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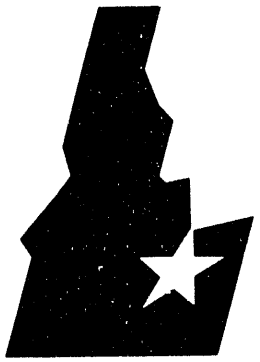

Idaho National Engineering Laboratory

Managed by the U.S. Department of Energy

INEL BNCT RESEARCH PROGRAM MAY/JUNE 1992

J. R. Venhuizen

EG&G Idaho

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ABSTRACT

This report presents summaries for two months of current research of the Idaho National Engineering Laboratory (INEL) Boron Neutron Capture Therapy (BNCT) Program. Information is presented on development and murine screening experiments of low-density lipoprotein, carboranyl alanine, and liposome boron containing compounds. Pituitary tumor cell culture studies are described. Drug stability, pharmacology and toxicity evaluation of borocaptate sodium (BSH) and boronophenylalanine (BPA) are described. Treatment protocol development via the large animal (canine) model studies and physiological response evaluation in rats are discussed. Supporting technology development and technical support activities for boron drug biochemistry and purity, analytical and measurement dosimetry, and noninvasive boron quantification activities are included for the current time period. Current publications for the two months are listed.

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TABLES

ACRONYMS AND ABBREVIATIONS

AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ANS	American Nuclear Society
B/C	Boron-to-carbon
BMRR	Brookhaven Medical Research Reactor
BNL	Brookhaven National Laboratory
BNCT	Boron Neutron Capture Therapy
BPA	Boronophenylalanine
BSH	Borocaptate Sodium ($Na_2B_{12}H_{11}SH$)
BSS	$B_{24}H_{22}SS^{4-}$
BSSO	$B_{24}H_{22}S_2O^{4-}$
CARBALA	Carboranyl Alanine
CHNS	Carbon-hydrogen-nitrogen-sulfur
CRH	Corticotropin releasing hormone
EM	Electron microscopy
FTIR	Fourier Transform Infrared Spectroscopy
GTRR	Georgia Institute of Technology Research Reactor
HPLC	High Performance Liquid Chromatography
ICP-AES	Inductively Coupled Plasma-Atomic Emission Spectroscopy
INEL	Idaho National Engineering Laboratory
ISU	Idaho State University
LDL	Low-density lipoprotein
LM	Light microscopy
LSD	Least significant difference
MRI	Magnetic resonance imaging
NMR	Nuclear magnetic resonance
OHSU	Oregon Health Sciences University
ORSU	Oregon State University
PAL	Phenylalanine ammonia lyase
PMT	Photo multiplier tube
PNL	Pacific Northwest Laboratory
PPM	Parts per million
RBE	Relative Biological Effectiveness
RSD	Relative standard deviation
SIMS	Secondary Ion Mass Spectrometry
SIRIS	Sputter-Initiated Resonance Ionization Spectroscopy
TBAS	Tetra bulyl ammonium sulfate
UCLA	University of California Los Angeles
UCSF	University of California San Francisco
UofR	University of Rochester
UofU	University of Utah
WSU	Washington State University

INEL BNCT RESEARCH PROGRAM
MAY/JUNE 1992

INTRODUCTION

This report contains an overview of progress for the Idaho National Engineering Laboratory (INEL) Boron Neutron Capture Therapy (BNCT) Research Program for May/June 1992, including information on the Research Programs, Technical Support Details, and Miscellaneous Project Information.

The following sections are the reports from the various investigators about their current research.

BORON DRUG DEVELOPMENT

Carboranyl Alanine (CARBALA) and Low-Density Lipoprotein (LDL) Development and Evaluation

A new batch of boron-loaded LDL has been prepared and sent to Washington State University (WSU) for animal testing. This sample contains elaidyl carborane carboxylate rather than alkyl or aryl carboranes. University of California/San Francisco (UCSF) researchers have decided this compound reconstitutes more reproducibly than simple carboranes, although with somewhat less boron per mg protein. Preparation of this material for animal experiments requires one additional setp: a dialysis against phosphate-buffered saline at pH 7.4 to remove the tricine buffer in which the reconstituted LDL is normally prepared. This procedure does not result in loss of material, as evidenced by similar protein concentrations in the final solution relative to the tricine-solvated precursor. The animal experiments will represent the first set of such experiments in the LDL program and, thus, represent a milestone in efforts to demonstrate that boronated LDL is a viable tumor-specific delivery system for BNCT.

Extensive and time-consuming work on the quantification of the stereochemical ratios of carboranyl alanine and its precursors have taken place during this reporting period. Initial attempts to obtain these data have been carried out using optical rotations and comparing them to literature values when available. The reported method proved not to be reproducible and was very sensitive to solution conditions. Elaboration of the amino acid by coupling with an optically active fluorine-containing carboxylic acid (Mosher's acid) at the α-amino terminus appeared to offer the next most accessible method of quantification. In general, however, the ^{19}F nuclear magnetic resonance (NMR) signals of the resultant diastereomers of the methyl, ethyl, and benzylic esters of N-amidated carboranylalanine showed little or no separation of the two ^{19}F signals at 242 MHz. Baseline separation was not possible and only a rough value of 95:5 could be obtained, even with the best-case methyl ester. In the end it was necessary to rely on high performance liquid chromatography (HPLC) separation on a chiral column. Each enantiomer or diastereomer should have a unique retention time dependent on its specific interactions with the chiral groups bound to the stationary phase that, in the present case, would need to be compatible with organic mobile phases. Therefore, the N-Mosher's amide C-methyl or benzyl ester derivatives of CARBALA and its precursor, an azido benzyl ester, were suitable for analysis.

Using a Machery-Nagel Nucleosil chiral-2 column, it became immediately apparent that these chiral separations were extremely touchy and subject to great disturbance with only minor changes in mobile phase. Even with moderate flowrates, true column equilibration was achieved only after 2-3 hours following very small changes in the polar solvent content. Bottle-to-bottle variation of HPLC-grade hexane precluded its use and forced the use of n-heptane, which is available as 99+% pure. Changes in ambient temperature also appear to greatly affect separations. The UCSF laboratory has recorded up to 9°C change from morning to afternoon. It seems that well-resolved separations could be eroded simply by running chromatograms at 29°C instead of 20°C.

Controlling all of these factors, reproducible, baseline separations using 0.05% isopropyl alcohol in n-heptane was finally achieved. Only the S-azido benzyl ester was initially available, but a deliberate, partially racemized sample, used as a first check of the separation, established the separation conditions. Results indicate that the azido benzyl ester normally isolated has a 98:2 ratio of enantiomers. This is an important result as it firmly establishes the maintenance of configuration at the optical centers, and confirms development of the first chiral synthesis of the target compound since hydrogenolysis to the amino acid should not cause any racemization.

To conclusively prove this, it was necessary to demonstrate that the Mosher's amide derivatives could be separated and quantified on the chiral column. Since these are diastereomers, not enantiomers, all four possible isomers must be prepared: (1) (L)-CARBALA ester-N-(S)-Mosher's amide, (2) (L)-CARBALA ester N-(R)-Mosher's amide, (3) (D)-CARBALA-N-(S)-Mosher's amide, and (4) (D)-CARBALA-N-(R)-Mosher's amide. These materials are currently being carried along in synthesis. To date, UCSF researchers have shown that the benzyl CARBALA esters of N-Mosher's amide derivatives are well-separated. This is especially useful for evaluation of the stereoselectivity of triphenylphosphine reduction of azides to amino groups in preparation of BOC-protected amino acids for eventual peptide synthesis.

Boronated Liposome Development and Evaluation

Liposome Encapsulation and Development: Murine melanoma screening experiments with liposomes containing $B_{10}H_{10}^{2-}$ have been completed at WSU. Tissue samples for this screening, as well as the tissues for the $(Co(C_2B_9H_{10}SH)_2)^{1-}$ screening are being held at WSU pending notification from INEL.

A new tumor line, P1798 murine lymphosarcoma, has been provided to WSU by Vestar, Inc. The first screening experiment with liposomes, prepared by University of California at Los Angeles (UCLA) researchers, containing $Na_3B_{20}H_{19}$ has been completed at WSU. This line should provide additional sensitivity not observed in the melanoma tumor line, allowing for better distinctions between boron compounds delivered by liposomes.

Biodistribution results for liposomes encapsulated with $Na_3B_{20}H_{17}NH_3$ and $NaB_{10}H_9NCO$ in the electron microscopy (EM)T6 tumor line at Vestar, Inc. have been analyzed by UCLA researchers (Figures 1 and 2, respectively). The biodistribution results for these compounds are extremely promising. The tumor-boron concentration for the encapsulated $Na_3B_{20}H_{17}NH_3$ compound at six hours is approximately 27 μg boron/gram tissue and rises over a period of 30 hours. The final tumor-boron level at 48 hours is 25.4 μg boron/gram tissue with a final tumor-to-blood-boron ratio of 5.3. The tumor-boron concentration for the encapsulated $Na_2B_{10}H_9NCO$ compound at six hours is approximately 20 μg boron/gram tissue, the highest observed for any ten boron compounds tested thus far. The boron level is maintained for approximately 30 hours and then decreased to 11.1 μg boron/gram tissue at 48 hours, where the tumor-to-blood-boron ratio is 5.0.

