a role (Folkow et al., 1970). Thus, cardiovascular effects might be expected secondary to the cholestatic actions of BCNU, or it might have other more primary actions of its own on the heart and/or blood vessels.

Based on these observations, several cardiovascular variables in rats pretreated with BCNU were measured. In vivo, whole animal studies were conducted to investigate effects of BCNU on heart rate (HR), mean blood pressure (BP), systolic blood pressure (SP), diastolic blood pressure (DP), pulse pressure (PP), and dose response relationships (DR) to NE, at weekly intervals through 5 weeks post-dosing. In view of the recently reported in vitro effects of BCNU on endothelial cells (Nicolson and Custead, 1985) and the important role they play in vascular reactivity (Furchgott, 1984), I looked for evidence of endothelial damage in caudal artery segments.
from BCNU treated rats. The rat caudal artery was also chosen as an *in vitro* model to assess effects of BCNU on vascular structural components since this artery had been observed to exhibit the aforementioned irregularities in vascular appearance. Collagen levels, contributing to the tensile and elastic properties of arteries, were determined in order to investigate the possibility that BCNU treatment would affect this arterial structural component.
Methods

Male Sprague-Dawley and Fischer 344 albino rats, weighing 200-350 g and 8-12 weeks of age were maintained on a 12 hour light/dark cycle at 22 ± 1°C. They were housed in groups of 5 animals to a cage (41 x 24 x 18 cm) in which they had free access to both food and water. Rats were randomly assigned to either control (corn oil vehicle) or BCNU treatment groups on day 0. Animals were dosed intraperitoneally with a single injection of vehicle (1.0 ml/kg) or BCNU (20 or 25 mg/kg) dissolved in corn oil. Previous pilot studies had demonstrated that administration of these doses of BCNU led to both dose- and time-dependent changes in vascular reactivity to norepinephrine in the rat caudal artery which will be discussed later (Appendix).

Sample differences were determined to be statistically significant by Student's t-test at the 95% confidence level. Some samples were composed of only two values however the t-statistic was still used in these cases.

Chemicals and Drugs

BCNU (NSC#409962) was obtained from the Drug Syntheses and Chemistry Branch, Division of Cancer Treatment of the National Cancer Institute. The sodium
Pentobarbital was purchased from the City Chemical Corp. Propylene glycol, ascorbic acid, NaCl, KCl, MgSO₄, CaCl₂, NaHCO₃, dextrose, CuSO₄, Na-K tartrate, sodium deoxycholate, NaOH, and citric acid were purchased from the J.T. Baker Chemical Co. Folin Phenol Reagent was purchased from Accra-Lab, Inc. and hydroxyproline was purchased from Nutritional Biochemicals, Inc. Heparin (Lipo-HepinR) was purchased from Riker Laboratories, Inc. and the norepinephrine (LevophedR) bought from Breon Laboratories. EDTA and perchloric acid were purchased from Mallinckrodt Chemical Works and Sigma Chemical Co. was the supplier for both the acetylcholine chloride (Ach) and the p-dimethylaminobenzaldehyde. Bovine serum albumin was purchased from Calbiochem and the Chloramine-T (sodium N-chloro-p-toluene sulfonamide) was purchased from Eastman Kodak, Co.

Solutions Preparation

Na Pentobarbital was prepared in the following manner:

1) 0.60g of Na pentobarbital
2) Add 1.0 ml of 95% ethanol, swirl to wet
3) 2.0 ml of propylene glycol
4) Q.S. to 10 ml with distilled water, shake to dissolve.

Heparinized Saline - Heparin 10,000 units/ml was
diluted 1000-fold with normal saline, .9%, to give 10 units/ml.

Concentrations of norepinephrine were made up to correspond with each rat's weight so that volumes for each dose would be the same from rat to rat. Doses of 0.1-4.0 μg/kg were used (5 - 200 μl volumes).

Solutions of norepinephrine (NE) and Ach were made fresh daily. A norepinephrine stock solution (10^-5 M) was made up in 1 mM HCl. Aliquots of this stock solution were added directly to make up dilutions using Krebs bicarbonate buffer. Ach was made as a stock solution in Krebs bicarbonate buffer, and dilutions were made in Krebs as well.

The chloramine-T solution was made by dissolving 1.41g of Chloramine-T in 10 ml of n-propanol and 10 ml of distilled water. Eighty milliliters of pH 6 buffer (25g citric acid x H2O; 6 ml glacial acetic acid; 60g sodium acetate x 3H2O; 17g sodium hydroxide to 500 ml) was added to 100 ml total volume. Aldehyde/Perchloric acid solution was made fresh daily. Fifteen grams of p-dimethylaminobenzaldehyde was suspended in 62 ml n-propanol, and 26 ml of 60% perchloric acid was added slowly to 100 ml total volume.
A. **In Vivo Studies**

Sprague-Dawley rats were anesthetized with Na pentobarbital, i.p., by gradual administration of the drug (initially 50mg/kg, wait 5-10 minutes, then give additional 5mg/kg injections until animal no longer responds to the pedal reflex test). This was neccessary to achieve equivalent levels of anesthesia for each animal. The rat was placed on a heating pad, the temperature of which was regulated by a rectal thermocouple probe (Tele-Thermometer, YSI model 73, Yellow Springs Instrument, INC.) and maintaining rectal temperature at 37°C. In general, less anesthetic was required to achieve anesthesia in BCNU-treated rats than in control rats. This effect was expected according to previous work done by Thompson and Larson (1969) which showed increased sensitivity to pentobarbital after BCNU treatment in rats.

Surgery was performed to isolate the right femoral artery and vein. Surface connective tissue, skeletal muscle, and fascia surrounding the vein, artery, nerve group were carefully dissected to separate them. The femoral vein was cannulated first with a short piece of heparinized PE 10 tubing with an injection cap attached to the distal end for the administration of drugs. The cannula was pushed in until resistance was met, approximately 20 mm, so that administration of drug was
closer to the vena cava and required less saline flushing volume. The femoral artery was cannulated with heparinized PE50 tubing coupled directly to a pressure transducer (Statham P23Gb) and through a Gould amplifier coupler connected to a Dynograph\textsuperscript{R} (Beckman type RB) physiological recorder. Heart rate (HR), Systolic Blood Pressure (SP), Diastolic Blood Pressure (DP), Mean Blood Pressure (BP) and Pulse Pressure (SP-DP=PP) were determined in controls and on day 7, 14, 21, 28, and 35 after dosing with 20mg/kg BCNU, i.p.. After cannulation was completed, a 20 minute equilibration period was allowed before recording responses. A control flush volume of heparinized saline (0.1 ml) for each rat did not cause any significant fluctuations in the measured variables. Two dose-response relationships were established for each rat with bolus doses of norepinephrine (NE), (0.1-4 \( \mu \)g/kg). At the end of each experiment, rats were sacrificed with an excess dose of Na pentobarbital, i.v., followed by exsanguination and thoracotomy. The hearts were quickly removed and weighed after flushing them thoroughly with 0.9\% saline.

B. Endothelial Integrity

Endothelium-dependent relaxation of precontracted arteries with Ach (acetylcholine) was used as an assay for endothelial functional integrity (Furchgott, 1980).
Male Sprague-Dawley rats were injected, i.p., with either corn oil vehicle (1.0 ml/kg) for controls or with 25 mg/kg BCNU dissolved in corn oil. Rats were sacrificed by cervical dislocation and thoracotomy on days 1, 2 and 7 after treatment. A proximal section of caudal artery, 1.5-2.0 cm in length, was carefully isolated. Before cannulation some arteries from control rats were rubbed 5 times on their inner surface with a stainless steel rod to remove endothelial cells (Furchgott, 1980). This treatment was called "control-rubbed" and served as a positive control for endothelial damage. The collateral vessels were tied off and both ends of the segment were cannulated with PE50 tubing. The cannulated arterial segment was then mounted in a 3.0 ml capacity glass tissue bath with insulating water jacket maintained at 37°C. The arterial segment was perfused intraluminally by use of a roller pump and was bathed extraluminally with Krebs bicarbonate buffer made fresh daily (122 mM NaCl, 5.2 mM KCl, 1.2 mM MgSO₄, 1.6 mM CaCl₂, 25 mM NaHCO₃, 0.03 mM EDTA, 0.11 mM Ascorbic Acid, 11.1 mM Dextrose, bubbled continuously with a 95% O₂, 5% CO₂ gas mixture to maintain the pH at 7.4). Once mounted, the arterial segments were allowed a 1 hour period for equilibration before beginning the experiment. This system allows solutions to be applied independently to the intraluminal or extraluminal surfaces of the artery. Perfusion flow rates were established to give a baseline perfusion pressure of 30 mmHg for each arterial segment. The rate
of flow remained constant for each preparation throughout the experiment. Changes in vascular resistance were recorded as changes in perfusion pressure by means of a Statham pressure transducer (model P23Gb) and recorded on a strip chart recorder. Measurement of the contractile responses were calculated by taking the maximum amplitude reached from the baseline during exposure to NE. Administration of NE \( (10^{-6} \text{M} - 5.6 \times 10^{-6} \text{M}) \) to the extraluminal surface of arterial segments permitted the intraluminal administration of Ach \( (10^{-8} \text{M} - 10^{-5} \text{M}) \). The percent relaxation with Ach was calculated as the change in pressure after Ach divided by the pre-relaxation pressure in mm Hg times 100.

C. Arterial Edema, Protein, and Collagen Content

Vascular endothelial damage by BCNU (Harlan et al., 1984) could result in edema or lead to other structural changes in vascular tissue that would affect arterial responsiveness (Folkow et al., 1970). The possibility of tissue edema was explored by determining the percent water content in arterial segments as well as the arterial percent protein composition, all based on the total artery wet weight. For these studies, both Sprague-Dawley and Fischer 344 rat strains were compared to demonstrate any differences in BCNU-induced changes in arterial collagen content. Lungs in Sprague-Dawley rats
had been observed to increase in collagen content following BCNU treatment while those of Fischer 344 rats did not after 28 days (Jarvi, 1986). Rats were sacrificed by cervical dislocation followed by thoracotomy. The caudal artery was isolated and carefully freed of surrounding connective tissue, rinsed with 0.9% saline and blotted lightly on gauze. The percent water content of each arterial segment was determined by placing pre-weighed segments of arterial tissue into dried and tared 10 ml beakers. The segments were then dried in an oven at 100°C and weighed at 1/2 hour intervals until no further changes in mass were noted. Percent water content was calculated by subtraction of wet and dry arterial weights then converting to a percent of total wet artery weight.

The protein content of each arterial segment was determined by first weighing the segment in 1.0 ml of deionized water. The artery was minced into small pieces with scissors then transferred to a ground glass homogenizer in a total volume of 5.0 ml. When no large pieces remained, the artery homogenate was then more finely ground with an Ultra-turrax homogenizer set at high speed for 30 seconds. A 1:5 dilution of this homogenate was used for the protein determination and for the collagen assay. Protein measurements were performed as described by Lowery et al. (1951). Each analysis was run in duplicate. A protein standard of 0.2mg/ml bovine serum albumin was used. A fresh solution of 0.01% CuSO₄,
0.02% Na-K tartrate and 2% Na$_2$CO$_3$ in H$_2$O (solution #1) was made fresh daily. Each sample contained 3.0 ml solution #1, 0.15 ml of 2 M NaOH, 0.15 ml 2% Na deoxycholate, and 0.5 ml of protein standard, arterial homogenate or H$_2$O blank. This was mixed by vortex then left to stand for 20 minutes. While mixing, 0.3 ml of phenol reagent was added to each sample then left to stand for an additional 45 minutes to permit color formation. The absorbance of each sample was read at 500 nm against a water blank reference using a UV-visible spectrophotometer (Bausch and Lomb, Spectronic 600). Protein concentrations of arterial homogenates were calculated by the following formula:

$$[\text{Protein}] = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times 0.2 \text{mg/ml} \times 5 \times (\text{the dilution factor})$$

Collagen was measured as the hydroxyproline content of arterial segments according to the method of Huszar et al. (1980). Aliquots of 0.2 ml of the tissue homogenate or of H$_2$O were added to pyrex test tubes. These samples were evaporated to dryness in an oven at 100$^\circ$C; 0.05 ml of 4N NaOH was added to each tube, mixed, then autoclaved for 10 min at 120$^\circ$C. At this point in the assay, the standards for hydroxyproline were started by the addition of 0.05 ml of 4 N NaOH without autoclaving. Excessive base hydrolysis of the hydroxyproline standards was found to disrupt color formation. To neutralize the sample, 0.05 ml of 1.4 N citric acid was added to bring the pH
to 6 as measured with pH indicator paper. One ml of chloramine-T solution was added and mixed thoroughly followed by a 20 minute incubation at room temperature. One ml of aldehyde/ perchloric acid solution was then added and, after vortexing, the samples were incubated for 15 min at 65°C in a water bath. The absorbance of each sample and standard were read at 550 nm against a reference blank on a UV-visible spectrophotometer (Bausch and Lomb, Spectronic 600). The sample contents of hydroxyproline were determined by linear regression of standard curves and extrapolation.
Results

A. In Vivo Studies

Treatment with BCNU led to decreases in whole body and heart masses as well as to alterations in the heart:body weight relationship (Table 1). Control rats maintained an average weight gain of 3.4 grams per day for the 28 day period. Treated rats continually lost weight through day 14 but they were able to gain weight by day 21. However, rats lost weight again during the fourth week following treatment with BCNU so that by day 28 they had lost an average of 3.7 grams per day with respect to controls (Table 1).

Control heart weights gradually increased through day 28 but not as rapidly as the body weights resulting in decreases in the heart:body weight ratios over time (Table 1). The heart:body weight ratios for treated rats fluctuated with respect to controls but by day 28 was not different from the control ratio. This observation is misleading because rats with significantly different body weights are being compared. A more meaningful comparison to make is that between treated and weight matched controls. When weight matched controls (200-260g ; x = 240 ± 6.9 g) were compared to day 28 rats (200-260g ; x = 237.5 ± 13.2 g) heart weights were still lower in the treated group, consequently a statistically significant
Table 1

Body Weight, Heart Weight, and Heart:Body Weight Ratios of Control and BCNU Treated Sprague-Dawley Rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Weight Matched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>244.3±9.7</td>
<td>281.4±3.4</td>
<td>311.7±4.6</td>
<td>340.0±5.0</td>
<td>240.0±6.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td>(14)</td>
<td>(6)</td>
<td>(2)</td>
<td>(7)</td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>253.3±7.6</td>
<td>229.5±7.6</td>
<td>276.7±6.7</td>
<td>237.5±13.2</td>
<td>253.3±17.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>(10)</td>
<td>(3)</td>
<td>(4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---A. Body Weights---

Control 0.77±0.04 0.83±0.02 0.86±0.03 0.95±0.01 0.72±0.04 (7) (14) (6) (2) (7)

Treated 0.73±0.03 0.66±0.03 0.75±0.03 0.63±0.04 (3) (10) (3) (4)

---B. Heart Weights---

Control 0.316±.018% 0.295±.006% 0.275±.007% 0.268+.001% 0.310+.013% (7) (14) (6) (2) (7)

Treated 0.291+.014% 0.329+.017% 0.272+.009% 0.268+.006% (3) (10) (3) (4)

---C. Heart : Body Weight Ratio---

Each value represents the mean ± s.e.m. of the number of animals in parentheses.

a) Untreated rats were weight matched with the 28-day BCNU treated rats for comparison.
b) Treated rats were injected, i.p., with a single dose of 20 mg/kg BCNU in corn oil on Day 0. Body and heart weights were determined on days 7-28 following treatment.
c) Body and heart weights are expressed in grams.
d) The percent of whole body weight contributed by the heart is calculated as heart weight (g) divided by whole body weight (g) x 100.

*Significantly different (p<.05) from the weight-matched group.
decrease in the heart: body weight ratio was evident (p<.05) (Table 1.). Grossly, hearts from treated rats on day 28 appeared dilated with thinner ventricular walls.

Basal cardiovascular variables of, BP, DP, SP, PP (Table 2, Figure 1) and HR (Table 3, Figure 2) were not significantly altered in rats by day 7 after a single i.p. injection of BCNU (20 mg/kg). Mean blood pressure, recorded prior to injecting rats with NE, was significantly higher (p<.001) by day 21 in rats treated with BCNU, however it decreased and was significantly lower than in controls on days 28 (p<.025) and 35 (p<.05) (Table 2, Figure 1). The components of mean blood pressure are the systolic and diastolic pressures. Systolic pressure became significantly lower than controls on days 28 (p<.025) and 35 (p<.005) after BCNU treatment. On the other hand, diastolic pressure was elevated on days 14 (p<.05) and 21 (p<.05) but had decreased again to control levels on day 28 and was lower, but not statistically different from controls on day 35. The early increase in diastolic pressure accounted for the increase in mean blood pressure on days 14 and 21 since no significant changes in systolic pressure were evident at these times. Pulse pressures were lower than controls at all times, from day 7 through day 35 after BCNU treatment. This is a reflection of higher diastolic
TABLE 2
Mean, Systolic, Diastolic and Pulse Pressures Measured In Vivo in Anesthetized Control and BCNU Treated Sprague-Dawley Rats.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A. Mean Pressure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>92.5 ± 12.5</td>
<td>105.0 ± 5.1</td>
<td>112.0 ± 3.0</td>
<td>89.5 ± 5.5</td>
<td>70.0 ± 5.0</td>
</tr>
<tr>
<td><strong>B. Systolic Pressure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>115.0 ± 25.0</td>
<td>121.5 ± 4.7</td>
<td>135.0 ± 5.0</td>
<td>102.8 ± 1.7**</td>
<td>82.0 ± 10.0*</td>
</tr>
<tr>
<td><strong>C. Diastolic Pressure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>80.0 ± 10.0</td>
<td>89.1 ± 5.5†</td>
<td>96.0 ± 1.0†</td>
<td>80.0 ± 5.7</td>
<td>63.5 ± 1.5</td>
</tr>
<tr>
<td><strong>D. Pulse Pressure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>35.0 ± 15.0</td>
<td>33.0 ± 3.3†</td>
<td>38.0 ± 3.5†</td>
<td>20.0 ± 6.4**</td>
<td>18.0 ± 5.7†</td>
</tr>
</tbody>
</table>

*Rats were injected, ip, with a single dose of BCNU (20 mg/kg) on day 0. They were anesthetized with 50 mg/kg of Na Pentobarbital, ip, on days 7,14,21,28, and 35 after dosing with BCNU.

Mean, Systolic, and Diastolic pressures (mmHg) were obtained by arterial pressure measurements from the femoral artery.

