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The pharmacokinetics and toxicology of a boronated porphyrin in dogs

Author
Tibbitts, Jack,

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The Pharmacokinetics and Toxicology of a Boronated Porphyrin in Dogs

by

Jack Tibbitts

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of the

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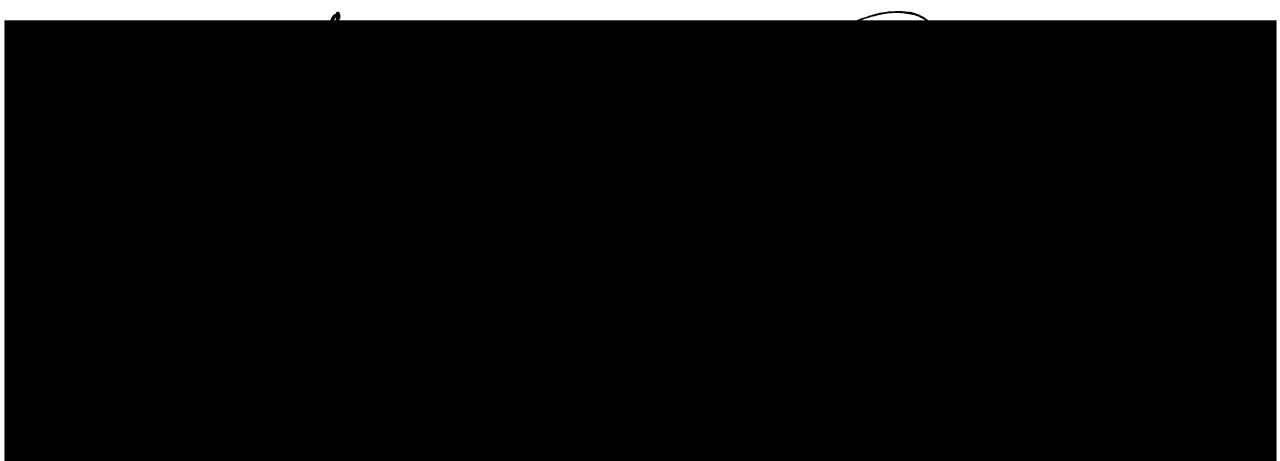

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Preface

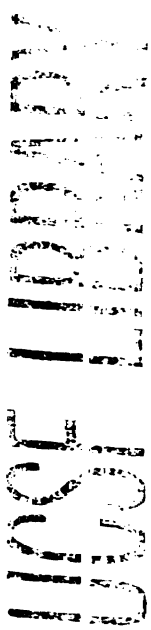
This document, and the work described herein, was possible only with the assistance and guidance of a number of people both here at UCSF and around the country. I would like to begin by thanking Dr. Svein Øie for his assistance in designing the pharmacokinetic studies and for providing suggestions toward making the studies more meaningful. Dr. Nancy Sambol was pivotal in interpreting the pharmacokinetic data and steering me through the tortures of NONMEM analysis. Dr. Joseph Zinkl, of the UC-Davis College of Veterinary Medicine, provided expert insight into the clinical pathologic data. The hemostatic analysis was made much easier with the assistance of Dr. Marjory Brooks of the Comparative Coagulation Section at Cornell University. Dr. Andy Bollen was a patient mentor in reviewing the tremendous number of histopathology samples. Dr. Glenn Gobbel provided not only his time and effort, but the use of much of his lab space to perform the cell culture and fluorescence microscopy experiments. Without his assistance, much of this work would not have been possible. Dr. Dennis Deen and the folks in his lab were generous with their advice and the use of their equipment. Dr. Meden Isaac, Larry Wainschel and especially Theresa Seilhan were those all-important extra hands that made the dog studies possible and a whole lot easier. My two principal advisors, Drs. John Fike and Stephen B. Kahl, were both supportive and generous. They were always available for discussion and provided valuable guidance in matters scientific and beyond. I learned a tremendous amount from all of the people listed above, but the most from my two "bosses".