Scheduling for murine the EMT6 screening experiments for liposomes containing $NaCo(C_2B_9H_{11})_2$, $NaCo(C_2H_{10}SH)_2$, $NaCo(C_2B_9H_{11}S)_2$, and $NaB_{10}H_9NH_3$ is in progress.

Compound Development: The synthesis of a series of derivatives of cobalt (III) *bis*-dicabollide(1-) has been completed. The series consists of the parent compound $[Co(C_2B_9H_{11})_2]^{1-}$, the sulfide derivative $[Co(C_2B_9H_{10}SH)_2]^{1-}$, the disulfide derivative $[Co(C_2B_9H_{10}S)_2]^{1-}$, and the disulfide monoxide derivative $[Co(C_2B_9H_{10}S)_2O]^{1-}$.

The water-soluble sodium salts of these metalloarboranes have been prepared for encapsulation in liposomes. An investigation is being initiated of the interaction of these species with cysteine and cystine.

The *n*-hexadecyl derivative of *ortho*-carborane, $C_2B_{10}H_{11}(CH_2)_{15}CH_3$, has been synthesized and purified. The compound can be degraded cleanly to the monoanion, $[C_2B_9H_{11}(CH_2)_{15}CH_3]^{1-}$ using KOH/EtOH. Deprotonation of the monoanion using NaH, followed by reaction with anhydrous $CoCl_2$, yields the cobalt complex of the dicarbollide, $[Co(C_2B_9H_{10}(CH_2)_{15}CH_3)]^{1-}$. This compound may be useful because of the potential to embed this amphipathic complex into the lipophilic membrane of the liposomes. Purification of the cobalt complex is in progress.

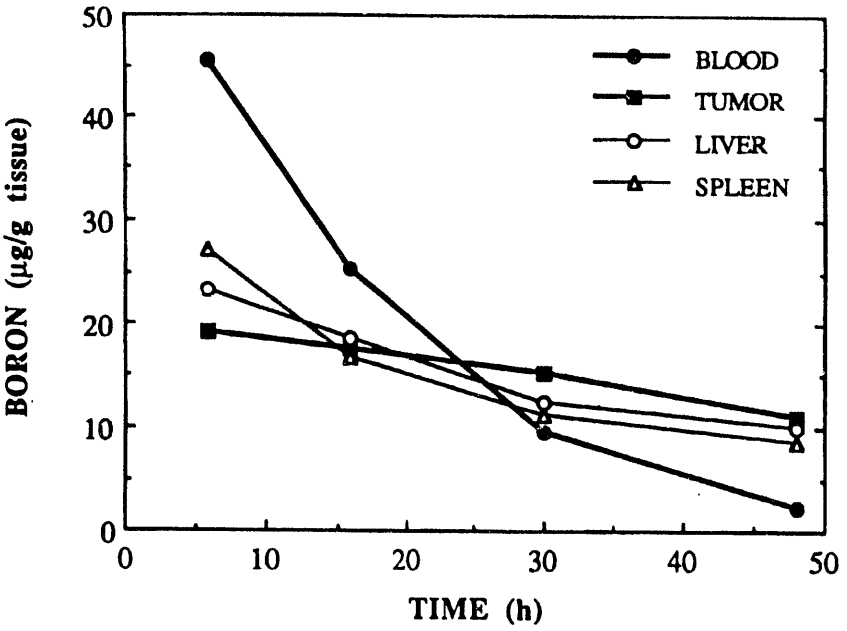

Figure 1. Biodistribution of liposomal $Na_3B_{20}H_{17}NH_3$ on tumor-bearing, EMT6 mice.

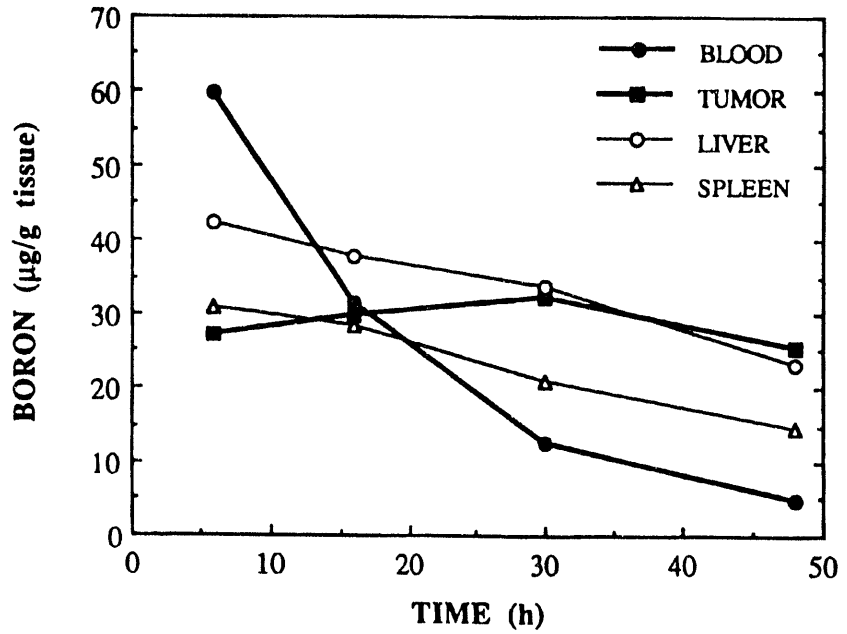

Figure 2. Biodistribution of liposomal $Na_2B_{10}H_8NHO$ on tumor-bearing, EMT6 mice.

Boron analyses have been completed for liposomes formed in the presence of *n*-hexylcarborane, a hydrophobic compound containing ten boron atoms. Vesicles were prepared with phospholipid containing *n*-hexylcarborane in concentrations varying from 1-10% boron. The high boron content found in the liposomal suspensions, which varied with the boron content of the lipid, indicates the boron compound has been successfully embedded into the liposome membrane rather than encapsulated in the aqueous core. Investigations are in progress to embed a 16 carbon chain carborane into the membrane. The longer chain should increase the stability of the resulting bilayer.

The promising biodistribution results obtained with encapsulated $Na_3B_{20}H_{18}NH_3$ has suggested that intracellular oxidation to the more reactive $NaB_{20}H_{18}NH_3$ may be occurring. The latter species is a substituted derivative of $Na_2B_{20}H_{18}$ and may react with intracellular moieties in the same manner, preventing the clearance of the compound from the tumor. To study this theory, $NaB_{10}H_8NH_3$ has been synthesized. If the oxidation theory is correct, this compound should not be retained by the tumor. If the tumor retention in $Na_3B_{20}H_{17}NH_3$ is a result of the amine functionality itself, the $NaB_{10}H_8NH_3$ should also be retained in the tumor. Additionally, a study of the oxidation potentials of the reduced species studied to date ($Na_3B_{20}H_{19}$, $Na_4B_{20}H_{17}OH$, and $Na_3B_{20}H_{17}NH_3$) is imminent.

Pituitary Tumor Evaluation

Oregon Health Sciences University (OHSU) researchers have taken AtT-20 rat pituitary tumor cells to the the Oregon State University (ORSU) reactor facility for exposure to neutrons. Cells were placed in sterile scintillation vials and exposed in the stringer port with the reactor running at 10% power for ten minutes. An identical control cell vial was prepared exactly as the experimental vial and placed outside the reactor during the exposure time. Background gamma radiation was approximately 650 rads. AtT-20 cells exposed to radiation survived similarly as control cells for 48 hours. All cells were exposed to 10^{-6} M corticotropin releasing hormone (CRH), frozen, and intracellular cyclic adenosine monophosphate (AMP) measured to assess endocrine function. These irradiation experiments are proceeding well. Results of this cyclic AMP assay are pending.

A second culture of AtT-20 cells were preexposed to either CRH-conjugated carborane or carborane cage alone for 15 minutes at 37°C prior to neutron exposure. Cells were then cooled to 4°C and washed to eliminate any nonspecific bound carborane. These cells were also exposed to neutrons (reactor at 10% power for ten minutes), washed to remove contaminating media, and replated to observe cell survivability over time and endocrine responsiveness. Results indicate that exposures at 100 kW for ten minutes do not cause the death of control AtT-20 cells; however, at 1000 kW for four minutes, control cells die in 5-6 days. Further experiments are underway to establish a dose response curve for cell death vs reactor radiation (neutrons plus gamma) for these cells. This data will enable subsequent experiments to evaluate the ability of carborane conjugated CRH to specifically kill pituitary tumor cells by BNCT beyond any effect attributed to contaminating gamma (or other) radiation. Weekly experiments are being conducted in this joint effort by OHSU and ORSU researchers.

Measuring Dosimetry: Additional bismuth has arrived and new 8-inch shielding constructed at the ORSU reactor facility to enhance the gamma shielding of the stringer port. In addition, ORSU researchers have enlarged the stringer port geometry by pulling the two adjacent stringers, one on the immediate left and one on the immediate right of the current stringer, and have increased the cell exposure area from a 4x4-inch to a 12x4-inch opening. This enlarged area will not only enable many more sample vials to be tested at one exposure, but will also enable small animal experiments to be conducted with relative ease. Thermal luminescence dosimeter and gold foil measures of gamma and neutron doses at current experimental runs suggest approximately 2000 rads of gamma exposure for $1x10^{13}$ neutrons. The new shielding (now in place) should reduce the gamma dose significantly while minimally affecting the neutron dose.