Pulse pressures (mmHg) were calculated as the difference between Systolic and Diastolic pressures. Each value represents the mean ± s.e.m.. Numbers in parentheses designate the number of animals used. Significantly different from the control group * (p < .005), ** (p < .025), † (p < .05), student's t-test.
FIGURE 1. Mean, systolic, diastolic and pulse pressures of anesthetized control and BCNU treated Sprague-Dawley rats. Values are taken from data contained in Table 2. Rats were injected, i.p., with a single dose of BCNU (20 mg/kg) on Day 0. They were anesthetized with 50 mg/kg of Na pentobarbital, i.p., on Days 14, 21, 28, and 35 after dosing with BCNU. Arterial pressure was recorded from a femoral artery cannulation. *Significantly different (p<.005) from the control group, Student's t-test. **Significantly different (p<.025) from the control group. †Significantly different (p<.05) from the control group.
Figure 1

Control

Day 14

Day 21

Day 28

Day 35

Mean Press

Systolic Press

Diastolic Press

Pulse Press

mmHg
TABLE 3
Heart Rates of Anesthetized Control and BCNU Treated Sprague-Dawley Rats.

<table>
<thead>
<tr>
<th>Heart Rates&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BCNU Treated&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>Day 7</td>
</tr>
<tr>
<td>394 ± 7</td>
<td>390 ± 21</td>
</tr>
<tr>
<td>(24)</td>
<td>(2)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Heart rates are expressed as beats per minute. They were obtained from arterial pressure measurements via the femoral artery.

<sup>b</sup>Rats were injected, ip, with a single dose of BCNU (20 mg/kg) on Day 0. They were anesthetized with 50 mg/kg Na Pentobarbital, ip, on days 7, 14, 21, 28, and 35 after dosing with BCNU.

Each value represents the mean ± s.e.m.. Number in parentheses represents the number of animals used.

*Significantly different (p<.005) from the Control group, student's t-test.
FIGURE 2. Heart rates of anesthetized control and BCNU treated Sprague-Dawley rats. Values are taken from data contained in Table 3 and are expressed as beats/minute. Rats were injected, i.p., with a single dose of BCNU (20mg/kg) on Day 0. They were anesthetized with 50 mg/kg of Na pentobarbital, i.p, on days 7, 14, 21, 28, and 35 after dosing with BCNU. Significantly different (p<.005) from the control group, Student's t-test.
Figure 2

Heart Rate (Beats/Min)

<table>
<thead>
<tr>
<th>Day</th>
<th>Heart Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>394</td>
</tr>
<tr>
<td>Day 14</td>
<td>390</td>
</tr>
<tr>
<td>Day 21</td>
<td>426</td>
</tr>
<tr>
<td>Day 28</td>
<td>331</td>
</tr>
<tr>
<td>Day 35</td>
<td>347</td>
</tr>
</tbody>
</table>

Time (Days) After 20 mg/kg BCNU

p < 0.005
pressures through day 21 and lower systolic pressures on
days 28 and 35.

Heart rates were significantly elevated on day 21
after BCNU treatment \((p<.005)\), but were lower than
controls on days 28 and 35 (Table 3, Figure 2). The
increase in heart rate on day 21 contributed to the
elevations in mean blood pressure seen at this time
(Figure 1). Representative tracings of physiograph
recordings are illustrated in Figure 3. Characteristics
of the arterial pressure wave were altered after
treatment with BCNU. The time required for arterial
pressure to rise from diastolic to peak systolic pressure
gradually increased with time after administration of
BCNU as can be seen in Figure 3. These tracings also
demonstrate the gradual decrease in pulse pressure
observed after BCNU treatment. Also, the time required
for the pressure to decrease, from peak systolic pressure
to the first shoulder of the pressure wave is increased.
These quantitative observations of cardiac performance
suggest that cardiac function has been compromised
following treatment with BCNU in these rats.

Dose response relationships with NE in vivo, were
evaluated by plotting the maximal systolic and diastolic
pressures attained after drug administration as a
function of NE dose (Figure 4, A. and B.). On days 7, 14,
and 21 after dosing with BCNU, no significant differences
in dose response relationships could be demonstrated with
FIGURE 3. *In vivo* arterial pressure tracings from the femoral artery of anesthetized control and BCNU treated Sprague-Dawley rats. Treated rats were injected, i.p., with a single dose of 20 mg/kg BCNU in corn oil on Day 0.
 CONTROL

 DAY 14 (20 mg/kg BCNU)

 DAY 21 (20 mg/kg BCNU)

 DAY 28 (20 mg/kg BCNU)

FIGURE 3
FIGURE 4. Dose response relationships with norepinephrine generated In Vivo as changes in both diastolic and systolic pressures of control and BCNU treated Sprague-Dawley rats. Bolus doses of norepinephrine were administered via the femoral vein and are expressed as μg norepinephrine per kg body weight. Figures 4A. and 4B. illustrate changes in diastolic and systolic pressures (mmHg), respectively, after the intravenous administration of norepinephrine. Data points represent the mean ± s.e.m. for n=19 control and n=2 Day 28 treated rats. Treated rats were injected, i.p., with a single dose of BCNU (20 mg/kg) on Day 0. All animals were anesthetized with 50 mg/kg of Na pentobarbital, i.p.. Both the femoral artery and vein were cannulated to measure arterial pressure and to administer norepinephrine, respectively. The systolic dose response curve from treated rats was significantly different (p<.05) from the control curve.
Figure 4A
Diastolic Pressure

Control  Day 28

Change in Pressure (mmHg)

Log Dose of NE (µg/kg)

Figure 4B
Systolic Pressure

Control  Day 28

Log Dose of NE (µg/kg)
respect to the controls (data not shown), however, by day 28 there were some changes evident. Increases in systolic pressure were consistently lower than controls in rats treated with BCNU after day 28 (p<.025) (Figure 4, panel B). This suggested, again, that cardiac performance had been compromised following treatment with BCNU since NE was unable to increase systolic pressure to the same extent in treated rats as was seen in controls.

B. Endothelial Integrity

Concentration response relationships for NE, administered to the extraluminal surface of caudal arteries, in control, control-rubbed, and BCNU pretreated rats (days 1, 2 and 7) are displayed in Table 4 and Figure 5. Arterial responsiveness to NE, was greater in control-rubbed than in control arteries. However, owing to large variability in the first group, these differences were not statistically significant. Similar changes in responsiveness were observed after one and two days after BCNU. By seven days, arteries were significantly more sensitive to NE (p<.05) when lines were analyzed by a 2-factor analysis of variance (2-ANOVA).

The degree to which Ach was able to relax arterial segments was dependent upon the concentration of NE
TABLE 4
Responsiveness of Intact Caudal Artery Segments From Control and BCNU Treated Rats to the Extraluminal Application of Norepinephrine.

<table>
<thead>
<tr>
<th>Norepinephrine (M)</th>
<th>Control Unrubbed</th>
<th>Control Rubbed</th>
<th>BCNU Treated Day 1</th>
<th>BCNU Treated Day 2</th>
<th>BCNU Treated Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 x 10^{-6}</td>
<td>11.4 ± 1.2</td>
<td>29.5 ± 17.6</td>
<td>11.1 ± 2.4</td>
<td>13.2 ± 4.8</td>
<td>20.6 ± 4.9</td>
</tr>
<tr>
<td>1.8 x 10^{-6}</td>
<td>22.1 ± 2.6</td>
<td>40.9 ± 16.5</td>
<td>41.1 ± 15.2</td>
<td>38.8 ± 1.8</td>
<td>39.5 ± 7.9</td>
</tr>
<tr>
<td>3.2 x 10^{-6}</td>
<td>71.9 ± 4.8</td>
<td>80.2 ± 18.8</td>
<td>97.0 ± 21.4</td>
<td>87.0 ± 3.0</td>
<td>99.2 ± 12.9</td>
</tr>
<tr>
<td>5.6 x 10^{-6}</td>
<td>152.2 ± 12.0</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>183.0 ± 10.0</td>
</tr>
</tbody>
</table>

a Changes in perfusion pressure were calculated as the difference between the plateau pressure after the application of norepinephrine and the basal perfusion pressure, in mmHg.

b Rats were injected, ip, with a single dose of BCNU (25 mg/kg) on Day 0. Arteries were collected on days 1, 2, and 7 after dosing with BCNU.

c Arteries were rubbed by passing a stainless steel rod through their lumen 5 times before cannulation. Each value is the mean ± s.e.m. of 4 animals except for day 2, where n=2.
FIGURE 5. Responsiveness of intact segments of caudal arteries taken from control and BCNU treated rats to the extraluminal application of norepinephrine. Values are taken from data contained in Table 2. Rats were injected, i.p., with a single dose of BCNU (25mg/kg) on Day 0. Arteries were collected from rats on day 7 after dosing with BCNU. **Individual points were significantly different (p<.05) from the corresponding control values, Student's t-test. *The BCNU treated curve was significantly different (p<.025) from the control line, 2-factor analysis of variance.
Figure 5

Control

Log Concentration NE (M)

Log Conc.Qtrati

1.0 x 10^-6  1.8 x 10^-6  3.2 x 10^-6  5.6 x 10^-6

Change in Perfusion Pressure (mmHg)

100

120

140

160

180

200

0

1.8 x 10^-6

3.2 x 10^-6

5.6 x 10^-6

1.0 x 10^-6

Control

Day 7*

0 0 0

--X--X--X--

**

**

**

**

0
applied to the extraluminal surface (Table 5, Figures 6 and 7). For a given concentration of NE (10^{-6} M) the ability for Ach to relax or contract arterial segments was dose dependent (Table 5A, Figure 6). Moreover, the ability for a given concentration of Ach (10^{-6} M) to relax arterial segments decreased as the level of tone in the artery, or the concentration of NE, was increased, except in rubbed arteries or those from BCNU treated rats on day 1 (Table 5B, Figure 7). Rubbing the intraluminal surface of caudal artery segments inhibited the relaxant effects of Ach in precontracted arterial segments, perhaps by removal of endothelial cells (Furchgott, 1984) whereas the effects of BCNU on the endothelium had altered its ability to respond to Ach, especially 1 day after dosing. A negative percent relaxation represents the percent contraction or increase in perfusion pressure with Ach (Furchgott, 1984).

All treatment groups were less responsive to the relaxant effects of Ach when compared with the control group except on day 2 owing to a larger variability and smaller sample number (n=2). Rubbed arteries, or those taken from rats on day 1 after 25 mg/kg BCNU were the least responsive to the relaxant effects of Ach (p<.01), and, in fact, most day 1 arteries only exhibited contractile responses to Ach. Days 2 and 7 after 25 mg/kg BCNU did not differ significantly when compared with each other, but on day 7 caudal arteries were significantly less responsive than the control group (p<.05) to the
### TABLE 5
Percent Relaxation of Pre-Contracted Arterial Segments With Acetylcholine, Perfused Intraluminally.

<table>
<thead>
<tr>
<th>Acetylcholine (M) or Norepinephrine (M)</th>
<th>Control Unrubbed</th>
<th>Rubbed&lt;sup&gt;b&lt;/sup&gt;</th>
<th>BCNU Treated&lt;sup&gt;a&lt;/sup&gt; Day 1</th>
<th>Day 2</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ach 1.0 x 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>77.2 ± 8.0</td>
<td>-2.9 ± 2.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-4.2 ± 13.2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>58.1 ± 16.9</td>
<td>51.5 ± 4.6&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.0 x 10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>89.2 ± 6.5</td>
<td>8.4 ± 5.7&lt;sup&gt;*&lt;/sup&gt;</td>
<td>-25.6 ± 26.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>68.3 ± 9.5</td>
<td>71.2 ± 12.8</td>
</tr>
<tr>
<td>1.0 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>95.1 ± 4.9</td>
<td>10.4 ± 7.0&lt;sup&gt;*&lt;/sup&gt;</td>
<td>-28.5 ± 43.7</td>
<td>77.0 ± 6.4</td>
<td>79.2 ± 14.3</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(4)&lt;sup&gt;**&lt;/sup&gt;</td>
<td></td>
<td>(4)</td>
<td>(4)&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>NE 1.0 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>95.1 ± 4.9</td>
<td>10.4 ± 7.0&lt;sup&gt;*&lt;/sup&gt;</td>
<td>-28.5 ± 43.7</td>
<td>77.0 ± 6.4</td>
<td>79.2 ± 14.3</td>
</tr>
<tr>
<td>1.8 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>70.9 ± 5.8</td>
<td>16.2 ± 7.2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>-7.5 ± 7.1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>21.7 ± 22.9</td>
<td>68.1 ± 7.8</td>
</tr>
<tr>
<td>3.2 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>40.6 ± 6.9</td>
<td>18.4 ± 7.6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>-2.4 ± 2.3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>19.6 ± 16.0</td>
<td>43.2 ± 8.4</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(4)</td>
<td></td>
<td>(4)</td>
<td>(4)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Rats were injected, i.p., with a single dose of BCNU (25 mg/kg) on Day 0. Arteries were collected on days 1, 2, and 7 after dosing with BCNU.

<sup>b</sup>Arteries were rubbed by passing a stainless steel rod through their lumens 5 times before cannulating.

<sup>c</sup>Cannulated arteries were bathed extraluminally with concentrations of norepinephrine until the response became constant. Acetylcholine was perfused intraluminally through these pre-contracted arteries.

<sup>d</sup>Satterthwaite's approximation was used \( (s_1^2 
eq s_2^2) \) for t-test.

Each value represents the mean ± s.e.m. of 4 animals except on Day 2, where n=2.

Significantly different \( *(p < .05) \) from corresponding controls, \( **(p < .025) \) from control group.
FIGURE 6. Percent relaxation of pre-contracted caudal artery segments from control, control-rubbed, and BCNU treated rats with the intraluminal perfusion of varying concentrations of acetylcholine. Values are taken from data contained in Table 5, part A. Arteries were rubbed by passing a stainless steel rod through their lumen 5 times before cannulation. Rats were injected, i.p., with a single dose of BCNU (25mg/kg) on Day 0. Arteries were collected on days 1, 2, and 7 after dosing with BCNU. Cannulated arteries were bathed extraluminally with norepinephrine \((10^{-6} \text{ M})\) until the response reached a plateau. Acetylcholine was perfused intraluminally in these pre-contracted arteries to elicit relaxation. *Significantly different \((p<.05)\) from corresponding controls, Student’s t-test.
FIGURE 7. Percent relaxation of caudal artery segments from control, control-rubbed, and BCNU treated rats with acetylcholine after pre-contraction with varying concentrations of norepinephrine. Values are taken from data contained in Table 5, part B. Arteries were rubbed by passing a stainless steel rod through their lumen 5 times before cannulation. Rats were injected, ip, with a single dose of BCNU (25mg/kg) on Day 0. Arteries were collected on days 1, 2, and 7 after dosing with BCNU. Cannulated arteries were bathed extraluminally with norepinephrine until the response reached a plateau. Acetylcholine \(10^{-6}\) M was perfused intraluminally in these pre-contraction arteries. *Significantly different \((p<.05)\) from corresponding controls, Student's t-test.
relaxant effects of Ach. BCNU acutely compromised the endothelium-dependent relaxant effects of Ach on precontracted caudal artery segments with 24 hours of its administration. However, recovery of this effect appeared to be occurring 2 to 7 days following treatment.

C. Arterial Edema, Protein, and Collagen Content

The percent water content of caudal arteries, indicative of edema formation, was not different from controls at any time assayed after BCNU treatment (20 mg/kg) except on day 28 (Table 6). At this time, a decrease in the percent water content of arteries was evident (-5%) which was statistically different (p<.05) from controls in Sprague-Dawley rats. No significant changes in percent protein content of caudal arteries from either Sprague-Dawley or Fischer 344 strains were evident at any time after dosing rats with BCNU (Table 7).

Collagen content of arterial segments, measured as μg hydroxyproline per mg protein, began to increase significantly (p<.05) by day 3 in Sprague-Dawley rats (Table 7, Figure 8). The levels of collagen in caudal arteries from Sprague-Dawley rats were significantly elevated (p<.05) from day 3 through day 28 as well. Collagen levels in caudal arteries from Fischer 344 rats were not elevated above their control levels on day 21 nor on day 28 after treatment with BCNU. They were,
TABLE 6
Comparison of the Percent Water Content of Caudal Arteries From Control and BCNU Treated Sprague-Dawley Rats.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent Water Content&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arterial dry weight was determined by drying arterial segments at 100°C then weighing until constant. Percent water content was calculated by taking the difference between the wet and dry arterial weights and dividing by arterial wet weight x 100.</td>
<td>82.3 ± 0.6%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>84.6 ± 0.9%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80.5 ± 1.4%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>83.3 ± 1.1%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78.4 ± 0.6%&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(5)</td>
<td>(2)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Rats were injected, ip, with a single dose of BCNU (20 or 25 mg/kg) on Day 0. Arteries were obtained 14, 21, and 28 days after dosing with 20 mg/kg BCNU and on day 7 after 25 mg/kg BCNU.

<sup>b</sup>Each value is the mean ± s.e.m. of 5 animals except for day 7, where n=2.

<sup>•</sup>Significantly different (p<.05) from the Control group.
### TABLE 7
Percent Protein and Hydroxyproline Content of Caudal Arteries From Control and BCNU Treated Sprague-Dawley and Fischer 344 Rats.