While the direct support of this work was important, Suzanne Russo provided both direct and indirect support to make this work possible. From helping me with dog studies in the wee hours of the mornings to being my best cycling partner, I couldn't have done it without her. She made a lot of sacrifices to make this possible, and I appreciate all of them. I'm glad I married her.

The Pharmacokinetics and Toxicology of a Boronated Porphyrin in Dogs.

Jack Tibbitts

Abstract: The genesis of this work was based on the promising results reported by Hill et al.[101] suggesting that BOPP, a boronated porphyrin under study for use in binary therapy, accumulates in brain tumors following systemic administration. To extend that work, these studies were performed to determine the pharmacokinetics and toxic effects of a 35 mg/kg dose of BOPP to dogs, a commonly used model in binary therapy studies. Following administration, plasma and tissue boron levels were measured at various time points and clinical and pathologic responses were monitored. BOPP was clinically well-tolerated at the administered dose although cephalic vein thrombosis was noted in some subjects. Plasma boron concentrations followed tri-exponential pharmacokinetics with a rapid but limited distribution and a large depot compartment resulting in a long terminal half-life. Tissue distribution studies suggested that the depot compartment is most likely the liver, with high concentrations of boron also found in the lymph nodes, spleen and adrenals. Low concentrations were found in most cranial tissues, with the exception of the carotid artery which showed porphyrin accumulation in the outermost layers of the vessel. Histopathologic evaluation of tissues found mild hepatocellular damage in 2/16 dogs and resolving cephalic thrombosis in 6/16 dogs. Cell culture studies with BOPP revealed that concentrations above 0.5 mg/ml for periods greater than 24 hours can cause endothelial cell injury, and may be involved in the formation of thrombosis. Some abnormalities in the hemostatic parameters were found following BOPP administration, inclucing prolonged prothrombin times and decreased fibrinogen levels, but no subject showed clinical evidence of systemic coagulopathy. *In vivo* and *ex vivo* studies of these phenomena suggested that



BOPP affects fibrinogen activation. BOPP was found to bind to fibrinogen, suggesting an interference with the action of thrombin on fibrinogen. The low toxicity of BOPP coupled with the favorable pharmacokinetics and tissue distribution provide evidence that BOPP is a promising compound for use as a sensitizing agent in binary therapy. Further study of the hemostatic effects and the accumulation in arterial tissues are indicated.

Stephen B. Kahl

Thesis advisor and committee chair

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CHAPTER 1.
PHARMACOKINETICS AND CLINICAL TOXICOLOGY

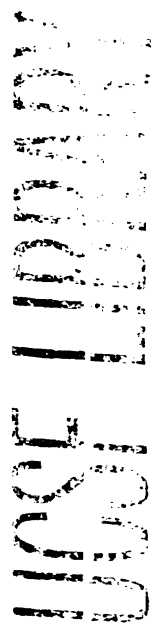
1.1 INTRODUCTION:

While the treatment of cancer has shown great advances over the past several decades, the treatment of malignant brain tumors has not progressed as rapidly. The mortality rate for primary malignant brain and other nervous system tumors has increased over the span of 1973-1993[1]. In addition, the five-year survival rate for patients with glioblastoma multiforme and anaplastic astrocytoma, which are the most malignant forms is reported to be 3.4 and 28%, respectively[1]. Primary brain tumors are also the second leading cause of cancer death in children[2], striking over 1200 children in the U.S. from 1990-1993[1]. The current therapy for brain tumors generally consists of surgical resection coupled with radiation and/or chemotherapy[2]. Since malignant brain tumors are highly invasive, surgery alone is not satisfactory for the removal of the entire tumor thus requiring the ancillary therapies to improve the elimination of cancerous cells. Unfortunately, this approach has led to only minor improvements in survival[2] and has precipitated an exhaustive search for alternatives.