BORON LOCALIZATION SCREENING

Boronophenylalanine (BPA) Evaluation Studies

Biodistribution of BPA: A second study, *in vivo* enhanced uptake of BPA, investigating the effect of limiting tyrosine and phenylalanine in the diet of tumor-bearing mice, has been completed by WSU researchers. Tissues are awaiting analysis by INEL.

Data have been received from INEL on the *in vivo* biodistribution of BPA study and are being analyzed by Analysis of Variance (ANOVA) with Fischer's Least Significant Difference (LSD) using repeated measures. Twelve (12) C57, melanoma tumor-bearing mice were administered 0.1 mg/mL BPA intravenously and sacrificed at 6 and 12 hours postinjection. Mean tumor levels during this time were approximately 0.8 μg/g.

Miscellaneous: Enzyme assays were conducted by WSU researhers to determine whether BPA is enzymatically degraded by phenylalanine ammonia-lyase (PAL). The assays were conducted under the following conditions: 2.8×10^{-2} M L-phenylalanine or 2.2×10^{-2} M BPA, 1×10^{-1} M Tris-HCl, pH 8.5, and enzyme (0.021 units of PAL) in a volume of 3.0 mL incubated at 37°C. The appearance of cinnamic acid is measured by the increase in optical density at 290 nm (Zucker, 1965). Results show that BPA is not degraded by PAL at 37°C (Figure 3).

Liposome Evaluation Studies

Data from INEL have been received by WSU researchers and are being analyzed by ANOVA with LSD using repeated measures. Tumor levels of $Na_3B_{20}H_{19}$ achieved levels of approximately 9 μg/g throughout the 48 hours of the screening. Blood levels were highest at six hours (65.786 μg/g) and then dropped through 48 hours. Ratios of tumor:liver and tumor:spleen at six hours were 0.09:1 and 0.16:1, respectively (Figure 4).

$Na[Co(C_2B_9H_{11})_2]$: Tumor levels peaked at six hours near therapeutic levels (9.505 μg/g). Blood levels at this time were lower (6.292 μg/g). Both blood and tumor levels decreased through 36 hours to 0.832 μg/g and 0.953 μg/g, respectively. Blood levels remained low (0.828 μg/mL), but interestingly, tumor levels rose again at 48 hours to 4.066 μg/g (Figure 5). At six hours, ratios of tumor:normal tissue were: tumor:brain 4.11:1, tumor:muscle 7.27:1, tumor:liver 0.85:1, and tumor:spleen 2.23:1.

$Na_3B_{20}H_{17}NH_3$: Tumor levels were relatively consistent from 6-48 hours and achieved levels of approximately 6 μg/g of boron. After 48 hours, tumor levels began to drop and continued to decrease through 72 hours. Blood levels peaked at six hour (41.433 μg/mL) then decreased through 72 hours (Figure 6). Tumor:normal tissue ratios were: tumor:brain 4.14:1, tumor:muscle 5.25:1, tumor:liver 0.21:1, and tumor:spleen 0.17:1.

$Na_4B_{20}H_{17}OH$: Data have been received from INEL and statistical analyses are being computed.

$Na_2B_{10}H_9NCO$, $Na[Co(C_2B_9H_{10}SH)_2]$ and $Na_2B_{10}H_{10}$: Tissues have been sent to INEL for analysis. Results are pending.

Miscellaneous: WSU researchers received a new tumor cell line, P1798, from Vestar, Inc. This new lymphosarcoma tumor cell was first allowed to grow *in vitro* and a preliminary study was instituted to define the most appropriate method of tumor innoculation. This tumor cell was used in the just-completed tenth boron screening. BALB-C mice were injected subcutaneously over the hip and the tumor allowed to grow for approximately seven days. The mice were then administered the boron liposome compound $Na_3B_{20}H_{19}$ intravenously and then sacrificed through time. Tissues were then sent to INEL for boron analysis. Results are pending.

WSU researchers also received some low-density lipoprotein boron compound from UCSF

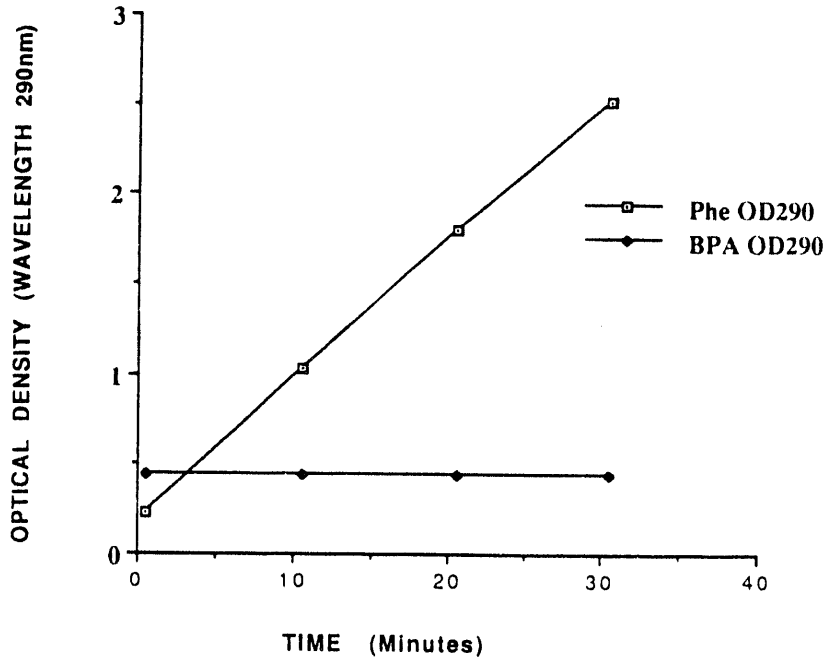

Figure 3. PAL does not degrade BPA at 37°C.

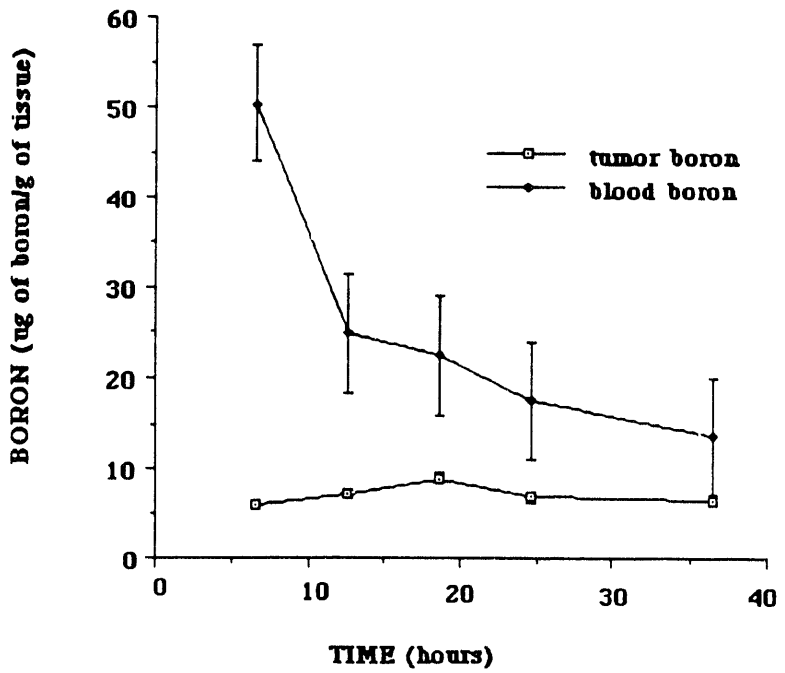

Figure 4. Accumulation of boron in tissues after intravenous injection of $Na_3B_{20}H_{19}$.

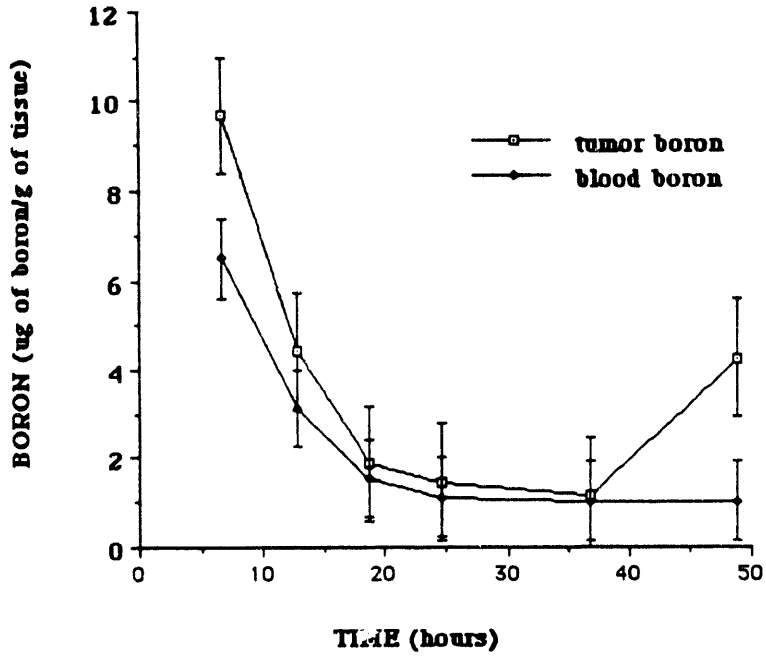

Figure 5. Accumulation of boron in tissues after intravenous injection of $Na[Co(C_2B_9H_{11})_2]$.