<table>
<thead>
<tr>
<th>Strain of Rat</th>
<th>Control</th>
<th>BCNU Treated</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.-D.</td>
<td>11.8 ± 0.9% (5)</td>
<td>10.9 ± 0.5% (5)</td>
<td>10.2 ± 0.6% (5)</td>
<td>10.5 ± 0.8% (5)</td>
<td>10.1 ± 0.6% (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fischer</td>
<td>12.5 ± 0.5% (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11.2 ± 0.4% (5)</td>
</tr>
</tbody>
</table>

---A. Percent Protein---

---B. OH-Proline---

<table>
<thead>
<tr>
<th>Strain of Rat</th>
<th>Control</th>
<th>BCNU Treated</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.-D.</td>
<td>46.0 ± 0.6 (5)</td>
<td>47.6 ± 3.6 (5)</td>
<td>65.3 ± 6.2 d* (5)</td>
<td>58.4 ± 2.5 d* (5)</td>
<td>60.3 ± 2.8 d* (5)</td>
<td>63.9 ± 3.2 d* (5)</td>
<td></td>
</tr>
<tr>
<td>Fischer</td>
<td>39.6 ± 2.0 d+ (6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>38.4 ± 2.4 f (5)</td>
<td>42.4 ± 1.4 f (5)</td>
</tr>
</tbody>
</table>

---Each value represents the mean ± s.e.m. Number in parentheses is the number of animals used. Treated rats were injected, ip, with a single dose of BCNU (20 mg/kg) on Day 0. Arteries were collected on days 1, 3, 14, 21, and 28 after dosing. Protein levels were calculated as the total arterial protein content (mg) by the Lowry protein assay (1951) divided by the total arterial wet weight (mg) x 100. Hydroxyproline levels are expressed as µg hydroxyproline per mg protein and reflect levels of collagen in these arterial segments. Satterthwaite's Approximation used (s^2_1 ≠ s^2_2) for the t-test. Significantly different (p < .05) relative to control of that strain, or *(p < .05)* from corresponding S.-D. group, student's t-test.---
FIGURE 8. Collagen levels in caudal arteries from both Sprague-Dawley and Fischer 344 rats after treatment with BCNU. Values are taken from data contained in Table 7, part B. Rats were injected, i.p., with a single dose of BCNU (20 mg/kg) on Day 0. Arteries were obtained 1, 3, 14, 21, and 28 days after dosing. *Significantly different (p<.05) relative to controls of that strain, Student's t-test. **Significantly different (p<.05) from corresponding Sprague-Dawley group, student's t-test.
Figure 8

Sprague Dawley

Fischer 344

OH-Proline μg/mg Protein

Control Day 1 Day 3 Day 14 Day 21 Day 28

Time (Days) After BCNU Treatment

Day 28

40

10

0

Day 1

Day 3

Day 14

Day 21

Day 28
however, significantly lower than the corresponding Sprague-Dawley values. The higher levels of collagen observed from day 3 forward in the Sprague-Dawley caudal arteries suggested that either an increased deposition or synthesis, decreased catabolism of collagen, or both were taking place in these arteries. The resistance of the Fischer 344 strain to a BCNU-induced elevation of arterial collagen suggests that these rats were not as susceptible to the instigatory effects of this drug.
Discussion

Sprague Dawley rats, pretreated with 20 mg/kg, BCNU, displayed decreases in body weight, and heart weight and changes in heart weight:body weight ratios which were all time-dependent. Bimodal fluctuations in all of these variables were evident (Table 1). In other studies liver and lung weight:body weight ratios generally stayed the same or increased after treatment with BCNU (Hoyt, 1984; Jarvi, 1986). However, in this study there was a statistically significant decrease in the heart:body weight ratio of BCNU treated rats by day 28 when compared with a final weight matched group. This indicates that even though decreases in body mass were evident, the percent decrease in heart mass was more significant. Upon gross examination, hearts from day 28 rats appeared dilated with thinner left ventricular walls. No direct measurements of heart performance were conducted, however in vivo determinations of cardiovascular variables such as pulse pressure and systolic pressure associated with cardiac performance suggested that the heart pumping activity had been compromised.

A decrease in dietary intake by BCNU treated rats led to whole body weight losses with respect to controls. Protein or calorie deficiencies in rat diets have been shown to alter cardiac performance and to decrease body and heart weights, but not in the same manner seen after
BCNU treatment. Benfey et al. (1983) fed Sprague-Dawley rats a protein deficient diet (23% control level). Body weights dropped to 37.2% of control weights, and hearts weights decreased, but heart:body weight ratios increased significantly, unlike the effects seen after BCNU treatment. Contractile properties of the heart tissue were not altered and the number of both alpha and beta binding sites in the heart tissue did not change. This suggests that the effect of BCNU on cardiovascular performance was probably not merely a result of decreased dietary intake, but due to some other drug related effect.

Sprague-Dawley rats, pretreated with 20 mg/kg BCNU, displayed alterations in cardiovascular variables measured in vivo, under pentobarbital anesthesia. Sleep times of rats with pentobarbital have been shown to increase after treatment with a single ip dose of BCNU in a dose-dependent manner (Thompson and Larson, 1969), therefore, great care was taken to induce equivalent levels of anesthesia in all rats by the gradual administration of the anesthetic. Sodium pentobarbital is one of the most widely used anesthetics for experimental cardiovascular studies (Manders and Vatner, 1976) and several studies have investigated its uses in this regard (Manders and Vatner, 1976; Vatner, 1978). The effects of pentobarbital on cardiac output, arterial pressure and total peripheral resistance are relatively minor, but it
will reduce stroke volume, myocardial contractility, and velocity of myocardial fiber shortening (Vatner, 1978). The distribution of cardiac output may also be affected by anesthetic leading to altered responses to pharmacological agents such as NE (Vatner, 1978). Analysis of cardiovascular variables both before and after treatment with BCNU must therefore reflect the characteristics of the pentobarbital anesthetic utilized.

Mean blood pressure (BP) is a product of both cardiac output (CO) and the total peripheral resistance (TPR). TPR is regulated mainly by the degree of tone in arterioles (Guyton, 1980). Changes in BP, therefore, are directly reflective of alterations in TPR, CO or both. Following treatment with a single dose of BCNU, the mean blood pressure of rats began to rise to a maximum on day 21, then fell through day 35. For purposes of discussion these two phenomena will be addressed separately as 2 phases; phase I = day 0-day 21, the period of rising mean blood pressure, and phase II = day 21-day 35, the period of falling mean blood pressure to clarify comparisons (Figure 1, Table 2).

On days 14 and 21 there were significant increases in diastolic blood pressures but no significant changes in systolic pressures. Therefore, the rise in mean blood pressure observed at these times was primarily due to increases in the diastolic pressure. The mean blood pressure is not an average of DP and SP, but is closer to DP because of the shape of the arterial pressure curve.
(Guyton, 1980). An increase in diastolic pressure therefore, would cause a more significant change in mean blood pressure than would a similar change in systolic blood pressure, especially since the decreases in systolic blood pressure were accompanied by a change in the shape of the arterial pressure curve in treated rats (Figure 3). Diastolic pressure changes are reflective of changes in total peripheral resistance (Guyton, 1980), thus the elevated DP on days 14 and 21, suggest that TPR was elevated. Cardiac output, the other variable determining BP, is an indicator of heart performance, and is the product of heart rate (HR) and stroke volume (SV):

\[ CO = SV \times HR \]
\[ BP = TPR \times SV \times HR \]

The significant increase in HR on day 21 following treatment with BCNU probably contributed another component to the elevated BP observed at this time (Figure 2), Since no alterations in HR were apparent on day 14, the variable responsible for increased BP at this time was an elevation in DP. It is difficult to predict the values for CO since no measurement of SV were obtained. Lower pulse pressures may infer smaller SV's in treated rats during both phase I and phase II. Therefore, the contribution of CO to BP in these rats is unsure. Nevertheless, even on day 14, where no increase in HR is observed, BP is still higher even if CO has decreased. This argues that an increase in the TPR has contributed
to the elevated mean blood pressure on days 14 and 21. Increases in DP as well as HR might be caused by an increase in the basal level of sympathetic nerve activity or increased sensitivity to released NE even under a normal sympathetic tone during phase I. Changes in vascular structural characteristics such as the observed elevations in collagen levels, may increase vascular resistance to flow and thus alter TPR which would also be reflected by changes in DP.

Whereas phase I reflects processes leading to a higher mean blood pressure, phase II is characterized by a decrease in these same indices (Figures 1 and 2). There was a steady decrease in mean blood pressure from day 21, on days 28 and 35, that were lower than control BP. Lower DP and HR, as well as a statistically lower SP (p<.025) contributed to lower mean blood pressures in these rats. Impairment of cardiac performance appeared to be the prevailing effect during phase II. Compromised cardiac contractility was inferred from statistically lower values of PP (40% of control) (p<.025), SP (65% of control) (p<.005) and lower HR (38% of control) on day 35. Certain bile acids have been reported to cause deleterious effects in beating rat heart cells in culture (Bogin et al., 1983). Hoyt and Larson (unpublished observation) found that within 72 hours after the administration of 25 mg/kg BCNU, ip, plasma from treated Sprague-Dawley rats had elevated levels of cholate and/or glycodeoxycholate which was not detected in the plasma
from control rats. One mechanism by which bile acids are thought to exert their deleterious effects on the contractility of heart cells is by inhibiting the activity of $\text{Na}^+, \text{K}^+-\text{ATPase}$ (Heper and Hofmann, 1973). A decrease in contractility of the heart would lead to lower stroke volumes and decreases in CO, causing lower SP leading to decreased PP. Lower HR and DP seen during phase II suggests that sympathetic nerve activity was depressed. This is in contrast with the higher HR and DP observed throughout phase I.

Dose response relationships generated in response to NE, in vivo, for DP and SP, were not different from controls on days 7, 14 or 21 after treatment with BCNU (data not shown). On day 28, however, statistically smaller changes ($p<.05$) in SP, but not in DP, were observed in response to the administration of NE (Figure 4, A. and B.). The maximum change in systolic pressure to NE also appeared to be lower than that seen in control rats (Figure 4, B.). Since changes in SP by NE are primarily the result of increased cardiac contractility leading to increased CO, the lower elevations in SP of treated rats might indicate compromised cardiac contractility. Decreases in cardiac contractility, also suggested by basal levels of PP and SP during phase II, as previously discussed, could be the result of structural changes in contractile proteins, depletion of energy reserves, lower Ca$^{++}$ availability, or alterations
of the nervous and hormonal control of the heart.

Changes in cardiac performance observed in vivo after BCNU treatment obscured any delayed effects that BCNU may have had on the vasculature. The caudal artery was chosen as a vascular model to assess possible structural changes as reflected by the percent protein, water and collagen content of the vessel wall. Changes in endothelium-dependent relaxation deleterious to Ach and vascular responsiveness to NE were also investigated after treatment with BCNU. Nicolson and Custead (1984), using cultured bovine aortic and corneal endothelial cells, revealed that after a 2 hour incubation with clinically relevant concentrations of BCNU (40 μg/ml), endothelial cells became rounded and detached from their matrix. At lower concentrations (10 μg/ml), adhesion of B16 melanoma cells or of platelets to the endothelial lining increased. Scanning electron-microscopic examinations of the endothelial cell monolayers confirmed these endothelial changes. Serum levels of angiotensin converting enzyme (ACE) were elevated by 24 hours after a single ip administration of 20 mg/kg BCNU in Sprague-Dawley rats (Jarvi, 1986). ACE is contained primarily in endothelial cells in the lung and vasculature so elevated serum levels indicate endothelial damage by BCNU (Nicolson and Custead, 1984). Jarvi (1986) reported that serum ACE remained elevated at 3 days, returned to control level by 7 days but then increased again 14 and 21 days after a single 20 mg/kg
dose of BCNU.

Furchgott (1980) found that the relaxation of isolated preparations of arteries by Ach was dependent upon the presence of endothelial cells on the intimal surface of the preparation. Loss of the capacity to relax in response to Ach was the result of removing endothelial cells during rubbing and was clearly demonstrated using scanning electron microscopy. This led to the discovery that many other agents, including some of the most potent known endogenous vasodilators, also require endothelial cells to produce relaxation in isolated arteries.

Ach is thought to act via a muscarinic receptor on endothelial cells initiating a $\text{Ca}^{++}$-dependent sequence of events in which arachidonic acid is liberated, then oxidized by lipooxygenase, to a short-lived compound(s) known as EDRF (endothelium-derived relaxing factor) (Furchgott, 1984). EDRF diffuses to the adjacent smooth muscle cells and activates a mechanism for their relaxation. This mechanism probably involves activation of smooth muscle guanylate cyclase, because there was a positive correlation found between the relaxation and elevated cGMP levels of vasculature (Furchgott, 1984). Endothelium-dependent increases in cGMP produced by Ach were found to produce a change in the pattern of phosphorylated proteins in aortic tissue that included the dephosphorylation of myosin light chains. This pattern of tissue phosphorylation was the same as that
found after administration of 8-Br cGMP or Na nitroprusside, which are not dependent on the endothelium to produce their relaxant effects (Furchgott, 1984). Known inhibitors of the endothelium-dependent relaxation of Ach include 5,8,11,14-eicosatetraynoic acid (ETYA), an inhibitor of both cyclo- and lipooxygenase, nordihydroguaretic acid (NDGA), an inhibitor of lipooxygenase, mepacrine and p-bromophenacylbromide (BPB) inhibitors of phospholipase A₂, and ouabain, an inhibitor of Na⁺, K⁺-ATPase (Furchgott, 1984).

Data presented in the present investigation demonstrate changes in the endothelial integrity of the caudal artery taken from rats pretreated with a single 25 mg/kg dose of BCNU. The assay utilized for these studies demonstrated the absence of an endothelium-dependent relaxation in response to stimulation with Ach. Failure of Ach-induced relaxation in pre-contracted caudal artery segments was observed 24 hours after the administration of BCNU (25 mg/kg), and did not recover fully by 7 days after treatment (Figures 5 and 6).

The ability of Ach to produce a contraction of smooth muscle in the absence of endothelium has been well documented (Furchgott, 1980). This has been demonstrated in several different kinds of artery segments from several different species (Furchgott and Jothianandan, 1983). Ach facilitated the contractile responses for some of the precontracted caudal artery segments from the control-rubbed group and arteries from
rats 24 hours after BCNU treatment. Thus, an acute lack of Ach-induced relaxation suggests that in the caudal artery BCNU is able to act either by selectively inhibiting any of the steps outlined above involving the action of EDRF on smooth muscle cells, or by its lethal effects on endothelial cells, possibly through its ability to inhibit glutathione reductase (Harlan et al., 1983; Nicolson and Custead, 1984).

Harlan et al. (1983) demonstrated that cultured endothelial cells were dependent upon the glutathione redox cycle as protection against lysis by enzymatically generated $H_2O_2$. The selective inhibition of glutathione reductase by BCNU reduced the levels of GSH required to detoxify $H_2O_2$ via glutathione peroxidase. Endothelial cell lysis by 6 hours was induced in a dose-dependent manner following a 10 min incubation with BCNU (Harlen et al., 1983). Endothelial cell lysis could be inhibited by catalase but not superoxide dismutase indicating the role of $H_2O_2$ in mediating cell lysis.

Responses of caudal arteries to extraluminally applied NE were significantly increased by day 7 after 25 mg/kg BCNU, and can only partially be explained by endothelial injury (Table 4, Figure 5). The highest degree of endothelial damage (day 1 after BCNU), according to the ability for Ach to induce relaxation, did not correlate with the highest degree of vessel responsiveness to NE. There were, however, increases in
the average response to NE in all of the other treatment groups, suggesting that endothelium also contributes to adrenergic responsiveness in this preparation. Besides EDRF, other arachidonic acid metabolites, the prostaglandins, are known to mediate vascular responses to NE (Armstrong, 1982). The major prostaglandin thought to be produced by vascular endothelium is prostacyclin (PGI$_2$). Recovery of the production of PGI$_2$ by de-endothelialized rabbit aorta was shown to precede the advent of endothelial restoration (Eldor et al., 1981). These researchers found that the aorta neointimal smooth muscle cells acquired the capacity to synthesize and secrete PGI$_2$ into the vascular lumen, but only after 35-70 days. However, on a short-term basis, de-endothelialized arteries produce much lower levels of PGI$_2$ (Eldor et al., 1981). Prostacyclin is a very potent inhibitor of platelet aggregation and usually relaxes vascular smooth muscle cells (Herman, 1982). The role of prostacyclin in regulating release of neurogenically-derived NE or in vascular smooth muscle responsiveness to NE has been found to vary depending on the species and vascular bed studied (Armstrong, 1982).

From these studies and the results here presented, it may be concluded that BCNU treatment has had an adverse effect on a particular endothelial function that may be contributing to the increased arterial sensitivity to NE. This may also reflect changes in other endothelial mechanisms if BCNU has acted to destroy the viability of
these endothelial cells.

Injury to the endothelial monolayer making up the intima of blood vessels with the subsequent exposure of subendothelial matrix is one way in which fibrosis may begin (Wight, 1985). Endothelial disruption might also lead to the development of edema and change the water and protein content of arterial smooth muscle. However, no evidence for edema was present in caudal arteries after treatment with BCNU. Examination of both protein and water contents of caudal arteries from Sprague-Dawley rats treated with BCNU showed no significant differences from control arteries except on day 28. A statistically lower water content in caudal arteries was observed at this time. Elevated levels of collagen were apparent by day 3 after a 20 mg/kg dose of BCNU and remained elevated through day 28 in the Sprague Dawley rat. Higher arterial collagen levels may cause changes in arterial structural integrity altering vascular responsiveness (Wight, 1985). Collagen levels did not increase after BCNU in Fischer 344 rats, even by day 28, in this strain. Neither did Fischer rats exhibit any of the outward appearances of BCNU toxicity as seen in the Sprague-Dawley strain such as weight loss, jaundice or unkempt coat. Strain differences are not uncommon when looking at a particular toxic effect and may indicate that BCNU is disposed differently in the system of the Fischer rat. Metabolism, distribution and/or
detoxification/repair processes may be different enough to make this strain of rat more resistant to the toxic insults of BCNU.

BCNU induced an initial decrease in endothelial derived functions which was followed by a gradual increase in arterial collagen content. We cannot conclude whether these two effects are directly related on the basis of the presented data. In atherosclerosis endothelial damage leads to platelet aggregation and the proliferation of fibrous material, primarily in the innermost layer of blood vessels. Several hypothesis have been proposed for the progression and etiology of atherosclerosis (Gryglewski and Szczekuk, 1982; Wight, 1985). Major events in the development of atherosclerosis seem to be hemodynamic stress and endothelial injury, arterial-wall-platelet interaction and smooth muscle proliferation, lipid entry and accumulation, fibrosis and finally ulceration, calcification and formation of aneurysms (Fuster, 1982). It is thought that injury of some form (mechanical, chemical, or viral) to the endothelial lining exposes the underlying connective tissues to formed elements of the blood. Platelets would adhere to and aggregate at this site to form a temporary plug and release their contents into the arterial wall. Aggregated platelets release a small cationic protein which is mitogenic and chemotactic for smooth muscle cells. The smooth muscle cells migrate into the intima, divide, and synthesize connective tissue which leads to the beginning
phases of an atherosclerotic lesion (Wight, 1985).

Reports of other antineoplastic agents such as bleomycin leading to subdermal vascular fibrosis following elevated ACE in plasma, (Nicolson and Custead, 1985), suggests that BCNU might have a role in the development of atherosclerosis. Future studies dealing with the characterization of BCNU-induced vascular injury would require a thorough histological examination for evidence of endothelial damage (i.e., changes in morphology, absence of cells) and for the localization of collagen deposition in vessels. Moreover, if fibrosis could be demonstrated in these vessels, characterization of the biochemical lesions and progression of steps leading to a fibrotic change might contribute to a more thorough understanding of the development of arterial fibrotic diseases in general.