One of these alternatives has been binary therapy. Binary therapy can be defined as the administration of a relatively inert and benign sensitizing agent followed by activation of the sensitizer to a cytotoxic agent using local administration of an activating agent. Two modes of binary therapy are photodynamic therapy (PDT) and boron neutron capture therapy (BNCT). The goal of binary therapy is to administer the sensitizing agent, usually a chemical compound, and allow it to localize predominantly in the target tissue, most commonly a tumor, but also other pathologies characterized by cellular proliferation such as macular degeneration[3]. With high concentrations of sensitizer in the target and low levels in the surrounding tissue, the activating agent can be administered to that region containing the target tissue. The advantages of a "binary strategy" are three-fold: (1) the sensitizing agent and the activating agent are of low toxicity by themselves, thus avoiding systemic toxicity due to treatment; (2) the individual therapies can be manipulated independently to

optimize their effectiveness; (3) since the activating agent is applied only in the area that contains the target tissue, the damaging effect is localized to that area.

In PDT, the sensitizing agent is a light absorbing chemical (a photosensitizer) which, when exposed to the activating agent, causes a photoreaction in the tissues in which it is present. The activating agent is light administered locally to the target tissue containing the photosensitizer. The result is the excitation of the photosensitizer to an excited singlet energy state followed by an intersystem crossover to a triplet energy state. The molecule in the triplet state can undergo a subsequent photoreaction and transfer an electron or hydrogen atom (Type I) or energy (Type II). The transfer of energy (Type II) to molecular oxygen (O_2) and the formation of singlet oxygen is thought to be the important mechanism for tissue damage in PDT. Type I transfers are also thought to occur and produce superoxide anions, hydroxyl radicals and O_2^-[4]. These products are short lived and are produced at the site of the photosensitizer reaction; thus the highly reactive nature of these products leads to tissue damage in a very limited area (radius 1-2μ)[5]. The intracellular damage associated with PDT can affect many parts of the cell and may vary depending on the precise subcellular localization of the photosensitizing agent. The mitochondria, cellular membranes and DNA have all been shown to be damaged as a result of PDT.[6] An important aspect of PDT success is damage to the vascular supply of the target tissue. Extensive damage to the local vasculature results in reduction in blood supply to the target tissue and decreased viability in that tissue[7, 8]. Thus direct damage to target tissue combined with damage to the vascular supply to that tissue lead to selective destruction of the target tissue.



BNCT utilizes a (^{10}B) boron-containing sensitizing agent followed by the activation by a low energy thermal neutron beam. Upon activation of the boron by the neutron beam, the boron nucleus absorbs a neutron (n_{th}) and undergoes a prompt fission reaction to release high energy particles (Figure 1.1).

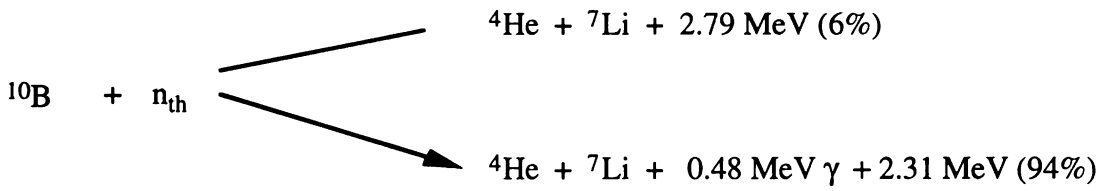


Figure 1.1.

These particles, a helium nucleus (^{4}He) and a lithium nucleus (^{7}Li), travel a very short distance (~10μ) but cause a large amount of damage in their path. This damage is due to the high amount of energy released by these particles. The alpha particles liberated in this reaction produce 200 keV per micron of their path[9]. The damage to cellular components, especially double stranded breaks in DNA, is sufficient to kill the cell. This damage is related to the location and concentration of the ^{10}B within the cell, thus higher concentrations of boron in regions of the cell critical to its survival (nucleus, mitochondria) will result in more efficient cell damage.