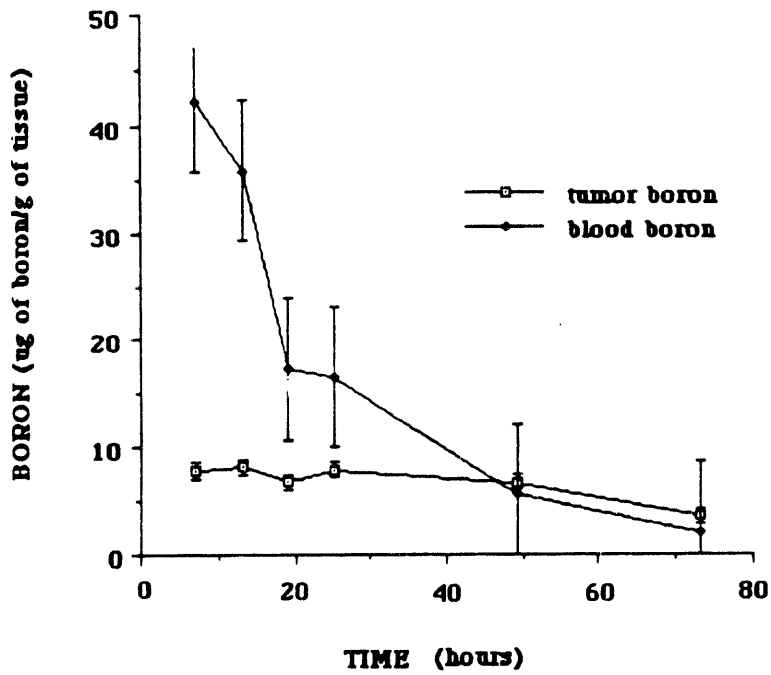

Figure 6. Accumulation of boron in tissues after intravenous injection of $Na_3B_{20}H_{17}NH_3$.

researchers. The screening of the compound will be performed the first week of July 1992.

Melanoma Detection and Boron Quantification by Scintigraphy

Uptake and Distribution of Radiolabeled Boron Compounds: Parallel experiments with the nonlabeled compound are still being planned and will be synthesized upon receipt of funding.

DRUG STABILITY, PHARMACOLOGY AND TOXICITY

Toxicological Evaluation of Borocaptate Sodium (BSH)

One of the respiratory parameters being monitored by Idaho State University (ISU) researchers is insufflation pressure (i.e., the pressure required to deliver a given volume of air into an animal's respiratory tree ("lungs"). Drug-induced increases in insufflation pressure suggest that the drug may be causing bronchoconstriction, while decreases in pressure suggest a possible bronchodilatory effect. Insufflation pressure is measured while artificially ventilating the rat. Because rats do not readily entrain to the rhythm of the ventilator, and because alterations in skeletal muscle tone might affect insufflation pressure, the anesthetized animals are typically paralyzed with gallamine. Initial experiments measuring BSH-induced effects on insufflation pressure have been unsuccessful. When BSH is administered to gallamine-paralyzed rats, the rats begin to spontaneously respire, interfering with the measurement of insufflation pressure. This reversal of gallamine's effect is not observed after administration of vehicle, suggesting that BSH is responsible for reversing the effect of the gallamine. Gallamine is a competitive antagonist of peripheral nicotinic cholinergic receptors (i.e., a neuromuscular blocking agent). The observation that BSH reverses gallamine's effect suggests that BSH may be altering cholinergic function. An effect on peripheral nicotinic cholinergic function was entirely unexpected, but such an action may be of physiological relevance since the literature (Hayashi, et al.) reports that toxic overdose with BSH was associated with paralysis of the limbs. Such paralysis could result either from overstimulation or blockage of nicotinic receptors of the somatic nervous system. These observations came as a surprise because there is no obvious similarity between the structure of BSH and those of nicotinic agonists or antagonists. Further experiments are planned to better understand the significance of these observations.

Toxicological Evaluation of BPA

As previously reported (March/April 1992), ISU researchers are using fructose to prepare pH 7.4 aqueous solutions containing 150-175 mg BPA/mL. These solutions have been infused intravenously to anesthetized rats at target delivery rates of 8.5, 12.8, and 17 mL/kg/hour and delivered approximately 1411, 2125, and 2822 mg/kg, respectively, over a one-hour infusion period. Tissue samples from these rats have been submitted to INEL for boron analysis. To date, boron data has been received for only a few samples, but the data is interesting. Rat #27 (345 g) received a one-hour intravenous infusion of 2115 mg/kg BPA (729.5 mg BPA containing 35.0 mg boron). Over a four-hour postinfusion period, 129.4 mg BPA was excreted in the urine (Table 1).

Assuming the rat had 12.6 mL of serum (Bijserbosch, Experientia 37:381, 1981), 1 mL of serum weighs 1.075 g, a hematocrit of 45%, and BPA does not bind to blood cells, then the serum boron concentration of 74.4 parts per million (ppm) at four hours postinfusion indicates a total blood load of 38.2 mg BPA. Thus, by four hours postinfusion, a total of 167.6 mg BPA (129.4 + 38.2) was either excreted in the urine or remained in the blood. The remaining 561.9 mg of BPA presumably entered tissues (enterohepatic excretion remains a possibility and gastrointestinal contents are being assayed for boron content). If the rat is modeled as a single, well-stirred compartment (an obvious oversimplification), then Rat #27 had a tissue weight of about 322 g (345 g body weight - 23 g blood) and an average tissue distribution of 1.75 mg BPA/g, for an average tissue boron concentration of 84 ppm. 20 ppm boron is considered adequate for BNCT. It seems unlikely that the average tissue concentration actually exceeds the blood concentration (84 vs 74 ppm). It may be that BPA is being excreted via the bile or research-

Table 1. BPA Infusion into Rat #27.

	Boron		Urine	
Time	In Serum (ppm)	In Urine (ppm)	Volume (mL)	Total BPA (mg)
Before Infusion	< 0.88	8.9	---	---
End of Infusion	74.6	1550.9	1.5	48.4
1 Hour Postinfusin	72.0	2161.6	0.2	9.0
2 Hours Postinfusion	74.7	1708.2	0.2	7.1
3 Hours Postinfusion	72.3	1631.0	0.1	3.4
4 Hours Postinfusion	74.4	1479.5	2.0	61.5

ers failed to collect all the urine from the bladder. However, the important point is that these data, while preliminary, suggest that intravenous formulations of BPA can deliver tissue levels of boron well in excess of that needed for BNCT.

TREATMENT PROTOCOL DEVELOPMENT

Large Animal Model Studies

Pharmacokinetics: No additional dogs have been infused.

Normal Tissue Tolerance (Neutron Irradiation): No dogs have been irradiated since the last reporting period. Three dogs, one at 100 μg/kg and 27 Gy, one at 25 μg/kg and 27 Gy, and one at 25 μg/kg and 23 Gy, had their six-month, postirradiation checkups. The magnetic resonance imaging (MRI) scan on the dog at 25 μg/kg and 27 Gy had changes consistent with edema in the right cerebral cortex of the brain. There are no clinical neurological signs in this dog. The other two dogs did not show any changes with the MRI scans. All of the dogs that have been irradiated are clinically normal.

Spontaneously-Occurring Brain Tumor Dogs: "Dudley" Fiset (#35447-151), a seven-year old, castrated male Golden Retriever, is still doing great.

"Muffy" Lower (#35447-140), a four-year old, spayed female Terrier cross, began pacing, not eating, and generally gave the impression of feeling pain during the middle of May 1992. She was taken to her local veterinarian and placed on high levels of Prednisone for three weeks. The medication did not provide her with any relief and her clinical signs did not improve. "Muffy" was brought back to WSU for a checkup. MRI revealed a slight increase in tumor size. She has been returned to her home and her condition is being monitored by her owners.

"Brandy" Hoff (#35447-94), a seven-year old, spayed female Golden Retriever, is doing great. "Brandy" was returned to WSU for her two and a half year checkup in June 1992. MRI revealed no change in the tumor. "Brandy" is clinically normal.

Physiological Response Evaluation and Interdiction

On Tuesday, April 7, 1992, BNCT Research Day was held at the University of Rochester (UofR) Cancer Center, Department of Radiation Oncology, Rochester, NY, with presentations by various researchers regarding biology and physics problems. Presentations were given by Mr. Merle Griebenow (INEL), Drs. Philip Rubin, Don Gash, John Hansen, and Mike Schell

(UofR), Dr. Jeffrey Coderre (BNL), and Dr. Howard Amols (Columbia University). This report primarily contains a synopsis of that meeting.

The primary focus of the meeting was on BNCT and normal tissue tolerance information, particularly with regard to the brain. The morning was devoted to biology with presentations by Dr. Jeffery Coderre of Brookhaven National Laboratory (BNL). Dr. Coderre indicated BNL's ability to achieve a therapeutic ratio with BNCT of the irradiation of the rat 9L tumor with longterm survival of the rat. This was true for both BNCT and 250 kVp x-ray. The UofR researchers presented their studies of normal brain pathophysiology with regard to blood-brain-barrier using horseradish peroxidase and ultrastructural correlative work utilizing electron microscopy. Dose response information is being generated with photon irradiation, which would provide a basis to compare damage produced by BNCT and determine the biologic equivalents ("the relative biologic effective dose") of BNCT and verify the accuracy of BNCT calculated doses.