Alkyl nitrosourea compounds, both carbamoylating and alkylating agents, with the exception of streptozotocin, have not been reported to alter cardiovascular performance in the rat. The data presented here illustrate a complex variety of time-dependent cardiovascular effects induced by the carcinostatic nitrosourea, BCNU. Indications of the greater sensitivity of the caudal artery from BCNU treated rats to NE provided the stimulus for additional investigations in this model.
Chapter 3
Mechanisms of Carmustine (BCNU)-Induced Vascular Supersensitivity

Introduction

Supersensitivity is defined as the phenomenon in which the amount of substance required to produce a given biological response is less than normal (Aprigliano, 1983). Caudal arteries from BCNU treated Sprague-Dawley rats were supersensitive to extraluminally applied norepinephrine (NE) after 7 days (Chapter 2; Table 4, Figure 5). This effect may have resulted, in part, from BCNU induced endothelial injury. Endothelial cells secrete vasorelaxant substances which act to mediate the vasoconstrictor responses to norepinephrine (NE). Pilot studies investigating both the time- and dose-dependent characteristics of BCNU induced supersensitivity to NE in the caudal artery are shown in the Appendix. Concentration response relationships, generated for intraluminally applied NE in caudal arteries, were shifted even further to the left than that seen with extraluminally applied NE (Table A1, Figure A1). Supersensitivity to NE was a time-dependent phenomenon which also depended on the dose of BCNU utilized in these caudal arteries. Fourteen to 17 days were required for supersensitivity to develop after a low dose (15 mg/kg) of BCNU. The intermediate dose (20
mg/kg) of BCNU only required 10 - 14 days to induce supersensitivity, and with the high dose (25 mg/kg) of BCNU supersensitivity was apparent by 7-10 days. The severity of the effect at each of these times was dose-dependent. The low dose produced a 1.5-fold change in sensitivity to NE (determined by potency ratio, Wilcoxon and Litchfield (1949)) while the intermediate dose shifted the concentration response curve 2.4-fold to the left. The highest dose of BCNU utilized caused a 4-fold shift in the concentration response curve to NE in caudal arteries from these rats by 7 days.

BCNU is a nitrosourea with both alkylating and carbamoylating activities. The availability of a carbamoylating nitrosourea, BHCNU (1,3 bis(4-hydroxycyclohexyl)-1-nitrosourea), allowed a means of comparison to determine whether the ability for BCNU to induce vascular supersensitivity was primarily an effect following alkylation, carbamoylation, or both. Shown in Table A4 and Figure A4 are the time-dependent effects of BHCNU on caudal artery reactivity towards NE. The dose of BHCNU used (20 mg/kg) corresponds, on an equimolar basis, to a dose of approximately 15 mg/kg BCNU. This dose of BCNU induced supersensitivity in caudal arteries by day 14 (Figure A2). BHCNU, on the other hand, did not increase the noradrenergic reactivity of caudal arteries on day 14, but appeared to cause a shift of the frequency response (FR) and concentration response (CR) curves to
the left on day 3 after treatment (Table A4, Figure A4). A similar shift of the FR curve on day 3 after 20 mg/kg BCNU was observed (Table A2, Figure A2), but no change in vascular reactivity to intraluminally perfused NE was evident at this time (Figure A1). These disparate yet similar effects of BCNU and BHCNU suggested that a carbamoylating component may exist for BCNU induced changes in the caudal artery. However, the alkylating component, missing in BHCNU treatment, may be responsible for the delayed increases in vascular reactivity following treatment with BCNU.

FR curves were shifted to the left of control curves after treatment with BCNU (Figure A2). The time- and dose-dependent characteristics of these shifts corresponded with the CR curve shifts except on day 3, as discussed above, and days 21 and 28 after 20 mg/kg BCNU. A significant increase in vascular reactivity to NE was seen on day 28 that exceeded the initial supersensitivity on days 10 - 14. On day 28, the CR relationship for NE was shifted 3-fold to the left of the control curves, however the FR curves were shifted to the right in the same arteries. These data suggested that the ability for nerves to release NE upon electrical stimulation had been compromised. Depressed nerve activity in vivo, as a result of delayed BCNU induced toxicity, may have led to the supersensitivity of vascular smooth muscle to NE.

Characterization of the initial increase in caudal
artery reactivity to NE seen on day 7 after 25 mg/kg BCNU or day 14 after 20 mg/kg BCNU, became the goal for investigation in these studies. The preliminary data (Appendix) suggested that a neural component may have contributed to the supersensitivity, as in denervation-supersensitivity, at later times after BCNU treatment. However, the increase in vascular reactivity to NE seen earlier in the time course was accompanied by an increase in responsiveness to nerve stimulation. This supersensitivity could be interpreted as either a post-junctional or a pre-junctional supersensitivity to NE.

In adrenergically innervated structures, such as the caudal artery, denervation supersensitivity has at least 2 components (Aprigliano, 1983). One is called pre-junctional and is due to impairment of neuronal uptake mechanisms or other processes able to regulate the concentration of agonist in the biophase. These include extraneuronal uptake and catecholamine metabolism by the mitochondrial monoamine oxidase enzyme (MAO) or the cytosolic enzyme catechol O-methyl transferase (COMT). Loss of any one of these mechanisms will cause an apparent increase in sensitivity of the effector organ to an adrenergic agonist which is a substrate for that process. The relative importance of each route of noradrenergic disposition would depend on the effector, its morphology, the distribution and density of innervation, and the width of the neuromuscular junction.
(Bevan, 1979).

The second type of supersensitivity has been named post-junctional, implying a change in the properties of the effector cells. This is a time-dependent and non-specific phenomenon, developing not only to the neurotransmitter, but to other agonists as well (Aprigliano, 1983).

Removal of the neuronal influence does not immediately induce post-junctional changes. According to the "Law of Innervation" (Flemming et al., 1973), "when functional nerve activity is chronically increased or decreased (surgically, physiologically, pathologically, or pharmacologically), the sensitivity of the most distal effectors to any process which initiates a response in the effector is slowly altered in a direction which will compensate for the altered neural input." Denervation induces metabolic alterations, changes in ion fluxes and resting membrane potential. Denervation supersensitivity, a generalized process, results in de novo synthesis of new receptor protein. However, altered membrane excitability, metabolic changes, alteration in calcium handling by the cell, and changes in contractile proteins may constitute additional mechanisms contributing to the supersensitivity of muscle cells to NE and other agents (Aprigliano, 1983). Denervation of an effector initially causes supersensitivity to its neurotransmitter that becomes greater with time and the development of post-junctional supersensitivity as shown
in several studies (Ishii, 1982; Yamada et al., 1982).

According to Hermsmeyer (1983), if determination of vascular muscle cell mechanisms is to be carried out, elimination or definition of the influence of nerve endings should be established. For most unequivocal results studies should demonstrate post-junctional reactivity with agonists unsusceptible to uptake or metabolism. Receptor binding studies, showing the number and affinity of receptor sites, would also be effective for classifying the type of adrenergic supersensitivity evident in rat caudal arteries following BCNU treatment. The following set of experiments was designed to examine possible mechanisms by which BCNU induced supersensitivity developed in rat caudal arteries during the first phase of increased vascular reactivity towards NE.
Methods

Caudal arteries from control and BCNU treated (20 or 25 mg/kg) Sprague-Dawley and Fischer 344 rats were cannulated and perfused as previously described in Chapter 2. Mesenteric arteries were isolated just proximal to the abdominal aorta. They were cannulated proximally with PE50 tubing and collaterals leading to the stomach and pancreas were tied off. These arterial preparations were set up in tissue baths identical to those already described for caudal artery preparations. The contractile responses of arteries to agonists: clonidine ($10^{-7}$M to $10^{-5}$M), methoxamine ($10^{-6}$M to $3.2 \times 10^{-5}$M), or norepinephrine ($5.6 \times 10^{-8}$M to $10^{-5}$M), were measured by taking the difference in perfusion pressure from the baseline to the maximal response plateau with each drug concentration. Drugs were perfused intraluminally unless otherwise stated. Each arterial response was converted to a percent of the maximal arterial response by dividing the response observed at each concentration of drug by the maximum response obtained in each artery for that particular drug.

The periarterial nerves of caudal arteries were stimulated by means of bipolar platinum ring electrodes. Square wave pulses of 0.3 millisecond duration and supramaximal voltage (100 V) were applied at 1.4, 1.9, 2.7, 3.7, 5.2, and 10.0 pulses/second (Hz) in 10 second
trains every three minutes by means of a Grass (S88) stimulator. Contractile responses were measured by difference between the baseline and the maximum amplitude reached during electrical stimulation. Complete inhibition of the vascular response in the presence of tetrodotoxin (10^{-6}M) (data not shown) confirmed the neurogenic origin of the vasoconstrictor responses observed.

Denervation

Denervation of arterial segments was performed \textbf{in vitro} by a 10 minute, extraluminal incubation with 6-hydroxydopamine (6-OHDA) (300 \text{ug/ml}) in Krebs without bicarbonate plus reduced glutathione (20 \text{ \textmu M}) at pH 4.9, followed by a 1 1/2 hour incubation in Krebs bicarbonate buffer, pH 7.4 as described by Aprigliano and Hermsmeyer (1976) for the rat caudal artery. Sham denervation of control caudal arteries (minus 6-OHDA) was shown not to alter concentration response relationships with NE (data not shown). Reduced glutathione (20 \text{ \textmu M}) was added to the unbuffered Krebs solution containing 6-OHDA in order to prevent its oxidation. Each denervated artery was field stimulated by means of platinum ring electrodes and Grass (S88) stimulator at 10 Hz using square wave pulses, 0.3 msec duration, in 10 second trains to test for loss of nerve activity. Caudal arteries did not elicit neurogenic responses after the \textbf{in vitro} denervation
procedure. Using this method, Aprigliano and Hermsmeyer (1976) demonstrated that rat caudal arteries, incubated with dl-NE-7-³H, did not accumulate radioactivity after denervation in vitro. The amount of radioactivity accumulated by 6-OHDA treated arteries was comparable to the amount accumulated by arteries which were incubated in the presence of cocaine or at 4°C, both of which block the active neuronal uptake of NE. This chemical method for in vitro denervation of caudal arteries was concluded effective for destroying nerve terminal activity.

Facilitation of Responses by Beta Blockade

Facilitation of the contractile responses initiated by intraluminal perfusion of norepinephrine (10⁻⁷M to 3.2 x 10⁻⁷M) by extraluminally applied propranolol (10⁻⁵M) was compared for caudal arteries from both control and BCNU treated (25 mg/kg, day 7) Sprague-Dawley rats. Responses were first determined for intraluminally perfused norepinephrine alone. Then arteries were bathed in a propranolol solution for 15 minutes before responses to norepinephrine were determined again. The percent facilitation of the response by propranolol was calculated by taking the difference in the response to NE in the presence and absence of propranolol, then dividing by the response to NE without propranolol x 100.
Alpha-1 Adrenergic Receptor Binding Assay

Tissue Collection

Male Sprague-Dawley control and BCNU treated (25 mg/kg) rats weighing 250-350 grams, were sacrificed by cervical dislocation and thoracotomy. The full length of the caudal artery was exposed and carefully dissected free of excess tissue then frozen immediately on dry ice. The tissue was stored in a freezer at -20 °C until binding studies could be performed.

Preparation of the Membrane Fraction

Caudal arteries from 10 control and 10 BCNU treated rats were each pooled to give approximately 400 mg of tissue per sample. Arteries were thawed at 1 °C, weighed, then finely minced with scissors to allow for a more uniform homogenization. The tissue was diluted in nine volumes of 0.32 M sucrose, 5 mM Tris (pH=7.6) and treated with three 15 second bursts (setting 6-7) in a Polytron (Brinkman Instr.) with 1 minute cooling periods between bursts. The homogenate was passed through several layers of gauze (4-5). This homogenate was then centrifuged at 45,000 x g and 4 °C for 15 minutes (Beckman JA-20.1 aluminum fixed angle rotor, Beckman J2-21 Centrifuge). The supernatant was discarded and 19 volumes of 0.25 M sucrose, 5 mM Tris (pH=7.6) were added to the pellet. The pellet was resuspended with a 15 second burst using a Polytron (setting 6-7). This
homogenate was centrifuged at 4000 x g and 4°C for 20 minutes. The pellet was discarded. It contained 30% of the displaceable specific binding sites but also had 65% of the nonspecific binding sites. The supernatant was decanted into another tube and centrifuged at 45,000 x g and 4°C for 15 minutes. This pellet contained 70% of the displaceable specific binding sites. It was resuspended to a volume of 5 ml in assay buffer (50mM Tris-HCl, pH=7.5 at 25°C) with a single 20 second burst (setting 6-7) with a Polytron.

**Binding Assay**

For the binding assay, 800 ul (89.6 μg/ml, Controls; 25.6 μg/ml, Treated) of tissue homogenate was added to glass tubes containing any unlabelled ligand or assay buffer in a total volume of 0.9 ml. The assay was started by the addition of 3H-prazosin, 0.1 ml (0.9 nM). Incubation was carried out for 30 minutes at 25°C in a temperature-controlled shaking water bath (Dubnoff). These conditions were previously shown to allow complete equilibration of these same ligands with the alpha-1 receptor (Sastre et al., 1984). The reaction was terminated by rapid filtration through glass fiber filters (Whatman GF/C) pre-soaked with poly-l-lysine (1mg/ml H₂O). Vacuum filtration was carried out using a single manifold (Hoefer Scientific Instr.). The assay tubes were rinsed with 4 ml of assay buffer (1°C) and the
filters were rinsed further with three 4 ml rinses of buffer (1°C). Assays were carried out in triplicate, with triplicate determinations of total and nonspecific binding (defined as the $^3$H bound to filters when the incubation medium contained 10 uM phentolamine).

Phentolamine displaceable binding to poly-l-lysine treated filters was determined to be < 5% of the total specific binding which was equivalent to < 1 fmole of $^3$H-prazosin (sp. act. 74.29 cpm/fmole). Total $^3$H-prazosin added to each tube was 970 fmoles (72,000 cpm). The specific binding was determined to be 80% of the total binding under these conditions. Samples were counted for 30 minutes, two cycles in a Beckman Liquid Scintillation Counter.

Protein determination was by a modified Lowery procedure (1951). Tissue homogenates were solubilized with 0.5 N NaOH. A standard curve was generated from 5,10,15,20, and 25 µg aliquots of a 1 mg/ml standard of bovine serum albumin in 50 mM Tris in triplicate. The curve was determined to be linear over this range by regression ($r^2 = 1.00$). Samples of tissue homogenate (25 µl) and blanks (25 µl of 50 mM Tris) were also run in triplicate. Samples were incubated for 2 hours before reading the absorbance at 700 nm on a spectrophotometer (Bausch and Lomb Spectronic 70).

Data from concentration response curves and facilitation by propranolol were analyzed by a 2-factor analysis of variance (Snedecor and Cochran, 1980).
Differences were considered significant at p < .05 for F values. EC50 values were calculated by the method of Wilcoxon and Litchfield (1949). Differences were tested by a Student's t-test and considered significant when p < .05.

Drugs used were as follows: 1-norepinephrine bitartrate (Levophed \(^R\)) Breon Laboratories, Inc.; Clonidine hydrochloride (Catapres \(^R\)) Boehringer Ingelheim; Methoxamine hydrochloride (Vasoxyl \(^R\)) Burroughs Wellcome and Co.; 6-hydroxydopamine (6-OHDA) Sigma Chemical Co.; Propranolol, Sigma Chemical Co.; Phentolamine (Lopressor \(^R\)) Sigma Chemical Co.; \(^{3}\)H-Prazosin, [7-Methoxy-\(^{3}\)H] (80.9 Ci/mmol), New England Nuclear, Dupont.
Results

Caudal arteries from Sprague-Dawley rats pretreated with BCNU (15-25 mg/kg) had been shown to become supersensitive to norepinephrine in a time-dependent manner in pilot studies (Appendix ). Studies demonstrating an endothelium-dependent component of this effect after the high dose of BCNU were discussed in chapter 2. Complete concentration response relationships with NE, expressed as the % maximum response and their corresponding EC50 values confirmed this effect (Table 8, Figure 9). EC50's for NE, generated in caudal arteries from BCNU-treated Sprague-Dawley rats, were significantly lower (p<.05) than those from controls (Table 8, Figure 10). The maximum responses seen in caudal arteries from both control and BCNU treated rats with NE were not significantly different (data not shown).

Maximal responses with NE were 50 percent lower in mesenteric arteries from BCNU-treated rats when compared with arteries from controls (data not shown). Concentration response relationships with NE generated in mesenteric arteries from BCNU-treated rats (25 mg/kg, day 7) were not significantly shifted from those of control arteries (Figure 11). EC50 values for concentration response curves with NE in mesenteric arteries were slightly lower for arteries from BCNU treated rats when compared with controls but this difference was not statistically different because of the large variability
Table 8
Percent of Maximal Response and EC50 Values For Intraluminally Perfused Norepinephrine in Caudal Artery Segments From Control and BCNU Treated Sprague-Dawley and Fischer 344 Rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of Norepinephrine(Μ)</th>
<th>---A. Sprague-Dawley---</th>
<th>---B. Fischer 344 ---</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5.6x10^-8</td>
<td>10^-7</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>3.8±1.0</td>
<td>10.6±1.9</td>
</tr>
<tr>
<td></td>
<td>(n=11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
<td>6.9±0.8</td>
<td>16.4±1.8</td>
</tr>
<tr>
<td></td>
<td>(n=10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 17</td>
<td></td>
<td>7.0±1.0</td>
<td>17.9±2.4</td>
</tr>
<tr>
<td></td>
<td>(n=13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td></td>
<td>8.0±2.4</td>
<td>25.2±4.6</td>
</tr>
<tr>
<td></td>
<td>(n=4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td></td>
<td>6.6±0.6</td>
<td>17.6±1.3</td>
</tr>
<tr>
<td></td>
<td>(n=15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 10</td>
<td></td>
<td>5.5±1.0</td>
<td>15.8±2.6</td>
</tr>
<tr>
<td></td>
<td>(n=7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denervated Control</td>
<td></td>
<td>8.4±1.6</td>
<td>23.9±0.5</td>
</tr>
<tr>
<td></td>
<td>(n=4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td></td>
<td>15.7±4.4</td>
<td>27.2±3.0</td>
</tr>
<tr>
<td></td>
<td>(n=4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>16.6±4.6</td>
<td>32.6±1.4</td>
</tr>
<tr>
<td></td>
<td>(n=2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td></td>
<td>9.2±2.0</td>
<td>21.6±2.0</td>
</tr>
<tr>
<td></td>
<td>(n=2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are represented as the mean ± s.e.m. of the number of animals in parentheses. Responses are expressed as the percent of the maximal response seen using 10^-7 M NE.

a) Caudal artery segments were bathed extraluminally with Krebs bicarbonate (37°C) and perfused intraluminally with varying concentrations of NE.
b) Rats were injected i.p. with a single dose of BCNU.
c) 20 mg/kg or
d) 25 mg/kg, on day 0. Arteries were collected on the days designated for each treatment.
e) Artery segments were denervated in vitro by bathing in 6-OHDA for 10 minutes.
f) EC50 values were calculated by the method of Litchfield and Wilcoxon (1949).
* Significantly different from corresponding control group (p<.05).
Figure 9. Responsiveness of caudal artery segments from control and BCNU treated Sprague-Dawley rats to intraluminally perfused norepinephrine. Values are represented as the mean ± s.e.m. of n=11 control and n=15 treated arteries. Rats were injected i.p. with a single dose of BCNU (25 mg/kg) on day 0. Arteries were collected from rats on day 7 after dosing with BCNU. The BCNU treated curve was significantly different (p<.05) from the control curve, 2-factor analysis of variance. EC50 values for curves are shown in Figure 10.
Figure 10. EC50 values for norepinephrine in caudal and mesenteric arterial segments from control and BCNU treated Sprague-Dawley rats. Values are represented as the mean ± s.e.m. Rats were injected i.p. with a single dose of BCNU (20mg/kg or 25 mg/kg) on day 0. Arteries were collected on days 7-10 after the 25 mg/kg dose and 14-18 days after the 20 mg/kg dose of BCNU. Caudal artery segments (control, n=5; days 14-18, n=23; days 7-10, n=22) and mesenteric artery segments (control, n=5; days 14-18, n=7; days 7-10, n=6) were perfused intraluminally with NE. EC50 values shown in boxes in bars were calculated for each artery by the method of Wilcoxon and Litchfield, and were obtained from data shown in Figures 9 and 11. * Significantly different (p<.05) from corresponding caudal arteries. Significantly different (p<.05) from corresponding controls, Students t-test.
Figure 10

Control

Days 14-18
(20mg/kg)

Days 7-10
(25mg/kg)

EC50 for NE (×10^-7M)

Caudal Arteries

Mesenteric Arteries

Type of Arterial Segment

+ 7.7
+ 3.8
+ 4.0

* 15.0
* 11.7
* 11.4
Figure 11. Responsiveness of mesenteric artery segments taken from control and BCNU treated Sprague-Dawley rats to the intraluminal perfusion of norepinephrine. Values are represented as the mean ± s.e.m. Rats were injected i.p. with a single dose of BCNU (20 mg/kg) on day 0. Arteries were collected on days 7-10 (n=6) after 25 mg/kg dose and 14-18 days (n=7) after the 20 mg/kg dose of BCNU. Concentration response curves generated in mesenteric artery segments from the 14-18 day or the 7-10 day treatment were not significantly different from the control segments (n=5) 2-factor analysis of variance. EC50 values are shown in Figure 10.
Figure 11

- **Control**
  - 0-0-0

- **Day 14-18**
  - (20 mg/kg BCNU)
  - 4-4-4

- **Days 7-10**
  - (25 mg/kg BCNU)
  - *---*

**Log Concentration NE (x10^-6 M)**

**% Maximum Response**
in each mean value (Figure 10).

Data in chapter 2 demonstrated that caudal arteries from Fischer rats treated with BCNU (20 mg/kg) did not accumulate hydroxyproline but those from Sprague-Dawley rats did (Figure 8). Rat strain differences in the effects of BCNU on vascular reactivity were also apparent in these studies. Caudal arteries from Fischer 344 rats treated with 20 mg/kg BCNU, did not become supersensitive to NE by day 28 (Figure 12). Concentration response curves and EC50 values for NE were not significantly different for caudal arteries from control and BCNU treated Fischer rats (Table 8). However, they were significantly different (p<.05) when generated in caudal arteries from control and BCNU treated Sprague-Dawley rats (Table 8, Figure 13). These data again demonstrated that the Sprague-Dawley rat strain was more susceptible to BCNU induced toxicity than was the Fischer 344 strain.

Frequency response curves from Sprague-Dawley caudal arteries, generated by field stimulation of periarterial nerves (Figure 14), followed the same general pattern of responsiveness as seen in the concentration response curve on day 7 after 25 mg/kg BCNU. Caudal arteries were supersensitive to intraluminally perfused NE on day 28 following 20 mg/kg BCNU; however, responses to periarterial nerve stimulation were smaller, especially at lower frequencies (1.4, 1.9, 2.7 & 3.7 Hz) in those
Figure 12. Responsiveness of caudal artery segments from control and BCNU treated Fischer 344 rats to intraluminally perfused norepinephrine. Values are represented as the mean ± s.e.m. for n=2 arteries. Rats were injected i.p. with a single dose of BCNU (20mg/kg) on day 0. Arteries were collected from rats on day 28 after dosing with BCNU. The BCNU treated curve was not significantly different (p<0.05) from the control curve, 2-factor analysis of variance. EC50 values for NE were 1.7 ± 0.01x10^{-7} M NE for control and 2.5 ± 0.10x10^{-7} M NE for treated caudal arteries. The treated EC50 was not significantly different from the control value, Student's t-test.
Figure 12

Fischer 344
Control

Fischer 344
Day 28 (20mg/kg)

% Response

Log Concentration NE (x10^-7 M)
Figure 13. Time-dependent effects of the responsiveness of caudal artery segments from control and BCNU treated Sprague-Dawley rats to intraluminally perfused norepinephrine. Values are represented as the mean ± s.e.m. of n=11 controls, n=10 day 14, and n=4 day 28. Rats were injected i.p. with a single dose of BCNU. Treated curves were significantly different (p<.05) from control curve, 2-factor analysis of variance. EC50 values were for control= 7.7 ± .9x10⁻⁷M, day 14= 3.8 ± 0.5x10⁻⁷M and day 28 = 2.6 ± 0.7x10⁻⁷ M. Treated EC50 values for NE were significantly different (p<.05) from the control value but not significantly different from each other, Student's t-test.
Figure 13

Control

Day 14 (20mg/kg)

Day 28 (20mg/kg)

% Response

Log Concentration NE (x10^-7 M)
Figure 14. Frequency response curves to periarterial nerve field stimulation in caudal arteries from control and BCNU treated Sprague-Dawley rats. Values represent the mean ± s.e.m. for n=7 arteries from control and treated rats. Treated rats were injected i.p. with a single dose of BCNU (25 mg/kg) on day 0. Arteries were collected on day 7 after dosing with BCNU. *Significantly different (p<.05) from corresponding controls, Student's t-test.
Figure 14

Control

Day 7 (25mg/kg)

Change in Pressure (mmHg)

Log Frequency (Hz)

0

10.0

20.0

30.0

40.0

50.0

60.0

70.0

0

1.0

10.0

Dashed line represents Day 7 (25mg/kg) data. 
same arteries (Tables A1 and A2, Figures Al and A2). Supersensitivity to exogenously applied neurotransmitter in the face of lower nerve reactivity to electrical stimulation suggests that the vascular smooth muscle became supersensitive as a consequence of denervation supersensitivity (Aprigliano, 1983). The supersensitivity of caudal arteries to NE observed days 7-10 (25 mg/kg BCNU), days 10-14 (20 mg/kg BCNU) and days 14-17 (15 mg/kg BCNU) was not accompanied by lower nerve reactivity (Table A3, Figure A3). To the contrary, arteries from these treatments all exhibited increases in nerve reactivity to electrical field stimulation. The mechanism for vascular supersensitivity at these times is less clear. Pre-junctional or post-junctional mechanisms are both possible explanations.

Concentration response relationships in caudal arteries with the alpha-1 agonist methoxamine (Figure 15), or the alpha-2 agonist clonidine (Figure 16), did not differ significantly when arteries from control and BCNU treated (day 7, 25 mg/kg) rats were compared. Methoxamine acted as a full agonist in the caudal artery while the action of clonidine resembled a partial agonist in this preparation. The maximum response attained with clonidine was 50% of the maximum response observed with either methoxamine or norepinephrine (data not shown). These alpha agonists are not subject to the same uptake and metabolic pathways as is NE. In light of the
Figure 15. Responsiveness of caudal artery segments from control and BCNU treated Sprague-Dawley rats to intraluminally perfused methoxamine. Values are represented as the mean ± s.e.m. for n=4 arteries. Rats were injected i.p. with a single dose of BCNU (25 mg/kg) on day 0. Arteries were collected from rats on day 7 after dosing with BCNU. The BCNU treated curve was not significantly different from the control curve, 2-factor analysis of variance.
Figure 15

CONTROL

DAY 7 (25 mg/kg)

% RESPONSE

LOG CONCENTRATION METHOXAMINE (x10^-5 M)
Figure 16. Responsiveness of caudal artery segments from control and BCNU treated Sprague-Dawley rats to intraluminally perfused clonidine. Values are reported as the mean ± s.e.m. of n=6 arteries. Rats were injected i.p. with a single dose of BCNU (25 mg/kg) on day 0. Arteries were collected on day 7 after dosing with BCNU. The BCNU treated curve was not significantly different from the control curve, 2-factor analysis of variance.
Figure 16

Log Concentration Clonidine (x10^-6 M)

% Response

Control

Day 7 (25mg/kg)
equivalent concentration response relationships with methoxamine and clonidine, a pre-junctional mechanism for noradrenergic supersensitivity in caudal arteries from BCNU treated Sprague-Dawley rats was supported.

Denervation of caudal arteries was performed in vitro by incubation with the neurotoxic agent 6-hydroxydopamine (6-OHDA). Caudal arteries contracted slowly in response to the 6-OHDA but relaxed after the compound was washed out. Approximately 15 minutes after the removal of 6-OHDA arteries began to contract once more. This time the change in perfusion pressure was smaller than the initial response to 6-OHDA. Following this second contraction arterial segments relaxed and remained quiescent until they were perfused with NE. Concentration response curves, generated by the intraluminal perfusion of caudal artery segments with NE (5.6 x 10^{-8} M to 10^{-5} M), were not significantly different when comparing the control curve with that from the BCNU treatment (day 7, 25 mg/kg BCNU)(Figure 17). The EC50 for NE in caudal artery segments from control rats was shifted from 7.7 x 10^{-7} M NE to 3.4 x 10^{-7} M NE after denervation with 6-OHDA. This EC50 was significantly different (p<.05) from the EC50 for NE in nondenervated control arteries (Figure 18). Denervated caudal artery segments from BCNU treated rats (25 mg/kg, day 7) had an EC50 for NE of 3.2 x 10^{-7} M NE. This EC50 was not significantly different from that observed for non-denervated arteries from BCNU treated rats.
Figure 17. Effects of in vitro denervation on the responsiveness of caudal artery segments from control and BCNU treated Sprague-Dawley rats to intraluminally perfused norepinephrine. Values are represented as the mean ± s.e.m. of n=4 arteries. Rats were injected i.p. with a single dose of BCNU (25 mg/kg) on day 0. Arteries were collected from rats on day 7 after dosing with BCNU. Incubation of artery segments in 300 µg/ml 6-OHDA in unbuffered Krebs for 10 min followed by 1.5 hour incubation in Kreb's bicarbonate buffer effectively denervated arteries. The BCNU treated curve and control curve were not significantly different, 2-factor analysis of variance. EC50 values for curves are shown in Figure 18.
Figure 17

Control

Day 7 (25mg/kg)
Figure 18. Effects of denervation on EC50 values for norepinephrine, intraluminally perfused in caudal arteries from control and BCNU treated Sprague-Dawley rats. Values are represented as the mean ± s.e.m. for n=11 control, n=15 day 7, n=4 denervated controls, and n=4 denervated day 7 and are from curves in Figures 9 and 17. Rats were injected i.p. with a single dose of BCNU (25 mg/kg) on day 0. Arteries were collected on day 7 after dosing with BCNU. Arteries were denervated by incubating in 300 μg/ml 6-hydroxydopamine in non-buffered Kreb's for 10 minutes. * Significantly different (p<.05) from nondenervated control, Student's t-test.
Figure 18

EC50 for NE (x10^-7 M)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EC50 Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>7.7</td>
</tr>
<tr>
<td>DAY 7</td>
<td>3.8</td>
</tr>
<tr>
<td>CONTROL DENERVATED</td>
<td>3.4</td>
</tr>
<tr>
<td>DAY 7 DENERVATED</td>
<td>3.2</td>
</tr>
</tbody>
</table>
(25mg/kg, Day 7) neither did it differ significantly from the EC50 for NE in denervated arteries from control rats. These data demonstrated that denervation of caudal artery segments from control rats made them supersensitive to NE in the same manner as seen 7 days after treatment with 25 mg/kg BCNU. Equivalent responses to NE after removal of periarterial nerves suggests that the supersensitivity of caudal arteries to NE after BCNU treatment was the result of pre-junctional supersensitivity.

An estimation of the number of alpha-1 adrenergic receptor binding sites was performed by a single point determination under equilibrium conditions. Specific binding, determined by subtraction of the nonspecific binding of $^3$H-prazosin (0.9 nM) in the presence of .01 mM phentolamine from the total binding (without phentolamine) was 82-85% of total binding. The affinity of prazosin for the alpha-1 receptor has been reported to be very high with a $K_D$ of approximately 90 pM (Sastre et al., 1984). The number of alpha-1 binding sites in caudal artery membrane preparations (10 arteries pooled) was 347±3 fmoles/g artery (mean ±s.e.m.) for arteries from control rats and 278±10 fmoles/g artery for arteries from BCNU treated rats. The amount of $[^3$H] prazosin bound per gram caudal artery taken from BCNU treated rats was significantly lower (p<.05) than that seen in controls. This decreased binding may represent a down-regulation of alpha-1 receptors in these arteries.
following treatment with BCNU. This observation also supported a pre-junctional mechanism for BCNU-induced supersensitivity to NE in rat caudal arteries since this post-junctional component would have led to decreased vascular responsiveness to NE by itself.

Many arterial beds are supplied with a population of beta-2 adrenergic receptors as well as alpha-1 and/or alpha-2 adrenergic receptors (Osswald and Guimaraes, 1983). Beta-2 receptor stimulation results in the relaxation of arteries by a cAMP-dependent process (Osswald and Guimaraes, 1983). Propranolol, a beta-adrenergic receptor antagonist, was applied extraluminally while norepinephrine, having some beta-2 activity, was perfused intraluminally in caudal arteries from control and BCNU treated rats. Figure 19 illustrates the compromised ability for propranolol (10⁻⁵M) to facilitate the responses to NE in caudal arteries from BCNU treated rats. The percent facilitation of responses to NE by propanolol did not vary significantly with respect to the concentration of NE used in either group (Figure 19). Propranolol was able to facilitate responses to NE (10⁻⁷ to 3.2x10⁻⁶M) by 17% in caudal artery segments from control rats. However it was not able to facilitate responses to NE (10⁻⁷M to 3.2 x 10⁻⁷M ) in caudal artery segments from BCNU treated rats. The percent facilitation in these arteries was significantly
Figure 19. The percent facilitation of responses to norepinephrine by propranolol in rat caudal arteries from control and BCNU treated Sprague-Dawley rats. Values represent the mean ± s.e.m. for n=4 arteries from control and treated rats. Treated rats were injected i.p. with a single dose of BCNU (25 mg/kg) on day 0. Arteries were collected from rats on day 7 after dosing with BCNU. Percent facilitation by propranolol \( (10^{-5} \text{M}) \) was determined by subtracting the response to NE without propranolol from the response in the presence of propranolol then dividing by the response without propranolol x 100. Negative values represent lower responses in the presence of propranolol. * Facilitation by propranolol was significantly different (p<.05) in the BCNU treated arteries from control arteries, 2-factor analysis of variance.
less (p<.05) than that seen for arteries from control rats when analyzed by a 2-factor analysis of variance. These data suggest that beta receptor dynamics were altered in caudal arteries 7 days after treatment with 25 mg/kg BCNU.
Discussion

Vascular supersensitivity to NE may be the result of one or more mechanisms involving the disposition of NE adrenergic receptor dynamics or changes in the sequence of events leading to contraction within smooth muscle cells (Aprigliano, 1983). These mechanisms have been broken down into two major categories; postjunctional and prejunctional (Aprigliano, 1983). Caudal arteries from BCNU treated rats become supersensitive to NE in both a time- and dose-dependent manner (Appendix). The low dose of BCNU (15 mg/kg) required the longest time (14-17 days) for the development of supersensitivity while the highest dose of BCNU (25 mg/kg) required the shortest time (7-10 days) for onset of supersensitivity. The effectiveness and importance of each of the noradrenergic inactivation pathways may vary from vessel to vessel due to such considerations as variability of media thickness and characteristics of innervation (e.g. junctional cleft width, nerve density) (Bevan and Su, 1973). The value of a vascular smooth muscle's true sensitivity to NE can be approached either by eliminating inactivation mechanisms or using alternative agonists unsusceptible to inactivation by these pathways (Hermsmeyer, 1983).

Alpha receptors agonists methoxamine and clonidine, alpha-1 and alpha-2 receptor agonists, respectively, are not inactivated by noradrenergic uptake or metabolism pathways. Methoxamine was a full agonist in caudal
arterial segments. Clonidine, on the other hand, was a partial agonist capable of eliciting only 1/2 of the change in perfusion pressure seen with NE or methoxamine at its peak concentration. The alpha$_2$ agonist TL99 also gave only 1/2 of the maximal response in Sprague-Dawley rat caudal arteries (Hicks et al., 1985), suggesting that this arterial preparation responded mainly to the activation of alpha-1 receptors. Concentration response curves generated with either of these agonists were not significantly different in caudal arteries from control or BCNU-treated (25 mg/kg, day 7) Sprague-Dawley rats (Figures 15 and 16). Thus, a postjunctional mechanism was not supported by these data, strongly suggesting that pre-junctional factors were responsible for BCNU induced supersensitivity. A single point determination of alpha-1 adrenergic binding showed that there was a significant change in the amount of prazosin bound per gram of caudal artery in the control and BCNU treated Sprague-Dawley rats. A decrease in the amount of prazosin bound per gram artery from BCNU treated rats again suggested that the supersensitivity induced by BCNU was pre-junctional. A more complete study of the binding kinetics over the entire dose and time course for BCNU is needed to verify these observations.

Neuronal uptake is the most important process in the inactivation of NE in many sympathetically innervated smooth muscle types (Iverson, 1973; Aprigliano and
Hermsmeyer, 1976). Increases in the capacity of neuronal uptake have been suggested to contribute to the subsensitivity to NE seen in mesenteric arteries after sinoaortic denervation in rats (Granata, 1984). The number of recognition sites for NE neuronal uptake may be altered by the prevailing level of neurotransmitter in the junctional cleft (Lee et al., 1983). Blockade of noradrenergic neuronal uptake by cocaine or desipramine causes sympathetically innervated effectors to become supersensitive to both exogenously applied neurotransmitter and to sympathetic nerve stimulation (Osswald and Guimaraes, 1983). Ishii et al., (1982) characterized a 2 phase supersensitivity to NE in young rat hearts after immunological or chemical sympathectomy. Changes in the sensitivity of the left atria to NE were related to the density of sympathetic innervation. Initially, sympathectomy produced pre-junctional supersensitivity to NE. This was characterized by showing increased sensitivity only to substances which could be taken up into the nerve endings which had been destroyed. The increase in sensitivity produced by pre-junctional mechanisms is generally moderate in degree. This developed into post-junctional supersensitivity over 4 weeks causing atria to become increasingly sensitive to NE after sympathectomy. Post-junctional supersensitivity requires a relatively long period of time to develop and shows an increased sensitivity to many substances including those which are not taken up by nerve endings.
This type of supersensitivity is often marked in degree (Ishii et al., 1982). An analogous sequence of events followed treatment with BCNU (20 mg/kg), however, the initial onset of supersensitivity was delayed suggesting that impairment of nerve function did not occur immediately after treatment with BCNU. In contrast with the acute effect of BCNU on endothelium seen in Chapter 2, onset of pre-junctional supersensitivity took more time to develop (7-10 days).