The afternoon was devoted to physics presentations by Merle Griebenow and Dr. Howard Amols, who reported both concurrent and divergent views as to the BNCT dose to the vascular endothelium.

The conclusion of the meeting was that an active collaboration should be initiated with BNL to provide BNCT irradition for the animals rather than waiting for the modification of the Buffalo facility, which will continue to be explored. This will allow researchers to proceed without undue delay to BNCT irradiation.

Active Collaboration with BNL: UofR researchers are in the process of forming an active relationship with Drs. Coderre and Joel at BNL and plan to visit the facility in the near future. The BNL reactor, although down for a short while, is now activated and ready to proceed with irradiation of animals. This will be dependent on availability of BSH or the BSS dimer. This has been discussed with the INEL representative (Merle Griebenow) and UofR researchers are awaiting materials from the manufacturer. The Long Evans rats will be provided and it may also be desirable to do this in Fisher rats. Funding for this project will be subcontracted out of the UofR funds once it is activated and is ongoing. UofR researchers are exploring the feasibility of transferance of funds for technical support through the INEL. Eventually, it is hoped that BNL will be able to negotiate their own support as the extent of their participation becomes evident.

Study of Coderre's Long-Term Surviving Rats: First, there is an inventory of 30-40 brains in formalin that can be processed. Secondly, and more important, there are nine animals that are alive at one year, or approaching one year, following "cure" of their 9L rat brain tumor. On May 5, 1992, Dr. Coderre drove to Rochester and provided the animals for sacrifice in a collaborative study with Drs. Gash and Hansen. This study involved 12 adult F344 rats in the following test groups:

Control (no tumor, no irradiation)	n = 3
9L tumor (BNCT irradiation, 370-day survival)	n = 4
9L tumor (BNCT + x-ray irradiation, 320-day survival)	n = 3
9L tumor (22.5 Gy x-ray irradition, 320-day survival)	n = 2.

The tumor cell implantation and irradiations were performed at BNL. The methods used in treating these animals were described in *Radiation Research*, Coderre, et.al., 1992. All of the animals appeared to be healthy and showed no signs of discomfort. The animals in the BNCT group showed signs of having unilateral or bilateral cataracts. There was no significant weight difference from the BNCT animals when compared to any other group.

Eleven (11) of the 12 rats were perfused with a 2% paraformaldehyde, 2% gluteraldehyde solution. After brains and pituitaries were removed, the skulls were placed in a buffered formalin solution. One rat died before perfusion and the skull fixed in formalin. Ten of the animals received a vascular infusion of horseradish peroxidase prior to sacrifice so blood-brain-barrier impairments could be evaluated.

The 9L tumor implants were placed in the rostral left cortex. Brain irradiation was focused on the left cortex and could have involved underlying soft tissues. While some brain pathology was evident on several of the treated rats, only animal #5 showed a growth

on the skull that appeared to be a bony or cartilagenous nature. Researchers will proceed to analyze the brains and pituitaries and diagnose the growth noted. In addition, the nasopharnx region, tongue, salivary glands, inner ear, and eyes of these animals will be evaluated.

Future Planned Studies: As soon as the BSH or the BSS dimer can be made available, UofR researchers will undertake boron loading in the blood. It is estimated the dose could be as high as 180 parts per million (ppm). It is recommended that once maximum boron serum dose is achieved, dose escalation of the BNCT irradiation proceed in terms of megawatt minutes. Since 7.5 MW-minutes in long-term surviving rats has been delivered, Dr. Rubin suggests that escalation be accomplished by a factor of 2.5 MW-min to levels of 10, 12.5, and 15 MW-min until acute toxicity is determined and death occurs because of total body lethality.

Researchers recognize there is a disparity in the physics calculation; however, according to the calculations of Drs. Coderre and Amols, with 180 ppm ^{10}B in the blood (0.034 Gy/MW-min/ppm yields 6.12 Gy/MW-min or 61.2 Gy to the blood for a 10 MW-min irradiation). BNL researchers use a sparing factor of 1/3 for dose-to-blood vessel wall resulting from boron in the blood. Since current established vascular geometry in the rat capillaries is comparable to human, it is estimated that the dose to the endothelium would be 20 Gy. A relative biological effectiveness (RBE) of 3 for the $^{10}B(n,\alpha)^{7}Li$ reaction would put the dose at 60 Gy equivalent. The concept is to achieve as high a dose as possible until once again death occurs. Thus, it is estimated that an equivalent dose to 60 Gy to the vasculature can be delivered with BNCT.

From the view of the INEL researchers (namely, calculations provided by Merle Griebenow), it is estimated that, with BNL's current BNCT system for irradiating rats, acute lethality will occur. The sparing factor of INEL is 1/5 the dose to the vasculature, i.e. less than calculated by BNL.

The pragmatic solution recommended for this dose calculation dimemma is that researchers proceed with small groups of animals to establish acute lethality as noted in the use of megawatt minutes. In the end, the biological equivalent dose to the photon dose will need to be established in correlations of biologic events and calculations. Theoretical modeling itself will not substitute for the actual biological experiments.

Forebrain Irradiation Model: Male Long Evans rats were irradiated with a Picker C8 cobalt source. Radiation exposure was limited to 30 Gy/hemisphere, resulting in a total dose of 60 Gy. A Lexan stereotaxic frame was used in conjunction with a collimator to restrict exposure to an area described by an inverted triangle between the eyes and ears. This technique allowed radiation exposure of the cortex, striatum, fimbria, corpus callosum, and hippocampus.

Animals were sacrificed at 2, 6, 12, and 24 weeks postirradiation. Five animals at each time point were treated with horse radish peroxidase ten minutes prior to sacrifice. Three animals were saline shams for electron microscopy (EM) analysis at each time point. Finally, three animals were paraffin embedded for basic hisotological analysis. To date, all time points have been completed. Analysis is nearly complete at both the light microscopy (LM) and EM levels. Analyses were primarily directed toward blood-brain-barrier integrity and vascular density. Preliminary results indicate a decrease in vascular density of 15-20% in both the cortex and corpus callosum. The most significant change noted was in the relative area of blood-brain-barrier leakage in the cortex, corpus callosum, fimbria, and hippocampus. Compared to control animals, a 5-10 fold increase in blood-brain-barrier leakage was observed in the 24-week time point animals. While some changes have been observed in the earlier time points, analyses must be completed before finalizing conclusions.

SUPPORTING TECHNOLOGY DEVELOPMENT

Task 1: Biochemistry of BSH and Its Oxidation Products

No activity to report.

Task 2: Noninvasive Boron Quantification

Using the tumor model developed by Salcman, et al., of the Johns Hopkins Hospital, two dogs were innoculated and, by 12 days later, had developed "classical" glioblastoma multiforme tumors suitable for boron imaging. Using the enhancement agent gadolinium (Gd-DTPA), the proton images of the tumors showed normal ring enhancement and a necrotic center. The boron imaging of BSH demonstrated boron uptake in tumors similar to muscle tissue. Surprisingly, the tissue outside the cranium surrounding the injection site received 1.5 times as much boron as either the tumor or muscle. There appears to be a wide range of boron concentration distribution within the head that has not been totally analyzed. Tissue analyses by ICP is pending.

Figures 7 and 8 show sample images taken approximately at the peak boron uptake. These figures compare the boron images with corresponding proton images. The field of view is 24 cm with a resolution of 7.5 x 7.5 x 15 mm (x,y,z).

Task 3: Real-Time Measurement Dosimetry Research

Gamma field measurements were made at the ISU Van deGraaf accelerator facility as part of a preliminary assessment of accelerator source characterization. The accelerator was producing a source of neutrons from 2 MeV protons impinging upon a lithium metal target. The moderator reflector assembly and target assembly are shown in Figure 9. Neutron yield measurements are shown in Figure 10. The gamma results are shown in Table 2. The gamma dose per source neutron ranges from 1.6E-13 to 2.7E-13 rads per neutron, depending on proton energy (Figure 11). There appears to be very little difference between the large buildup cap gamma dose data and the small buildup cap gamma dose data, suggesting the major contribution to the gamma dose arises from the 478 keV gamma emission from proton capture in lithium. The dose contribution from the 13-17 MeV gamma emissions appears to be weak in comparison.

Task 4: Analytical Dosimetry

Task 4A: Macrodosimetric Model Development

This task was suspended as of the end of May 1992 because of funding restrictions. An initial X-port version of the bnct_edit medical image processing and reconstruction software has been installed on the INEL BNCT Program's HP-750 computer and is in usable condition. All enhancements, benchmarking, and quality assurance activities planned for the remainder of the year have been postponed until funding becomes available.

Task 4B: Microdosimetric Model Development

No activity to report.

Task 4C: Microdosimetric Cellular Response Study

The SVEC4-10 endothelial cells remain healthy. Pacific Northwest Laboratory (PNL) researchers have now completed three time-lapse experiments, each with varying degrees of success. Researchers have extracted cell-cycle time data (time between successive mitoses), but have not yet begun the analysis of these data.