Denervation of control artery segments shifted concentration response curves for NE to the left when NE was intraluminally perfused (Figure 17). Since responses to the intraluminal administration of NE could be facilitated by denervation, this suggested a significant role for neuronal uptake in the disposition of NE in these caudal artery segments. Aprigliano and Hermsmeyer (1976) found that contractile responses of rat caudal arteries and portal veins were potentiated to extraluminally applied NE after denervation. Denervation of caudal arteries with 6-hydroxydopamine (6-OHDA) inactivated the uptake of NE in rat caudal arteries (Aprigliano and Hermsmeyer, 1976). Hamilton and Reid (1980) studied the effects on adrenergic activity after i.v. infusion of 50 mg/kg 6-OHDA in rabbits. Twenty four hours after infusion no changes were found in the affinity or number of alpha₁ adrenoceptors in the heart or spleen, but there was a shift to the left in dose
response curves to NE. Concentration response curves for NE in caudal artery segments from BCNU treated rats were not shifted further to the left after denervation with 6-OHDA. EC50 values for NE in these arteries were not significantly different from the non-denervated BCNU treated arteries nor were they significantly different from the control-denervated arteries (Figure 18). Thus, caudal artery segments from BCNU treated rats behaved as if neuronal uptake of NE had been impaired.

No significant supersensitivity was seen in mesenteric arteries to NE after treatment with BCNU. On day 7, after 25 mg/kg BCNU, a time when significant supersensitivity was apparent in caudal artery segments, there was only a slight shift to the left of the concentration response curve for NE in mesenteric arteries (Figure 10). These mesenteric arterial segments from BCNU treated rats ballooned at lower perfusion pressures than those from control rats suggesting that the structural integrity of these arteries had been affected. Consequently, the maximum response generated by NE in these arteries was lower than control arteries. When responses were converted to a percent of maximum response, concentration response curves for NE were not significantly different for mesenteric arteries from either group. The pre-junctional mechanism for NE disposition may not be as important for its termination of action in this segment of mesenteric artery. In general, the density of innervation and junctional cleft
width in an artery will determine the significance of this pathway (Bevan and Su, 1974). The mesenteric arteries were not responsive to field stimulation of periarterial nerves (data not shown). The degree of innervation of the aorta and regions of its collateral vessels most proximal to it are not as densely innervated as regions of these same vessels more distal to the aorta (Bevan, 1979). Such observations tend to be supportive of a pre-junctional mechanism for the supersensitivity caused by BCNU to NE in caudal arteries, which are densely innervated vessels (Bevan and Su, 1974). Another explanation for the lack of supersensitivity in the mesenteric artery may be that the time required for development of supersensitivity in this vessel does not coincide with that seen for the caudal artery. Agents causing peripheral neuropathies generally affect neurons with longer axons first and affect those with shorter axons later and/or at higher doses (Clark and Schmidt, 1984).

This denervation-like effect of BCNU has not previously been reported in the literature. However, streptozotocin, an alkylating methyl nitrosourea, has been shown to induce peripheral sympathetic neuropathies in rats after a single injection (Clark and Schmidt, 1984). Streptozotocin is used experimentally to induce insulin-dependent diabetes in laboratory animals in order to study the deleterious effects of prolonged
hyperglycemia (Agarnol, 1980). A single dose of streptozotocin (65 mg/kg), administered i.v., will selectively destroy beta cells in the Islets of Langerhans (Uchigata et al., 1982). Diabetic autonomic peripheral neuropathies, associated with alterations in carbohydrate metabolism, are present 6 months to 1 year following a single dose of streptozocin. Clark and Schmidt (1984) saw markedly dilated unmyelinated axons containing accumulations of both normal and abnormal axoplasmic organelles. Transport of $^{125}I$-Nerve Growth Factor was abnormal in ileal mesenteric nerves. Decreases in catecholamine histofluorescence and dopamine hydroxylase activity in selected end organs were also observed (Schmidt et al., 1984). Only the most distal portions of the mesenteric nerves to the terminal ileum were affected. The shorter axons to the proximal jejunum were spared, suggesting that this process was a distal axonopathy. It is unclear whether this phenomenon is related in kind to the effects observed in this study following treatment with BCNU. BCNU has not been associated with onset of diabetes or hyperglycemia and peripheral neuropathies following streptozotocin are reported to be reversible with insulin therapy or islet cell transplantation (Schmidt et al., 1983). Similar peripheral neuropathies were seen following treatment with alloxan, a non-nitrosourea diabetogenic agent (Mueller et al., 1982).

McCleod and McNeill (1981) did a comparative study
on the effects of a single dose of alloxan (65 mg/kg, i.v.) or streptozotocin (50 mg/kg, i.v.) on the sensitivity of thoracic aorta and hepatic portal vein to NE after 3 months. Three months after treating rats with a single dose of streptozotocin the pD2 values for NE were lower in aortas but not when alloxan was used. Both streptozotocin and alloxan had induced hyperglycemia and reduced serum insulin levels to less than 50% of control. Scarbourgh and Carrier (1984) demonstrated that increases in the sensitivity of rat aorta to NE was related to an increase in alpha-2 adrenoceptor activity. The increased aortic contractile responses induced by NE were probably due to an increased influx of extracellular calcium through nifedipine-sensitive ion channels associated with activation of alpha-2 adrenoceptors. They suggested that other mechanisms may be playing a role in the streptozotocin induced vascular supersensitivity besides the diabetogenic effect. Recently, Ramanadham et al. (1984) found that four weeks following a single i.v. dose of 65 mg/kg streptozotocin, caudal arteries from Sprague-Dawley rats were supersensitive to both NE and methoxamine. The NE content of these arteries was decreased as well. These authors suggested that streptozotocin had induced denervation supersensitivity in the rat caudal artery. Thus, the autonomic peripheral neuropathy induced by streptozotocin may be related to the effects observed after BCNU administration in these
studies. A direct correlation of their effects can not be made as the timeframe and histological evidence currently available can not be directly compared for either treatment. Both agents are alkylating nitrosourea compounds and both appear to have similar effects on cardiac performance as well as vascular reactivity to NE (Dillman, 1980; Vadlamude and McNeil, 1981; Vadlamude et al., 1982).

Xylamine $(N-(2\text{-chloroethyl})-N\text{-ethy}1\text{-2-methylbenzylamene})$ and its brominated derivative DSP 4, both nitrogen mustard alkylating agents, have been shown to selectively impair noradrenergic neuronal uptake after binding with the NE active carrier system (Zieher and Jaim-Etcheverry, 1980; Fischer et al., 1983). These agents were shown to be co-transported with $Na^+$ into adrenergic varicosities where they accumulated over time producing a selective degeneration of NE nerve terminals in the rat and mouse (Jonsson et al., 1981). Protection against their neurotoxic effects was afforded by co-incubation in the presence of noradrenergic uptake inhibitors, desipramine or cocaine. This suggested that uptake or interaction with the uptake transporter protein was required for their neuronal toxicity. A large portion of the accumulated xylamine was found to be irreversibly bound to intracellular tissue components and some bound with membrane lipid components (Fischer et al., 1983). Structural and mechanistic similarities between these chloroethyl alkylating agents and BCNU suggests that BCNU
may be inducing supersensitivity to NE in rat caudal arteries by a neurotoxic mechanism of action.

Docherty and McGrath (1980) investigated the influence of noradrenergic uptake on pressor (change in diastolic blood pressure) and cardiac responses to intravenous NE in the pithed rat. Blockade of neuronal uptake, with cocaine or desipramine, potentiated only the pressor effects. The pressor effects of NE were potentiated to the same degree (150%) by extraneuronal or neuronal uptake blockade. Other investigators (Bonaccorsi et al., 1970; Guimaraes et al., 1971; Osswald and Branco, 1973; Webb et al., 1980) have shown that denervation of effectors, in vitro, led to a 150% increase in the sensitivity of the effector to its neurotransmitter.

Impaired neuronal uptake or leakage of endogenous NE following treatment with BCNU could have resulted in the higher levels of diastolic pressure and heart rate seen in vivo on days 14 and 21 by increasing the level of NE in the biophase (Figures 1 and 2, Chapter 2). Decreases in the basal levels of heart rate, diastolic pressure, and systolic pressure during phase 2 may reflect a lack of adrenergic activity to the heart and vasculature. This hypothesis is supported by the decreased field stimulated nerve activity in the caudal artery at lower frequencies observed in vivo by day 28 (Table A2, Figure A2). Lower frequencies of nerve stimulation (2-8 Hz) are thought to resemble endogenous levels of nerve activity.
A smaller facilitation of alpha adrenergic contractile responses by beta receptor antagonism with propranolol after BCNU treatment suggests that these receptors or their transducing properties may be desensitized in caudal arteries. Facilitation of noradrenergic responses by propranolol can be explained by two mechanisms. Blockade of beta receptors in the presence of propranolol may allow more of the norepinephrine in the biophase to interact with alpha receptors; or, beta receptor blockade may interfere with the stimulation of beta receptors by NE which lead to relaxation processes and therefore facilitate contraction by the interaction of NE with alpha receptors. Norepinephrine is not a very potent agonist at beta-2 receptors and concentration response curves with NE were not significantly different in caudal arteries after denervation suggesting that beta receptors dynamics did not contribute significantly to adrenergic supersensitivity.

These studies, investigating time- and dose-dependent supersensitivity of rat caudal arteries to NE induced by BCNU treatment, suggest that this nitrosourea is acting via a pre-junctional mechanism which results in the impairment of neuronal uptake processes for the disposition of NE. This hypothesis is supported by other studies demonstrating the selective neurotoxic effects produced by structurally similar alkylating agents.
(xylamine, DSP 4, and streptozotocin). Impairment of neuronal disposition of NE would lead to higher levels of this neurotransmitter in the biophase and cause an apparent supersensitivity as observed. Therefore, a more detailed study of the uptake and biotransformation of NE in caudal arteries from BCNU treated rats was undertaken.
Chapter 4

Uptake and Metabolism of Norepinephrine

Introduction

Norepinephrine (NE) is subject to several routes of disposition each resulting in its metabolism or redistribution into neuronal vesicles to terminate its action in the biophase (Graefe and Henseling, 1983). Neuronal uptake (uptake₁), extraneuronal uptake (uptake₂) by vascular smooth muscle and endothelial cells, nonspecific binding, and enzymatic destruction all contribute to the inactivation of NE. The primary enzymes important for degradation of NE are monoamine oxidase (MAO) and catechol O-methyltransferase (COMT). These enzymes are widely distributed throughout the body and appear to be located in both non-neuronal tissue and the sympathetic neuron (Graefe and Henseling, 1983). The extent of a steady-state contraction of a noradrenergically innervated smooth muscle is determined by the concentration of NE in the biophase. This contraction is therefore affected by the activity of those mechanisms which tend to lower agonist concentration in the biophase (Wyse, 1976). These inactivation processes, if impaired, would lead to the accumulation of higher levels of NE in the biophase, thus producing a response that is increased in proportion to the impairment of the contribution made by that inactivating process (Wyse, 1976).
The relative roles played by the two compartments (uptake₁ or uptake₂) may depend on the substrate (i.e., norepinephrine) concentration. Osswald and Branco (1973) found that the removal of NE by the autoperfused dog hind-limb was attributed primarily to neuronal uptake and intraneuronal oxidative deamination when low levels of NE were perfused. Extraneuronal uptake and inactivation played an important role only when neuronal mechanisms were saturated or impaired. The majority of the work examining uptake and metabolic pathways for the termination of NE has utilized heart, vas deferens, spleen, and aortic tissues, which are all capable of producing a high metabolic turnover of NE. Most studies examining the importance of these pathways in small blood vessels often rely more on pharmacological techniques and the examination of a response. A direct comparison of work in other arterial beds or in other types of sympathetically innervated tissues does not seem feasible. Reports on the ability for each removal route to affect sensitivity towards NE are often contradicting. Evidence exists that neuronal uptake is the predominant mechanism of NE inactivation in small vessels which possess dense adrenergic innervation, a relatively narrow junctional cleft (1,000 - 2,000 A), and a thin media (<50 μm) (Whall et al., 1980).

Blockade of extraneuronal uptake was shown to potentiate only the pressor responses to sympathetic
nerve stimulation in vivo, but inhibition of neuronal uptake led to the potentiation of both pressor and cardiac activity in the pithed rat (Docherty and McGrath, 1980). Recently, more evidence for a significant extraneuronal component in vitro was demonstrated. Sasake et al. (1984) showed that naloxone, an opioid antagonist used for the treatment of shock, increased the sensitivity of canine arterial smooth muscle to NE by inhibiting extraneuronal uptake, but failed to augment phenylephrine induced constriction. Phenylephrine is a poor substrate for uptake properties.

Previous results (Chapter 3) suggested that BCNU-induced adrenergic supersensitivity in rat caudal arteries was occurring by a pre-junctional mechanism. Chemical denervation of caudal artery segments from control rats led to a significant decrease in the EC50 value for NE in those preparations (Chapter 3). Failure of denervation to induce a similar decrease in the EC50 found in caudal artery segments from BCNU treated rats (25 mg/kg, day 7), and the similarities of EC50 values for control denervated, treated denervated and treated nondenervated artery segments, suggested that BCNU treatment had impaired the neuronal disposition of NE in caudal arteries. Pilot studies (Appendix) and studies assessing cardiovascular performance in vivo (Chapter 2, part A.) also suggested that nerve reactivity had been compromised by day 28 following treatment with BCNU. These studies, pharmacological and physiological in
nature, led me to investigate the characteristics of $^3$H-NE uptake and metabolism in the rat caudal artery. Supportive biochemical evidence for this effect would help to substantiate this hypothesis for BCNU-induced vascular supersensitivity.
Methods

Methods were adapted from those of Levin (1973) for determination of the amount of $^3$H-NE and its five major metabolites by paper chromatography.

Materials

Tritiated NE (DL-$(7-^3$H-Norepinephrine)) (13.274 Ci/m mole) was purchased from New England Nuclear. It was purified according to the methods of Crout (1961) and Neff et al. (1971). Aluminum oxide (alumina) was prepared by boiling 3g of alumina (Neutral, Brockman Activity 1 (80-200 mesh) Fischer Scientific CO., Merck) in 10 ml of 2 N HCL for 30 minutes in a reflux apparatus. The cloudy supernatant was poured off and 10 ml of distilled water was added to the alumina. This was stirred briefly, allowed to settle for 5 minutes, then decanted. The process was repeated 12-15 times until wash water cleared after 5 minutes of settling and was pH 4-5. The alumina was collected in a large Buchner funnel under vacuum and allowed to dry overnight at room temperature. The next day the dry alumina was heated in an oven at 100°C for 2 hours. The pH of the $^3$H-NE was adjusted to 8 with NaOH. One gram of processed alumina was added and gently mixed for 10 minutes, then centrifuged at low speed (2000 rpm) for 5 minutes. The supernatant was removed and counted by liquid scintillation counting. The alumina was washed with 3 ml of distilled water two times, and this wash was also counted. The $^3$H-NE was eluted from the alumina by
adding 3 ml of 0.2 N acetic acid with 0.03 mM EDTA and 1 mg/ml ascorbic acid. An aliquot of this extract was spotted on a cellulose phosphate layer chromatography plate and developed in solvent #1. Ninety percent of the radioactivity in this extract co-chromatographed with NE on thin layer chromatography plates. The specific activity of the $^3$H-NE was determined to be 7.8 Ci/mmmole (stock solution = 30 μCi/ml).

Unlabelled metabolites: DL-normetanephrine (NMN), 4-hydroxy-3-methoxy mandelic acid (VMA), 3,4-dihydroxy mandelic acid (DOMA), 3-methoxy-4-hydroxyphenylethylene-glycol (MOPEG), and DL-3,4-dihydroxy phenylglycol (DOPEG) were purchased from Sigma and stored at -5°C in a dessicator until used. Standards were made up in methanol (analytical grade) at a concentration of 1 mg/ml and stored at -5°C.

Tissue Preparation and Incubation

Male Sprague-Dawley rats, 250-350 g, were injected i.p. with either corn oil (1.0 ml/kg) (controls) or with BCNU (25 mg/kg, in corn oil). On day 7, 5 control and 5 treated rats were sacrificed by cervical dislocation and thoracotomy. The caudal artery was carefully dissected out and incubated in Krebs bicarbonate buffer bubbled with a 95% O$_2$ - 5% CO$_2$ gas mixture at 37 °C for 15-30 minutes. Each piece of tissue was then placed in 0.9 ml of Krebs solution in a glass disposable test tube.
Tritiated norepinephrine (0.1 ml, 7.8 Ci/m mole) was added to constitute a concentration of $4 \times 10^{-7}$ M NE to start the incubation, and the incubate was bubbled continuously with 95% $O_2$-5% $CO_2$ at 37 °C for 30 minutes. Steady state levels of accumulated [$^3$H] NE had been observed at this time under similar conditions in caudal arteries (Rawson, 1983). At the end of the incubation the tissue was blotted lightly on filter paper and weighed.

**Extraction of Radioactivity**

The radioactivity was extracted by placing each caudal artery in 2.0 ml of 1 N acetic acid containing 0.03 mM disodium EDTA. After 30 minutes, the tissue was transferred to a fresh 2.0 ml portion of the same solution for an additional 30 minutes. The extraction was performed in disposable glass test tubes and the samples were kept on ice during the extraction procedure. The two portions of tissue extract were combined in a 30 ml beaker and 50 µg each of cold norepinephrine and the five metabolites (normetanephrine (NMN), 4-hydroxy-3-methoxy mandelic acid (VMA), 3,4-dihydroxy mandelic acid (DOMA), 3,4-dihydroxy phenylglycol (DOPEG), and 3-methoxy-4-hydroxy phenylethylene glycol (MOPEG)) (dissolved in 50 µl of methanol) were added as a carrier. An aliquot (100 µl) of the combined extract was taken for determination of total radioactivity in the extract. The remainder of the extract was evaporated to dryness in a vacuum.
dessicator at room temperature (22 °C). The dried residue was redissolved with 1.0 ml of methanol:distilled water (1:1).

Chromatography and Counting

Cellulose phosphate thin layer chromatography (TLC) plates (250 μm thickness) were prepared by dissolving 10 g of Cellulose phosphate in 60 ml of distilled water. This suspension was mixed in a Waring blender at high speed for 60 seconds. The Cellulose phosphate mixture was quickly poured into a commercial TLC plate-preparing device and spread at uniform velocity onto 20x20 cm glass plates. The plates were air dried overnight and examined for irregularities. Only those plates with a uniform appearance were selected for chromatography.

The concentrated tissue extract was spotted on two TLC plates in several 0.5 μl aliquots. The Krebs solution used to incubate each tissue with \( ^3H \)-NE was also assayed for NE metabolites. The radioactivity in a 20 μl aliquot of the Kreb's solution was counted directly in 10 ml of counting solution (Instagel, Beckman Instruments) on a Beckman model 6800 Liquid Scintillation Counter. Two chromatography plates were each spotted with 20 μl of the Kreb's solution and 10 μg each of the five metabolites as carrier. Samples of \( ^3H \)-NE (4x10^{-7}M) in Kreb's solution containing no tissue were incubated along with the tissue samples. Aliquots of this Kreb's solution were spotted in
the same manner as described above. In addition, a tissue extract blank was run along with the other tissue extracts. Tritiated NE was added directly to 4.0 ml of the 1 N acetic acid extract solution. This extract blank was handled in the same manner as the other tissue extracts.

The chromatograms were equilibrated with the solvent for at least 2 hours and then developed for 17 cm by ascending chromatography. One chromatogram from each sample was developed with n-butanol:pyridine:distilled water = 14:3:3 (solvent 1); the other chromatogram was developed with n-butanol:95% ethanol:glacial acetic acid: distilled water = 2:1:1:1 (solvent 2). After drying at room temperature, the chromatograms were stained by spraying them lightly with a fresh solution containing 0.5% potassium ferricyanide and 10% ferric chloride in distilled water. Norepinephrine and all five metabolites appeared as blue spots on a green background. Each chromatogram was divided into 6 consecutive segments corresponding to the location of the spots (Figure 20). Each segment was scraped into a liquid scintillation counting vial. The radioactivity was eluted from the cellulose phosphate by adding 1 ml of 0.3 N HCL and shaking the vial for 60 minutes. Ten millimeters of counting solution (Instagel, Beckman Instruments) was added directly to the vial and shaken.
Figure 20. Migration of $[^3\text{H}]$ NE and its five primary metabolites on cellulose phosphate thin layer chromatography plates. This drawing illustrates the relative position of each metabolite and NE after development with solvent #1 (n-butanol:pyridine:distilled water = 14:3:3) or solvent #2 (n-butanol:95% ethanol:glacial acetic acid:distilled water = 2:1:1). After development, plates were divided into 6 sections. Each section was scraped into a scintillation vial and eluted with 0.3 N HCL. $R_F$ values were 0.03 (NE and NMN), 0.28 (DOMA), 0.5 (VMA), 0.7 (DOPEG), and 0.9 (MOPEG) when developed in solvent #1 or 0.14 (NE), 0.4 (NMN), 0.65 (DOMA and DOPEG), and 0.8 (VMA and MOPEG) when developed in solvent #2.
FIGURE 20

Solvent Front

MOPEG

MOPEG + VMA

DOPEG

DOPEG + DOMA

VMA

NMN

DOMA

NE

NE + NMN

Site of Origin

Solvent #1

Solvent #2
Calculations and Data Analysis

All samples were corrected for counting efficiency by signal channel ratio and a quench curve generated in the same manner with standardized quenched samples. Samples were counted for 30 minutes or until sigma=2.00. The net disintegrations per minute in each segment of the chromatogram were expressed as a percent of the total radioactivity on that chromatogram. A correction was applied for the amount of cross-contamination in each segment by $[^3\text{H}]$-NE from the corresponding tissue blanks for Krebs extract (Levin, 1973). The total disintegrations per minute in the original tissue extract (and Krebs solution) were determined from the aliquot that was counted separately. The amount of exogenous $[^3\text{H}]$-NE and each of its five metabolites, in nanomoles/gram tissue, was then calculated from the formula:

$$C_m = \frac{(\% \text{net dpm in chromatogram} - \% \text{cross-contam. with } [^3\text{H}]\text{NE})}{\text{total dpm in tissue extract or Kreb's solution}} \times \frac{(\text{Tiss. wt.})(\text{Sp. Act. of } [^3\text{H}]\text{NE}(\text{Ci/mmole})(2.2 \times 10^6)(100)}}$$
Results

BCNU treatment (25 mg/kg, day 7) had been shown to cause supersensitivity to NE (Chapters 2, 3 and Appendix). The uptake of NE into caudal arteries from BCNU treated rats amounted to only 28% of the uptake found in control arteries (Table 9, Figure 21).

Neuronal and extraneuronal uptake are the primary disposition pathways for NE in sympathetically innervated tissues. Metabolism of NE by COMT results primarily in the formation of NMN. DOMA and DOPEG result from the conversion of NE by MAO. MOPEG and VMA are formed by combinations of these enzymatic processes.

The metabolites NMN, DOMA, and VMA, were all found at significantly lower levels (p<.05) after BCNU treatment. Levels of MOPEG and DOPEG were not statistically different in arteries from control and BCNU treated rats, but this was because of their small contribution even in control arteries. The total conversion of $^3$H-metabolites from $^3$H-NE was significantly less (p<.05) in arteries from BCNU treated rats. The conversion of $^3$H-NE to its metabolites was very active in caudal arteries from control rats. The total amount of $^3$H metabolites was 97% of the total tissue level of $^3$H-NE in control tissue but only 40% of the $^3$H-NE tissue level in arteries from BCNU treated rats. This suggests that not only uptake, but some metabolic processes and perhaps
Table 9. Uptake and Metabolism of $[^3$H] NE by Caudal Arteries from Control and BCNU Treated Sprague-Dawley Rats.

<table>
<thead>
<tr>
<th></th>
<th>Tissue (nmoles/g)</th>
<th>Krebs (nmoles/g)</th>
<th>Total (nmoles/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Control</td>
</tr>
<tr>
<td><strong>Total^c</strong></td>
<td>1.854±0.255</td>
<td>0.526±0.035</td>
<td>11.306±0.911</td>
</tr>
<tr>
<td><strong>Metab.</strong></td>
<td>0.097±0.027</td>
<td>0.002±0.002</td>
<td>1.621±0.494</td>
</tr>
<tr>
<td><strong>NE</strong></td>
<td>1.768±0.237</td>
<td>0.524±0.035</td>
<td>9.686±1.277</td>
</tr>
<tr>
<td><strong>NMN</strong></td>
<td>0.050±0.020</td>
<td>0.000±0.003</td>
<td>1.110±0.419</td>
</tr>
<tr>
<td><strong>DOMA</strong></td>
<td>0.030±0.003</td>
<td>0.000±0.003</td>
<td>0.222±0.021</td>
</tr>
<tr>
<td><strong>VMA</strong></td>
<td>0.008±0.008</td>
<td>0.000±0.008</td>
<td>0.120±0.037</td>
</tr>
<tr>
<td><strong>DOPEG</strong></td>
<td>0.006±0.002</td>
<td>0.002±0.002</td>
<td>0.144±0.072</td>
</tr>
<tr>
<td><strong>MOPEG</strong></td>
<td>0.005±0.002</td>
<td>0.000±0.000</td>
<td>0.025±0.019</td>
</tr>
</tbody>
</table>

Each value represents the mean ± s.e.m. for n=5 caudal arteries. Values are expressed as nmoles of compound found per gram of caudal artery.

a) Caudal arteries were incubated in Krebs in the presence of $[^3$H] NE (4x10^-7 M) for 30 minutes. Tissues were extracted with 1N acetic acid for 60 minutes. The extract was evaporated to dryness then redissolved in methanol:water (1:1). Extract were spotted and developed on thin layer chromatography plates.
b) Krebs incubation medium was also spotted and developed on TLC plates. Levels of NE and its metabolites in Krebs are expressed per gram of incubated caudal artery.
c) Total radioactivity of NE and all metabolites.
d) NMN, DOMA, VMA, DOPEG, and MOPEG combined.
e) Abbreviations are defined in Methods section.

* Significantly different from the control value (p<.05), Student's t-test.
Figure 21. Uptake and metabolism of $^{3}$H NE by caudal arteries from control and BCNU treated Sprague-Dawley rats. Data are represented as mean ± s.e.m. in Table 9. Caudal arteries were incubated in the presence of 4.0x10^{-7} M $^{3}$H NE for 30 minutes. Tissue radioactivity was extracted with acetic acid. Analysis of tissue extract and Krebs incubation medium was performed by thin layer chromatograph by two developing systems. * Significantly different from corresponding control value (p<.05), Student's t-test.
Figure 21

Control

BCNU Treated

NE Tissue Metabolites Total NMN Total DOMA Total VMA Total DOPEG Total MOPEG Total

Metabolism or Uptake (nmol/g tissue)

0.0 0.3 0.6 0.9 1.2 1.5 1.8 2.1
metabolic tissues themselves (e.g., nerves), were impaired in the treated arteries.

Uptake, and possibly metabolism of norepinephrine were impaired in caudal arteries taken from rats treated with BCNU. This experiment supports a pre-junctional mechanism for BCNU induced noradrenergic supersensitivity in caudal arteries from Sprague-Dawley rats.
Discussion

Uptake and metabolism are the most important routes for the disposition of NE in sympathetically innervated tissues (Graefe and Henseling, 1983). The tissue uptake and conversion of $[^3H]$ NE to its 5 primary metabolites, measured by thin layer chromatography, indicated that uptake processes for NE had been impaired in caudal arteries from BCNU treated rats (Table 9, Figure 21). Levels of tissue-extractable $[^3H]$ NE were 3-fold lower in caudal arteries from BCNU treated rats. Aprigliano and Hermsmeyer (1976) demonstrated that in vitro denervation of proximal caudal artery segments decreased tissue uptake of $[^3H]$ NE more than 10-fold. Other studies have implicated a principal role for neuronal disposition of NE in rat caudal arteries as well (Bonaccorsi et al., 1970; Webb et al., 1980; Wyse, 1976).

Since the entire length of the caudal artery was used for this study it is difficult to make comparisons with other studies in which only proximal caudal artery segments were used. The density and pattern of sympathetic innervation, and consequently its proportional contribution to the total uptake of NE in this artery, is not homogeneous from its proximal to distal end (Bevan, 1979). Distal segments of caudal artery were not as responsive to electrical field stimulation of periarterial nerves as were proximal segments (data not shown, personal observation).
suggesting that distal segments were less densely innervated. Thus, the results presented here do not clearly define which of the uptake processes is primarily inhibited. However, based on the distribution of metabolites, extraneuronal disposition appeared to be an important process in caudal arteries from control rats. NMN levels, the primary COMT mediated metabolite of NE, are thought to be derived primarily from extraneuronal tissues and was significantly higher than were the MAO-mediated metabolites DOMA and DOPEG (Figure 21).

Levels of both COMT- and MAO-mediated metabolism were depressed in caudal arteries from BCNU treated rats. Lower metabolite levels were probably, in part, the result of impaired tissue uptake of NE. However, NE uptake was decreased 3-fold while total metabolite levels were 8-fold lower indicating that there might also have been a decrease in tissue metabolism of the compound in caudal arteries from BCNU treated rats. The results of these studies implied that supersensitivity to NE in rat caudal arteries after BCNU treatment was due to a pre-junctional mechanism.

A decrease in the number of uptake sites would lead to a decreased capacity for adrenergic tissue uptake. Lee et al. (1983) have shown that the number of uptake binding sites could be regulated by synaptic levels of NE. When levels of NE were depressed by chronic administration of reserpine the number of uptake sites decreased by 50% after 6 days, but increasing synaptic
levels of NE by treatment with MAO inhibitors induced an increase in the number of uptake sites thereby constituting a homeostatic mechanism to keep synaptic levels of NE within a selective range. Synaptic levels of NE may have been depressed in rat caudal arteries after treatment with BCNU but this speculation is unlikely in light of the observed alpha and beta receptor dynamics. A decrease in the number of alpha-1 receptors and a depressed beta receptor-mediated relaxation of BCNU-treated caudal arteries both indicate that neurotransmitter levels in the junctional cleft were most likely elevated than lowered because a downregulation of receptor number is often indicative of higher levels of neurotransmitter in the biophase (Ishii et al., 1982; Sun and Hanig, 1983; Aaron et al., 1983). If BCNU treatment produced an increase in synaptic NE, this should have led to an increase in the number of uptake binding sites as well which would have resulted not in a decreased uptake as observed but an increased level of tissue uptake.

It is still unclear how the administration of BCNU might produce these effects. Bile acids, which may be elevated in the blood in association with the cholestasis produced by BCNU (Hoyt, 1984), are inhibitory towards the activity of Na⁺, K⁺ATPase and Mg⁺⁺ ATPase (Meijer et al., 1978). Inhibition of Na⁺ K⁺ ATPase would depress neuronal uptake of NE. The detergent properties of bile acids acting on plasma membranes could alter their fluidity and
possibly perturb the activity of membrane proteins (Lowe and Coleman, 1981). Alkylating agents themselves may act through alkylation of membrane components such as Na\(^+\), K\(^+\) ATPase leading to its inactivation, cross-linking of membrane lipids. Alternatively they could induce changes in protein-lipid interactions (Goldenberg and Begleiter, 1979). Nitrogen mustard alkylating agents with structural and mechanistic similarities to BCNU (e.g., xylamine and DSP-4) have been shown to produce delayed sympathetic autonomic neuropathies (Ross, 1976; Cho et al., 1980; Zieher and Jaim-Etcheverry, 1980; Jonsson et al., 1981; Fischer et al., 1983; Landa et al., 1984). These agents were transported into sympathetic nerves via uptake and alkylated several cellular constituents including proteins, lipids and DNA. Streptozotocin, an alkylating nitrosourea agent, also produced a delayed sympathetic autonomic neuropathy in rats (Aqarwal, 1980; MacLeod and McNeil, 1981; Schmidt et al., 1981; Mueller et al., 1982; Schmidt et al., 1983; Clark and Schmidt, 1984), and was shown to increase caudal artery reactivity to NE and methoxamine 4 weeks following a single i.v. administration in male Sprague-Dawley rats (Ramanadham et al., 1984). Although the mechanistic details of the action of BCNU are unclear, the drug did produce a delayed pre-junctional supersensitivity to NE in caudal arteries of Sprague-Dawley rats which appears to have some analogous properties with the effects of the alkylating nitrosourea, streptozotocin. This pre-junctional
supersensitivity in caudal artery segments might be the result of a delayed sympathetic neuropathy which remains to be defined.
Chapter 5
Summary and Conclusions

BCNU is a chemotherapeutic nitrosourea with alkylating and carbamoylating properties the clinical effectiveness of which is limited and complicated by several dose- and time-dependent toxicities. The studies presented in this thesis were performed in order to characterize the cardiovascular effects of this drug. Other antineoplastic agents such as adriamycin (Van Stee, 1982) have been shown to exhibit cardiovascular toxicity, but BCNU with antineoplastic and cytotoxic properties has not previously been reported to affect cardiovascular physiology.

Vascular endothelial cells have been reported to be deleteriously affected by BCNU in cell culture. Harlan et al. (1983) and Nicolson and Custead (1984) have recently demonstrated lethality and altered structural characteristics of cultured vascular endothelial cells after incubating with BCNU. Jarvi (1985) found that serum from BCNU treated Sprague-Dawley rats contained elevated levels of ACE, an index of endothelial cell injury in vivo (Nicolson and Custead, 1984). Caudal arteries were used to assess the endothelial-dependent relaxation by acetylcholine (Furchgott, 1984) of precontracted arterial segments. BCNU acutely inhibited the ability for acetylcholine to cause this relaxation by 24 hours following a single dose (25 mg/kg). A
substantial but incomplete recovery followed this effect by day 7. Altered endothelial integrity may lead to changes in arterial structural composition such as edema or collagen deposition. No edema was apparent in these arteries, however collagen levels were significantly elevated by day 3 and remained at this high level through day 28 following a single dose (20mg/kg) of BCNU.

Changes in arterial structural composition could lead to altered responsiveness of these arteries by changing their resistance properties to flow (Folkow, 1970). Irregularities in vascular appearance were noted in rats after treatment with BCNU. Mesenteric arteries from BCNU treated rats were found to balloon at lower perfusion pressures than those from control rats suggesting that this vessel had altered structural integrity.

Cardiovascular variables measured in vivo were altered and fit into 2 phases. Phase I, from day 7 to day 21, was characterized by higher levels of HR, DP, and BP suggesting elevated basal levels of sympathetic activity or increased sensitivity of the heart and vasculature to endogenous catecholamines. Phase II was characterized by decreased HR, BP, and DP but was affected primarily by depressed indices of cardiac function. It cannot be determined with certainty that changes observed in vivo were a direct result of BCNU since these responses were greatly delayed and other
BCNU-induced effects, such as delayed increases of plasma bile acids, may have contributed to the observed alterations in cardiovascular function. The effects observed in vitro are reflective of these conditions experienced in vivo, which are subject to several homeostatic mechanisms to maintain BP, and so must be interpreted with an appropriate recognition of cardiovascular dynamics.

Pilot studies demonstrated that there was a time- and dose-dependent onset of supersensitivity to NE in the caudal artery of the Sprague-Dawley rat following a single dose of BCNU (15 to 25 mg/kg, i.p.)(Appendix). These studies characterized the time-dependent vascular effects of BCNU, from 1-28 days post dosing, and illustrated a pattern of vascular reactivity to exogenously applied NE and periarterial nerve stimulation. These data suggested that BCNU was acting to increase vascular sensitivity by a denervation supersensitivity-like effect. Arterial segments were most responsive to exogenously applied NE on day 28 yet were least responsive to periarterial nerve stimulation at this time.

Experiments were designed to examine the nature of this supersensitivity on day 7 after a single 25 mg/kg intraperitoneal injection of BCNU. The contribution of a post-junctional and/or pre-junctional component(s) was determined by pharmacological and biochemical criteria. Concentration response curves were generated for NE,
methoxamine, and clonidine. Concentration response relationships for methoxamine or clonidine were not different in caudal artery segments from control and BCNU treated rats. Concentration response relationships for NE were shifted to the left in caudal artery segments from BCNU treated rats. Preliminary radioligand binding studies suggested that the number of alpha-1 adrenergic receptors had decreased slightly. Together, these data supported a pre-junctional mechanism for adrenergic supersensitivity. 

In vitro denervation of caudal artery segments from Sprague-Dawley rats was performed by incubating them in 6-OHDA. The EC50 of NE for control denervated caudal artery segments was significantly lower (p<.05) than the EC50 for the control nondenervated caudal artery segments and was not different from the EC50 values of denervated or nondenervated caudal artery segments from BCNU treated rats. These data demonstrated that a pre-junctional mechanism was contributing to noradrenergic vascular supersensitivity in the caudal artery. This hypothesis was also supported by studies examining the uptake and metabolism of [3H] NE by caudal arteries. Significantly lower levels of uptake and metabolism of NE by caudal arteries from BCNU treated rats would lead to higher levels of NE in the biophase and result in supersensitivity to exogenously applied or neuronally-released NE by a pre-junctional mechanism.