PNL researchers have designed, built, and installed a slider mechanism to replace the rotating lens turret by which both the objective lens and the photomultiplier tube (PMT) were attached to the Zeiss Axiomat® microscope. (This microscope is located directly above the exit window of the beam line, and problems with alignment and indexing of the motor-driven turret have prevented accurate alignment of the physical point at which the cell nucleus would be visualized and thr corresponding point through which the beam of protons would be sent.) This problem has been eliminated with the new positioning apparatus, allowing the lens to be moved as a positive stop.

Task 4D: Proton-Induced Biological Damage Study

A final report has been submitted to the INEL BNCT Program office for this study. This task is complete.

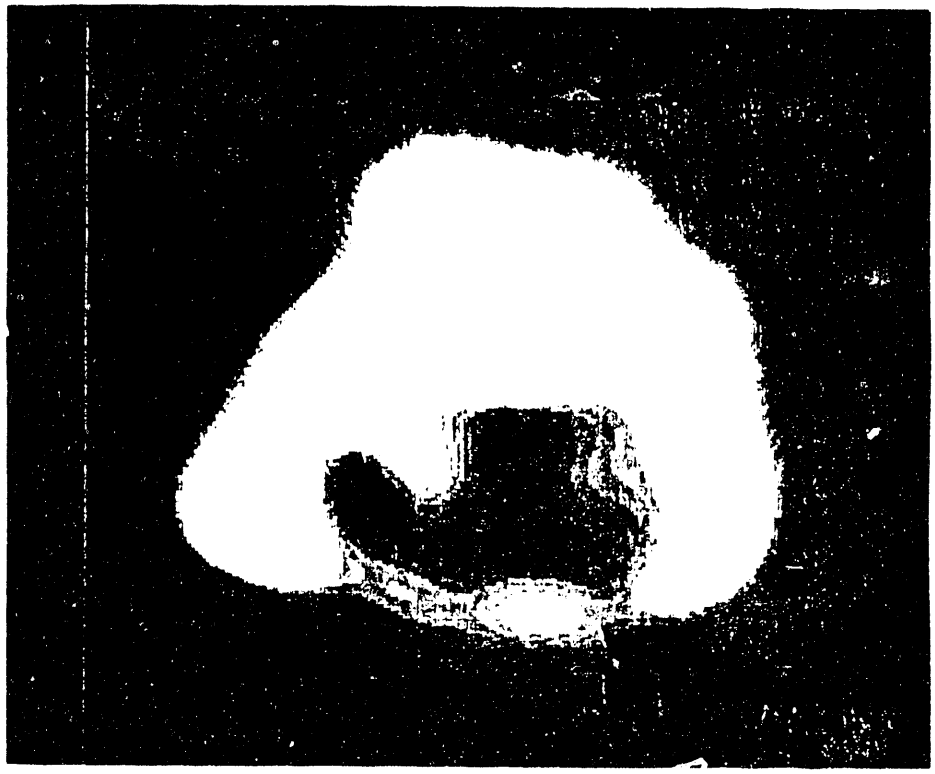

Figure 7. Sample image taken approximately at peak boron uptake.

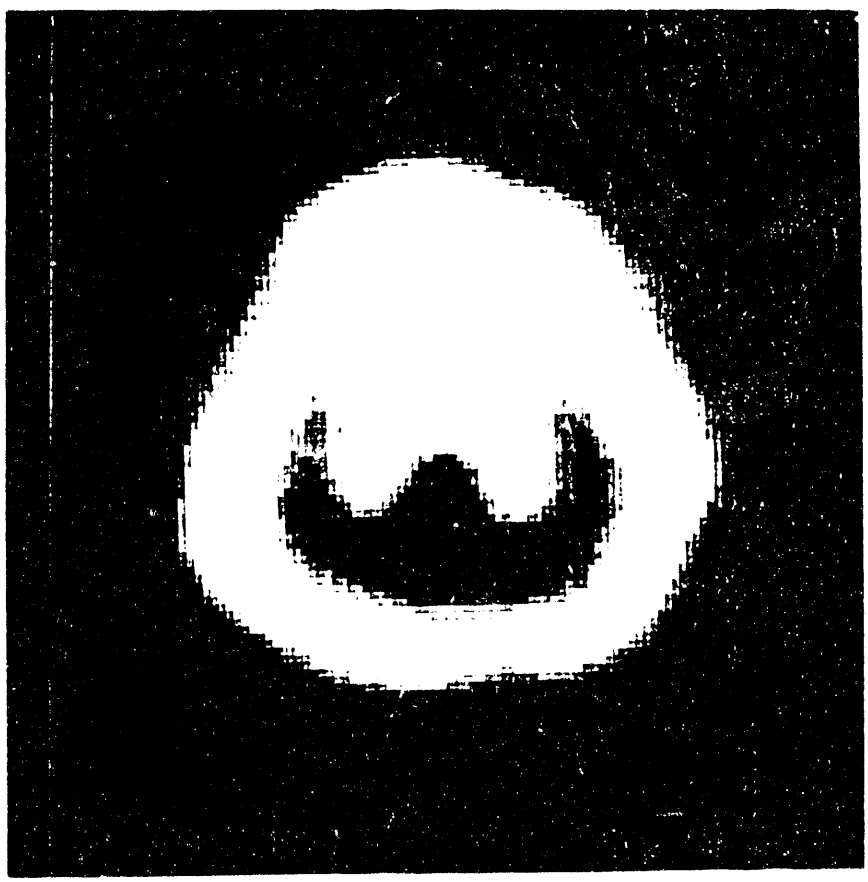

Figure 8. Sample image taken approximately at peak boron uptake.

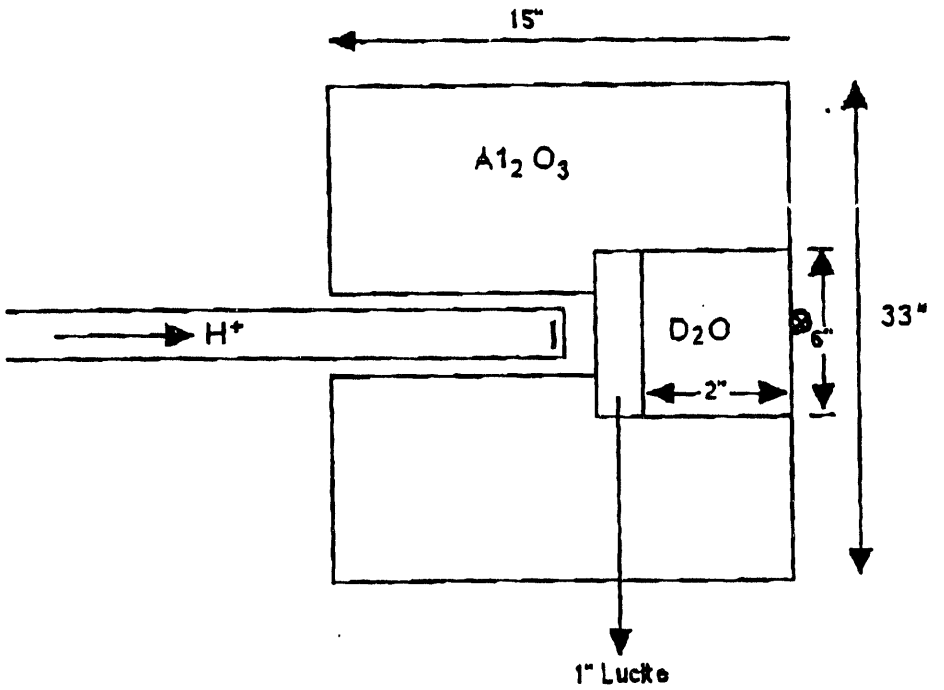

Figure 9. Benchmark BNCT experiment: gamma dose measurement position.

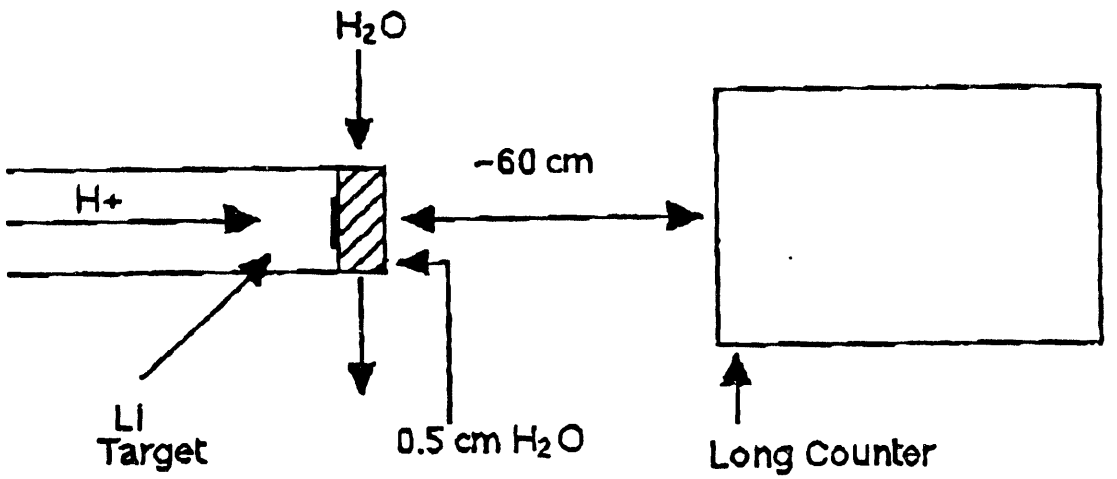

Figure 10. Yield experiment (not to scale).