Catacholamines play an important role in regulating
the function of their own receptors (Sun and Hanig, 1983). According to the "Law of Innervation" (Flemming et al., 1973) "when functional nerve activity is chronically increased or decreased (surgically, physiologically, pathologically, or pharmacologically), the sensitivity of the most distal effector to any process which initiates a response in the effector is slowly altered in a direction which will compensate for the altered neural input." Evidence for downregulation of alpha-1 receptors as well as for depressed neuronal uptake of NE following BCNU treatment could be the result of increased neurotransmitter leakage from sympathetic varicosities following injury to these nerves.

These data provide evidence for the development of a delayed autonomic sympathetic toxicity following treatment with a single dose of BCNU.
Bibliography


Herman, A.G., "Introductory Remarks About the Nomenclature of Prostaglandins and Their Biosynthesis and Metabolism", Cardiovascular Pharmacology of the Prostaglandins (Herman, A.G., ed.), pp.1-5, 1982.


Hoyt, D.J., "Characterization of Cholestasis Induced by 1,3-bis(2-chloroethyl)-1-Nitrosourea in Rats", M.S. Thesis, Oregon State University, 1984.


Levin, V.A., Stearns, J., Byrd, A., Finn, A. and Weinkam, R.J., "The Effect of Phenobarbital Pretreatment on the Antitumor Activity of 1,3-Bis(2-chloroethyl)-1--3-Cyclohexyl-1-Nitrosourea (CCNU) and 1-(2-Chloroethyl)-3-(2,6-Dioxo)-3-Piperidyl-1-Nitrosourea (PCNU), and on the Plasma Pharmacokinetics and Biotransformation of BCNU", J. Pharmacol. Exp. Ther., 208: 1-6, 1979.


APPENDIX
Table A1

Sensitivity of Caudal Arteries From Control and BCNU Treated Sprague-Dawley Rats to Norepinephrine.

<table>
<thead>
<tr>
<th>Group^b</th>
<th>Concentration of Norepinephrine^a (M)</th>
<th>5.6x10^-8</th>
<th>10^-7</th>
<th>1.8x10^-2</th>
<th>3.2x10^-7</th>
<th>5.6x10^-7</th>
<th>10^-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=16)</td>
<td>25.2±5.40</td>
<td>51.4±7.40</td>
<td>86.3±10.3</td>
<td>137.6±14.1</td>
<td>193.1±17.5</td>
<td>237.1±0.9</td>
<td></td>
</tr>
<tr>
<td>Day 1 (n=3)</td>
<td>36.7±9.80</td>
<td>73.7±20.5</td>
<td>130.0±38.3</td>
<td>175.0±46.0</td>
<td>219.7±55.4</td>
<td>262.3±60.1</td>
<td></td>
</tr>
<tr>
<td>Day 3 (n=4)</td>
<td>28.6±11.0</td>
<td>63.8±20.7</td>
<td>114.1±30.4</td>
<td>169.2±36.6</td>
<td>229.0±40.9</td>
<td>273.2±35.6</td>
<td></td>
</tr>
<tr>
<td>Day 7 (n=5)</td>
<td>24.4±8.00</td>
<td>56.1±13.0</td>
<td>105.2±21.3</td>
<td>151.1±26.0</td>
<td>199.4±30.9</td>
<td>245.6±34.5</td>
<td></td>
</tr>
<tr>
<td>Day 10 (n=7)</td>
<td>46.5±6.80</td>
<td>92.0±11.9</td>
<td>150.1±17.0</td>
<td>209.7±18.5</td>
<td>268.3±21.6</td>
<td>315.7±15.1</td>
<td></td>
</tr>
<tr>
<td>Day 14 (n=7)</td>
<td>32.8±6.00</td>
<td>73.7±12.5</td>
<td>131.7±16.4</td>
<td>195.7±19.6</td>
<td>257.6±21.2</td>
<td>309.1±19.1</td>
<td></td>
</tr>
<tr>
<td>Day 17 (n=5)</td>
<td>26.2±6.00</td>
<td>59.5±10.9</td>
<td>113.6±16.0</td>
<td>168.6±24.4</td>
<td>228.4±33.2</td>
<td>271.8±40.3</td>
<td></td>
</tr>
<tr>
<td>Day 21 (n=2)</td>
<td>41.5±12.5</td>
<td>83.0±24.0</td>
<td>128.5±23.5</td>
<td>183.0±9.00</td>
<td>235.0±7.00</td>
<td>272.0±6.00</td>
<td></td>
</tr>
<tr>
<td>Day 24 (n=2)</td>
<td>40.5±6.50</td>
<td>80.8±14.8</td>
<td>116.0±5.00</td>
<td>173.5±51.5</td>
<td>223.0±58.0</td>
<td>244.8±1.20</td>
<td></td>
</tr>
<tr>
<td>Day 28 (n=2)</td>
<td>55.8±12.8</td>
<td>111.0±11.0</td>
<td>184.0±4.00</td>
<td>243.0±3.00</td>
<td>333.0±5.00</td>
<td>&gt;400</td>
<td></td>
</tr>
</tbody>
</table>

Each value represents the mean ± s.e.m. of the number of animals in parentheses. Responses represent the increase in perfusion pressure measure in mmHg.

a) Constricted arterial segments were bathed extraluminally with Krebs bicarbonate (37°C) and perfused intraluminally with varying concentrations of norepinephrine.
b) Rats were injected, i.p., with a single dose of BCNU (20mg/kg) on Day 0. Arteries were collected on Days 1, 3, 7, 10, 14, 17, 21, 24, and 28 after dosing with BCNU.
Figure A1. Responsiveness of rat caudal artery segments from control and BCNU treated Sprague-Dawley rats to intraluminally perfused norepinephrine on days 1-28 after dosing. Values are taken from data contained in Table A1. Responses represent the increase in perfusion pressure measured in mmHg. Rats were injected, i.p., with a single dose of BCNU (20 mg/kg) on Day 0. Arteries were collected on Days 3, 14, 21, and 28 after dosing with BCNU. Cannulated arterial segments were bathed extraluminally with Krebs bicarbonate (37°C) and perfused intraluminally with varying concentrations of norepinephrine (5.6 x 10^{-8} M to 10^{-6} M).
Figure A1

Control Day 3 Day 14 Day 21 Day 28

Response (mmHg)

Log Concentration NE (M)
Table A2

Sensitivity of Caudal Arteries From Control and BCNU Treated Sprague-Dawley Rats to Field Stimulation of Periarterial Nerves.

<table>
<thead>
<tr>
<th>Group</th>
<th>Frequency Response (Hz)</th>
<th>1.4</th>
<th>1.9</th>
<th>2.7</th>
<th>3.7</th>
<th>5.2</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16</td>
<td>6.10±1.3</td>
<td>11.2±1.9</td>
<td>18.7±2.7</td>
<td>27.0±3.4</td>
<td>37.7±4.4</td>
<td>62.0±6.7</td>
</tr>
<tr>
<td>Day 1</td>
<td>3</td>
<td>9.20±4.8</td>
<td>14.7±6.9</td>
<td>21.5±9.0</td>
<td>29.2±11.1</td>
<td>37.5±13.1</td>
<td>58.7±17.6</td>
</tr>
<tr>
<td>Day 3</td>
<td>4</td>
<td>16.9±8.6</td>
<td>28.0±13.8</td>
<td>40.4±18.0</td>
<td>51.6±21.6</td>
<td>64.6±24.4</td>
<td>90.6±28.3</td>
</tr>
<tr>
<td>Day 7</td>
<td>5</td>
<td>3.7±1.3</td>
<td>7.5±1.7</td>
<td>14.8±2.0</td>
<td>22.6±2.2</td>
<td>32.3±2.3</td>
<td>52.2±2.5</td>
</tr>
<tr>
<td>Day 10</td>
<td>7</td>
<td>8.4±3.2</td>
<td>15.6±4.9</td>
<td>26.9±6.9</td>
<td>37.8±7.7</td>
<td>51.1±7.8</td>
<td>78.7±9.1</td>
</tr>
<tr>
<td>Day 14</td>
<td>7</td>
<td>10.0±2.8</td>
<td>18.7±4.0</td>
<td>31.6±5.2</td>
<td>43.2±5.7</td>
<td>56.7±5.7</td>
<td>84.4±7.9</td>
</tr>
<tr>
<td>Day 17</td>
<td>5</td>
<td>12.2±4.2</td>
<td>20.3±6.6</td>
<td>31.5±9.6</td>
<td>42.1±12.0</td>
<td>53.8±15.3</td>
<td>82.2±22.7</td>
</tr>
<tr>
<td>Day 21</td>
<td>2</td>
<td>0.50±0.5</td>
<td>1.80±0.2</td>
<td>9.8±1.8</td>
<td>20.5±4.5</td>
<td>33.0±7.0</td>
<td>56.8±7.8</td>
</tr>
<tr>
<td>Day 24</td>
<td>2</td>
<td>11.2±10.8</td>
<td>17.8±15.2</td>
<td>28.5±18.0</td>
<td>38.0±15.0</td>
<td>52.8±11.2</td>
<td>80.0±10.6</td>
</tr>
<tr>
<td>Day 28</td>
<td>2</td>
<td>0.0±0.0</td>
<td>0.8±0.2</td>
<td>4.5±1.5</td>
<td>13.8±4.8</td>
<td>28.8±8.8</td>
<td>58.8±12.2</td>
</tr>
</tbody>
</table>

Each value represents the mean ± s.e.m. of the number of animals in column. N. Responses represent the increase in perfusion pressure measured in mmHg.

a) Cannulated arterial segments were bathed extraluminally and perfused intraluminally with Krebs bicarbonate (37°C). Periarterial nerves were electrically stimulated by means of bipolar platinum ring electrodes with square wave pulses of 0.3 msec duration in 10 second trains at supramaximal voltage (100V) from 1.4 to 10.0 Hz.

b) Rats were injected, i.p., with a single dose of BCNU (20 mg/kg) on Day 0. Arteries were collected on Days 1, 3, 7, 10, 14, 17, 21, 24, and 28 after dosing with BCNU.
Figure A2. Responsiveness of rat caudal artery segments from control and BCNU treated Sprague-Dawley rats to periarterial nerve stimulation on days 1-28 after dosing. Values are taken from data contained in Table A2. Responses represent the increase in perfusion pressure measured in mmHg. Rats were injected, i.p., with a single dose of BCNU (20 mg/kg) on Day 0. Arteries were collected on Days 3, 14, 21, and 28 after dosing with BCNU. Cannulated arterial segments were bathed extraluminally and perfused intraluminally with Krebs bicarbonate (37°C). Periarterial nerves were field-stimulated by means of bipolar platinum ring electrodes with square wave pulses of 0.3 msec duration in 10 second trains at supramaximal voltage (100V) from 1.4 to 10.0 Hz.
Figure A2

Control  Day 3  Day 14  Day 21  Day 28

Response (mmHg)

Frequency (Hz)
Table A3

Sensitivity of Caudal Arteries From Control and BCNU Treated Sprague-Dawley Rats to Norepinephrine and Field Stimulation of Periarterial Nerves.

<table>
<thead>
<tr>
<th>Group b \nGroup b</th>
<th>Concentration of Norepinephrine a</th>
<th>Control 5.6x10^-8</th>
<th>5.6x10^-7</th>
<th>18x10^-7</th>
<th>3.2x10^-7</th>
<th>5.6x10^-7</th>
<th>10^-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 16</td>
<td>25.2±5.4</td>
<td>51.4±7.4</td>
<td>86.3±10.3</td>
<td>137.6±14.1</td>
<td>193.1±17.5</td>
<td>237.1±20.9</td>
<td></td>
</tr>
<tr>
<td>Day 7 25mg/kg</td>
<td>2 63.5±23.5</td>
<td>121.5±38.5</td>
<td>211.5±72.5</td>
<td>294.0±104.</td>
<td>326.0±74.0</td>
<td>345.0±55.0</td>
<td></td>
</tr>
<tr>
<td>Day 10 20mg/kg</td>
<td>2 49.8±7.20</td>
<td>102.2±8.80</td>
<td>177.0±7.00</td>
<td>245.5±6.50</td>
<td>311.5±6.50</td>
<td>371.0±3.00</td>
<td></td>
</tr>
<tr>
<td>Day 14 15mg/kg</td>
<td>3 33.2±7.50</td>
<td>72.0±16.0</td>
<td>126.8±19.7</td>
<td>198.0±31.4</td>
<td>248.3±29.2</td>
<td>310.7±31.2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Frequency Response (Hz) c</th>
<th>Group b</th>
<th>1.4</th>
<th>1.9</th>
<th>2.7</th>
<th>3.7</th>
<th>5.2</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 16</td>
<td>6.1±1.3</td>
<td>11.2±1.90</td>
<td>18.7±2.70</td>
<td>27.0±3.40</td>
<td>37.7±4.40</td>
<td>62.0±6.70</td>
<td></td>
</tr>
<tr>
<td>Day 7 25mg/kg</td>
<td>2 11.8±2.8</td>
<td>25.4±0.10</td>
<td>44.5±6.00</td>
<td>59.2±10.8</td>
<td>74.5±15.5</td>
<td>101.8±23.2</td>
<td></td>
</tr>
<tr>
<td>Day 10 20mg/kg</td>
<td>2 21.0±8.0</td>
<td>37.5±6.50</td>
<td>59.0±4.00</td>
<td>77.0±1.00</td>
<td>91.0±5.00</td>
<td>121.0±9.00</td>
<td></td>
</tr>
<tr>
<td>Day 14 15mg/kg</td>
<td>3 7.0±2.0</td>
<td>16.7±1.20</td>
<td>32.2±1.40</td>
<td>45.2±2.60</td>
<td>59.7±4.30</td>
<td>85.7±3.80</td>
<td></td>
</tr>
</tbody>
</table>

Each value represents the mean ± s.e.m. of the number of animals in column N.

Responses represent the increase in perfusion pressure (mmHg).

a) Cannulated arteries were bathed extraluminally with Krebs bicarbonate (37°C) and perfused intraluminally with varying concentrations of norepinephrine.

b) Rats were injected, i.p., with a single dose of BCNU (15, 20, or 25 mg/kg) on Day 0. Arteries were collected on Day 7 (25 mg/kg), Day 10 (20 mg/kg), or Day 14 (15 mg/kg) after dosing with BCNU.

c) Periarterial nerves were stimulated by means of bipolar platinum ring electrodes with square wave pulses of 0.3 msec duration in 10 second trains at supramaximal voltage (100V) from 1.4 to 10.0 Hz.
Figure A3. The dose- and time-dependent effects of BCNU on the sensitivity of caudal artery segments to intraluminally perfused norepinephrine. Values are taken from data contained in Table A3. Responses represent the increase in perfusion pressure (mmHg). Sprague-Dawley rats were injected, i.p., with a single dose of BCNU (15, 20, or 25 mg/kg) on Day 0. Arteries were collected on Day 14 after 15 mg/kg, Day 10 after 20 mg/kg, and Day 7 after 25 mg/kg BCNU. Cannulated arteries were bathed extraluminally with Krebs bicarbonate (37°C) and perfused with varying concentrations of norepinephrine (5.6 x 10^{-8} M to 10^{-6} M).
### Table A4

Sensitivity of Caudal Arteries From Control and BHCNU Treated Sprague-Dawley Rats to Norepinephrine and Field Stimulation of Periarterial Nerves.

<table>
<thead>
<tr>
<th>Group</th>
<th>(N)</th>
<th>Concentration of Norepinephrine (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(5.6 \times 10^{-8})</td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
<td>51.4±7.40</td>
</tr>
<tr>
<td>Day 1</td>
<td>2</td>
<td>28.0±6.00</td>
</tr>
<tr>
<td>Day 3</td>
<td>4</td>
<td>99.0±21.5</td>
</tr>
<tr>
<td>Day 7</td>
<td>2</td>
<td>47.8±15.2</td>
</tr>
<tr>
<td>Day 14</td>
<td>2</td>
<td>69.5±16.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>(N)</th>
<th>Frequency Response (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(1.4)</td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
<td>6.1±1.30</td>
</tr>
<tr>
<td>Day 1</td>
<td>2</td>
<td>1.5±0.50</td>
</tr>
<tr>
<td>Day 3</td>
<td>4</td>
<td>9.1±5.20</td>
</tr>
<tr>
<td>Day 7</td>
<td>2</td>
<td>13.5±10.5</td>
</tr>
<tr>
<td>Day 14</td>
<td>2</td>
<td>8.2±3.4</td>
</tr>
</tbody>
</table>

Each value represents the mean ± s.e.m. of the number of animals in column \(N\). Responses represent the increase in perfusion pressure (mmHg).

a) Cannulated arteries were bathed extraluminally with Krebs bicarbonate (37°C) and perfused intraluminally with varying concentrations of norepinephrine.

b) Rats were injected, i.p., with a single dose of BHCNU (20 mg/kg) on Day 0. Arteries were collected on Days 1, 3, 7, and 14 after dosing with BHCNU.

c) Periarterial nerves were stimulated by means of bipolar platinum ring electrodes with square wave pulses of 0.3 msec duration in 10 second trains at supramaximal voltage (100V) from 1.4 to 10.0 Hz.
Figure A4. Comparison of the time-dependent effects of BHCNU and BCNU on the sensitivity of caudal artery segments from Sprague-Dawley rats to intraluminally perfused norepinephrine. Values are taken from data contained in Table A4 and Table A1. Responses represent the increase in perfusion pressure (mmHg). Sprague-Dawley rats were injected, i.p., with a single dose of BHCNU (20 mg/kg) or BCNU (20 mg/kg). Arteries were collected on Days 3 and 14 following treatment with BHCNU or BCNU. Cannulated arteries were bathed extraluminally with Krebs bicarbonate (37°C) and perfused with varying concentrations of norepinephrine (5.6 x 10^{-8} M to 10^{-6} M). #, * symbols = BCNU treatment; X, + symbols = BHCNU treatment.
Figure A4

Response (mmHg) vs. Log Concentration NE (M)

- Control
- Day 3
- Day 14

Symbols indicate statistical significance:
- *: p < 0.05
- #: p < 0.01

Graph shows a linear increase in response with increasing log concentration for each day.
Figure A5. Comparison of the time-dependent effects of BHCNU and BCNU on the sensitivity of caudal artery segments from Sprague-Dawley rats to periarterial nerve stimulation. Values are taken from data contained in Table A4 and Table A2. Responses represent the increase in perfusion pressure (mmHg). Sprague-Dawley rats were injected, i.p., with a single dose of BHCNU (20 mg/kg) or BCNU (20 mg/kg). Arteries were collected on Days 3 and 14 following treatment with BCNU or BHCNU. Cannulated arteries were bathed extraluminally and perfused intraluminally with Krebs bicarbonate (37°C) and periarterial nerves were field-stimulated by means of bipolar platinum ring electrodes with square wave pulses of 0.3 msec duration in 10 second trains at supramaximal voltage (100V) from 1.4 to 10.0 Hz.

#,,* symbols=BCNU treatment ; X,+ symbols=BHCNU treatment