Table 2. Gamma Dose Measurements on ISU Lithium Target Missouri assembly 2 MeV Van de Graaf, 4/10/92. Neutron data from Frank Harmon (ISU); reported to INEL BNCT Program Office. Radcal Model 1515 calibrated ion chamber, converter model #1550, chamber model 10x5-0.6.

Proton Energy (MeV)	Accumulated Charge (coulombs x 10^{-8})	Gamma Exposure (millirads)	Elapsed Time (min:sec)	Neutron Emission Rate (neutrons/ μ coulombs) x 10^{6})	Gamma Dose/μ coulombs (rads/ μ coulombs)	Gamma Dose/Source Neutron (rads/neutron)
Small buildup cap mod 1085-4						
1.90E+00	6649	2.00	---	---	---	---
2.00E+00	5918	2.14	1:28	135	3.62E-05	2.68E-13
2.10E+00	5200	2.02	1:13	232	3.88E-05	1.67E-13
2.20E+00	4700	2.13	1:00	284	4.53E-05	1.60E-13
2.25E+00	3288	2.07	0:56	---	---	---
Large buildup cap mod 1085-6						
2.00E+00	5951	2.11	0:59	135	3.55E-05	2.63E-13
2.10E+00	5177	2.09	0:53	232	4.04E-05	1.74E-13
2.20E+00	5502	2.05	0:56	284	3.73E-05	1.31E-13[a]
2.25E+00	4350	2.08	0:40	---	4.78E-05	---
2.20E+00	4371	2.07	0:40	284	4.74E-05	1.67E-13

[a] Bad charge reading

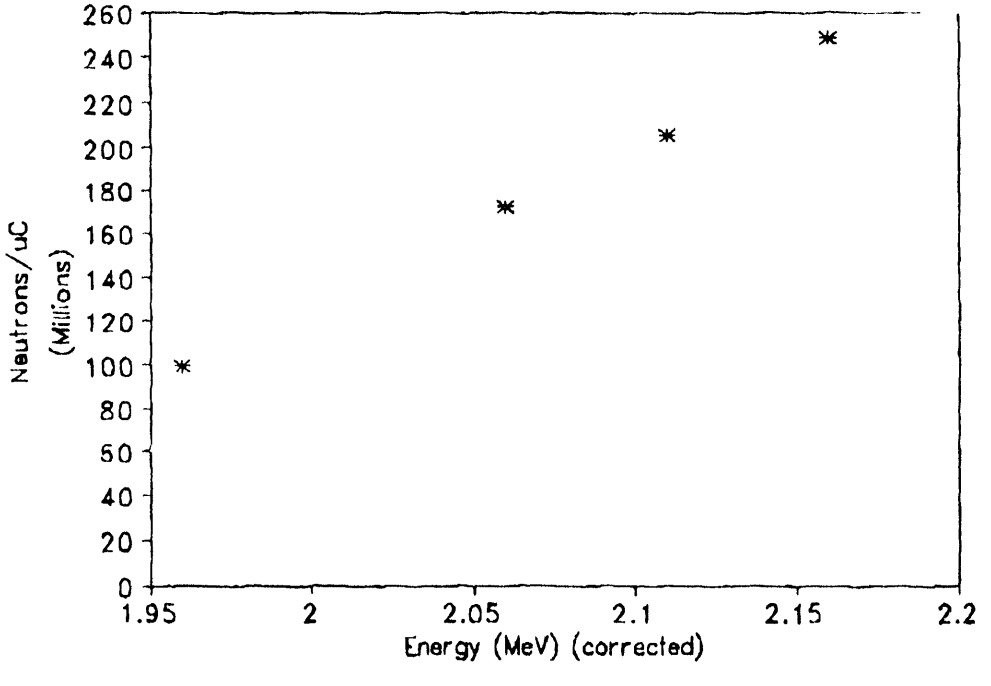

Figure 11. Neutron yield vs incident proton energy, (Li target).

TECHNICAL SUPPORT CORE ACTIVITIES

Task 1: Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) Analyses of Boron in Biological Samples

Samples received	1521
Samples prepared for analysis	~ 591
Samples analyzed	~ 510
Backlog:	
Awaiting preparation	~ 2288
Prepared, awaiting analysis	~ 158

Both ICP-AES instruments are fully operational. The new samples received this month were from ISU, WSU, and University of Utah (UofU).

Task 2: Boron Compound Purity Determinations

One batch of $Na_2B_{12}H_{11}SH$ (Na_2BSH) was received in the month of May from Callery Chemical Company. The Na_2BSH shipment contained a total of 30 grams packaged in five-gram quantities. One vial, representing Callery Lot #2351-82-1, was opened for purity investigations. Two grams of the opened vial were transferred under nitrogen to a crimp top vial for archive purposes, one gram was used for the analyses and the remainder was stored for other uses. A laboratory specific number was assigned for tracking purposes (BNCT-430). The purity of BNCT-430 was determined by HPLC, NMR, carbon-hydrogen-nitrogen-sulfur (CHNS) analysis, and ICP-AES.

HPLC Analyses: The chromatography was performed on a 250x3 mm column packed with 5 μm particles of Nucleosil® C_{18}. The mobile phase was approximately a 50% methanol, 50% water solution with 5mM tetra bulyl ammonium sulfate (TBAS) ion-pairing reagent flowing at 0.4 mL/min. The described conditions are ideal for evaluating the BSS^{-4} and $BSSO^{-4}$ component concentrations and overall sample integrity. A slower flowrate and changes in the methanol concentration enhance the separation of the $B_{12}H_{12}^{2-}$ from the BSH parent peak.

The HPLC analysis results for the new BSH sample are listed in Table 3. A sample chromatogram of BNCT-430 is displayed with the reference sample, BNCT-202, in Figure 12.

The chromatographic analysis scheme included preparation of the new sample (BNCT-430) in triplicate, an individual preparation of the reference sample (BNCT-202), and duplicate chromatograms of all samples. The sample chromatogram indicates the presence of the late eluting peaks, although at a somewhat lower level. At this time, the identity of these products is still unknown. The HPLC analyses were unclear regarding the presence of the $B_{12}H_{12}^{2-}$ component in the BCNT-430 sample. The NMR spectrum was unremarkable, but may hint at the presence of $B_{12}H_{12}^{2-}$ in the sample.

ICP-AES Analysis: Elemental analysis for boron, sulfur, and nitrogen by ICP-AES were performed on aliquots of all samples and standards prepared for HPLC. The results of these analyses are presented in Table 4. The weight percent boron, sulfur, and nitrogen for many of the samples indicated a slight over-recovery, once again demonstrating a possible error in weighing milligram quantities of the sample. The calculated mole ratios are unaffected by weighing/preparation errors. The repeatability of weighing the BNCT-430 sample appears to be quite good since the results agreed to within 1.2% (RSD). Boron and sulfur appear to be consistently over recovered.

CHNS Combustion Analysis: A nitrogen atmosphere was utilized as much as possible for preparation of the samples used in the combustion analyses. The results of these analyses are presented in Table 5. Sulfur results are not reported since they are irreproducible and usually low. The weight percent for hydrogen may provide evidence that the older sample (BNCT-202) may be "wet," most likely enhanced by its frequent handling. Carbon is also present and notable in all of the new materials, although the carbon present is lower in concentration than in previous samples.

NMR Spectroscopy: The natural abundance boron NMR of the new BSH batch indicates that BSH is the primary constituent. However, there was some possibility that $B_{12}H_{12}^{2-}$ may be

Table 3. Oxidation Products in new BSH (BNCT-430) and the Reference Sample (BNCT-202) as determined by HPLC.

Sample	Compound	BSS^{4-} (wt %)	$BSSO^{4-}$ (wt %)
202	$Na_2B_{12}H_{11}SH$	1.36 ± 0.18	0.145 ± 0.007
430	$Na_2B_{12}H_{11}SH$	0.93 ± 0.15	0.7 ± 0.1

present at low levels. The $B_{12}H_{12}^{2-}$ component was not confirmed by HPLC analysis.

Fourier Transform Infrared Spectroscopy (FTIR) Spectroscopy: The new and reference samples were prepared for FTIR analysis entirely under nitrogen. The actual quantity of sample in each pellet was not accurately determined, consequently, some pellets may have had more sample material in them than others. Thus, the spectra in Figure 13 have been normalized for clarity and to offset the sample mass differences. All single beam infrared spectra were ratioed to a single-beam spectrum of a blank KBr pellet. The spectrum of the BNCT-313 sample, used as a reference, indicates a significant presence of water represented by the peaks at ~3500 cm^{1-} and ~1640 cm^{1-}. Organic contamination is also notable in the BNCT-313 spectrum because of the peaks between 2900 and 3000 cm^{1-}. The new sample, BNCT-430,appears to be quite dry and free from significant organic contamination by comparison.

New Compound Analysis: A new batch of Na_2BSH was received late in the month of June 1992 and is currently undergoing analysis.

Task 3: Intra- and Intercellular Boron Analyses

Secondary Ion Mass Spectrometry (SIMS): No activity to report.

Sputter Initiated Resonance Ionization Spectroscopy (SIRIS): No activity to report.

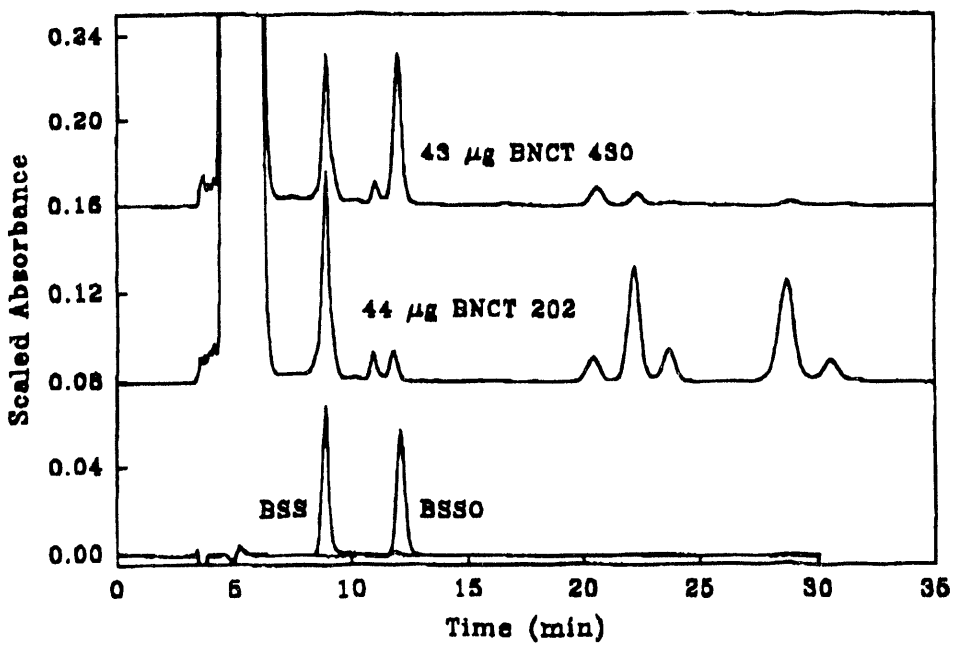

Figure 12. Reversed phase, ion-pair HPLC chromatograms of BNCT-430, BNCT-202, BSS^{4-}, and $BSSO^{4-}$.

Table 4. ICP-AES Analysis of BSH Compounds.

		Weight Percent			Mole Ratios		
Sample	Compound	S	B[a]	Na	B/S	B/Na	Na/S
202	$Na_2B_{12}H_{11}SH$	12.7	57.2	17.7	13.3	6.8	1.9
210	$Cs_4{}^{10}B_{24}H_{22}S_2$	8.8	33.4	0.58	11.8	---	---
212	$Cs_4{}^{10}B_{24}H_{22}S_2$	8.3	30.4	0.21	11.3	---	---
430A	$Na_2B_{12}H_{11}SH$	15.6	86.8	19.4	12.7	7.3	1.7
430B	$Na_2B_{12}H_{11}SH$	16.5	66.4	20.1	11.9	7.0	1.7
430C	$Na_2B_{12}H_{11}SH$	16.6	67.5	20.0	12.0	7.1	1.7

[a] All samples have been corrected for isotopic abundance.

Table 5. CHNS Combustion Analysis Results.

Sample	Weight Percent C	Weight Percent H	Weight Percent N
202[a]	0.79 ± 0.05	6.89 ± 0.27	0
430	0.26 ± 0.11	5.87 ± 0.14	0
Expected	---	5.5	---

[a] Stock vial.

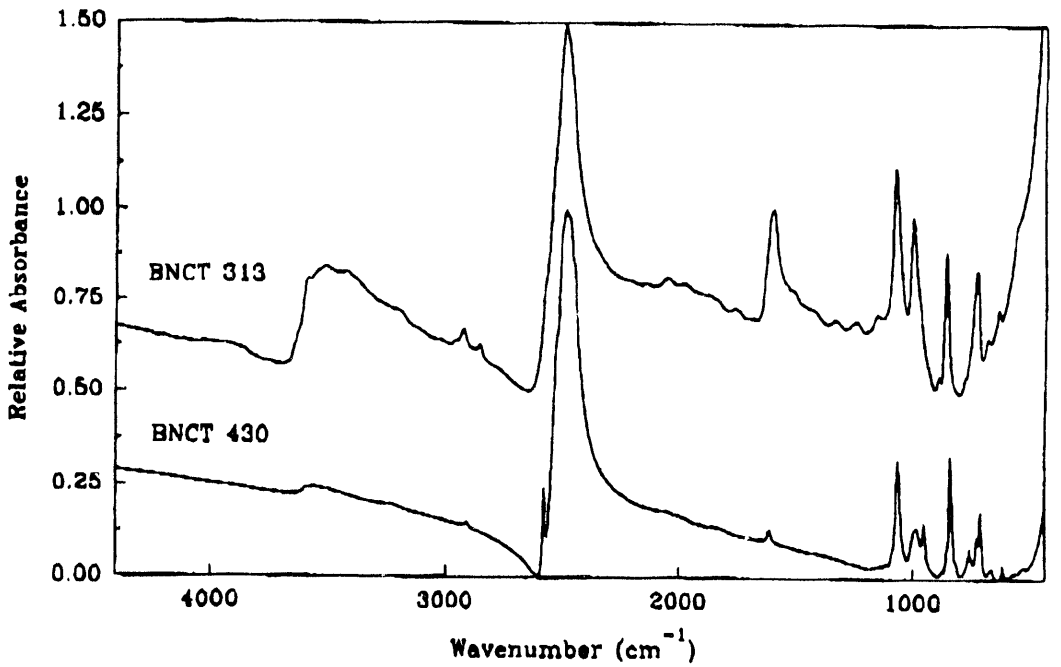

Figure 13. FTIR spectra of BNCT-430 (Na_2BSH) and BNCT-313 ($Na_2{}^{10}BSH$).

Task 4: Neutron Beam Measurement Dosimetry

Current plans include irradiation of two healthy dogs with reflected scalps at Brookhaven Medical Research Reactor (BMRR) in late July 1992. It is believed that reflection of the scalp and top head musculature will cause the position of maximum thermal neutron flux to occur deeper into the brain and reduce scalp/muscle dose so dose-tolerance effects on the central nervous system can be observed. INEL dosimetry researchers visited WSU Veterinary Department in June 1992 to observe practice scalp reflection surgery on dogs and/or dog cadavers to determine the best method for attaching and locating dosimeters, the dosimeter sterilization methods to be used, and assess the problem of positioning the dog's head for irradiation and locating fiducial marks.

Task 5: Canine Dosimetry Calculations

The canine dosimetry optimization study for four tumor dogs at BMRR was completed and documented. The results of this study will be presented at the Fifth International Symposium to be held in Columbus Ohio September 13-17, 1992. Optimization studies for rat irraditaions in the BMRR thermal-neutron beam were begun and largely completed. The results will be documented during the next reporting period. It was determined that some dose distribution improvements can be obtained by redesigning the rat holder and the beam delimiter that will be used for these irradiations.

Task 6: BNCT Database Management System

No activity to report.

Task 7: Georgia Tech Research Reactor (GTRR) Physics Support

Work on this task has been completed for this year. A paper discussing the conceptual physics design results for an epithermal-neutron filter at the GTRR was presented at the June 1992 American Nuclear Society (ANS) Annual Meeting in Boston, MA.

Task 8: Research Reactor/Accelerator Physics Support

Scoping physics calculations to explore various possible accelerator-based, epithermal-neutron source designs for BNCT were begun. Analytical methods will be benchmarked in an attempt to duplicate published results for an accelerator-based source developed at Ohio State University. Possibilities for improved systems will then be explored based on different target materials (possibly beryllium) and on different filter configurations.

Miscellaneous:

INEL BNCT Program researchers presented three invited papers at the special BNCT session held during the June 1992 American Nuclear Society Annual Meeting in Boston, MA. One paper concerned the proposed epithermal-neutron filter at GTRR, the second covered macroscopic tissue heterogeneity work (also to be published in journal article form in the June 1992 issue of *Medical Physics*), and the third covered radiobiological compound factor measurements and calculations.

Australian Collaboration: MRI images of the Florida patient, the recipient of BNCT treatment in Japan in May 1991, were received and processed into a form suitable for input to the bnct_edit medical image reconstruction software. Information has also been obtained on the Japanese thermal-neutron beam used for the BNCT treatment on this patient. Receipt of the images and the beam information will allow researchers to perform detailed posttreatment dosimetry calculations for this patient using the INEL analytical dosimetry software system, providing valuable beta testing data at minimal cost to the INEL BNCT Program.

European Collabortion: Preliminary arrangements for transfer of a developmental version of the INEL radiation transport software (rtt MC) module of the analytical dosimetry software system to the European Collaboration were initiated. This will involve a cost-sharing arrangement between INEL and the European Collaboration. Under the existing export au

thority, INEL can provide a binary developmental version of this module. The European Collaboration will be willing, in principle, to share in travel and incidental costs. INEL will also assist the European Collaboration in arranging for a use license for the bnct_edit reconstruction software, part of which (mainly the spline mathematics routines) is still proprietary to the UofU.

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