Binary therapy relies fundamentally on the quality of the sensitizing agent. The ideal agent should localize in high concentrations in the target tissue, with concomittantly low concentrations in surrounding tissues. Also, the agent should be of low toxicity at therapeutic doses when administered alone. For PDT, the sensitizing agent should have a high triplet quantum yield (relative amount of excited molecules that achieve triplet energy state) and a long half-life in the triplet state[10]. The compound should have adequate absorbance of light energy at a wavelength of 500-700 nm to take advantage of an increased tissue penetration of light in this range[10]. For BNCT, the sensitizer should have a high boron-carrying capacity so that the number of sensitizing molecules required to be taken in by the cell to cause cell damage upon activation is minimal. Since the actual sensitizing agent in BNCT is the ^{10}B atom, a wide array of carriers of boron have been designed to optimize the target tissue uptake of the sensitizer[11]. This allows for more flexibility by using a sensitizer that is best suited for accumulation in a particular target.

The history of PDT can be traced back to as far as 1400 BC, but the modern history of photodynamic therapy is attributed to the pioneering of the scientific concept of

photosensitization by Raab[12], who discovered the increased toxicity of acridine on paramecium in the presence of light. In 1903 Raab's mentor, von Tappeiner, collaborated with Jesionek using topical and intratumorally injected eosin as a photosensitizer followed by sunlight to treat skin tumors in humans. von Tappeiner published a book in 1907 and therein used the term "photodynamic therapy" to describe such treatment[12].

Porphyrin-based photosensitizing agents have a shorter history. Hausmann described the use of hematoporphyrin, which had been discovered in the 1840's, in mice and noted photosensitivity associated with its administration and subsequent light exposure. The localization of porphyrin compounds to tumors was noted by Policard in 1924[12] and Auler and Banzer[13] described the ability of hematoporphyrin to exhibit photodynamic action in tumors. Rasmussen-Taxdal et al.[14] demonstrated uptake of hematoporphyrin in a variety of carcinomas in humans following intravenous administration. At that time, Schwartz et al.[12] found that "hematoporphyrin" was a heterogeneous mixture of porphyrins with a range of chemical properties. Subsequently, Schwartz et al.[15] were the first to produce a more purified form of hematoporphyrin. Unfortunately, the pure hematoporphyrin was found to be a poor tumor localizer. In their effort to isolate the more active tumor localizing fractions of the impure hematoporphyrin, Schwartz et al.[15] attempted to extract non-hematoporphyrin compounds with ethyl acetate/acetic acid followed by sulfuric acid. The resulting product improved tumor localizing ability over hematoporphyrin[12], and the method for the production of this new product was subsequently published by Lipson and Baldes[16]. The currently used method for producing this product, called hematoporphyrin derivative (HpD), is the treatment of hematoporphyrin with a 19:1 mixture of acetic/sulfuric acid[17] followed by treatment with base[10]. HpD is a complex mixture of dimers and oligomers. Lipson et al.[12] subsequently did much of the pioneering work on the use of HpD for the detection and eventually the treatment of cancer[18].

Hematoporphyrin and HpD have become the model PDT sensitizing agents and have been used by Dougherty and other groups to study the efficacy of PDT in both human and animal tumors[12]. Dougherty et al.[19] found that the administration of HpD and red light could produce long-term cures in mice and rats bearing several types of tumors. This work was followed by human clinical trials with HpD in 1978. Diamond and colleagues were the first to demonstrate the effectiveness of hematoporphyrin in the destruction of brain tumor cells, both *in vitro* and *in vivo*[20, 21]. Perria et al.[22] first used photodynamic therapy to treat human gliomas and later work by Kaye et al.[23] demonstrated good uptake and retention of HpD in cranially implanted rat gliomas with minimal HpD uptake by normal brain. Hill et al.[24] showed a similar selectivity of HpD for brain tumors in humans. HpD has continued to be used successfully in the photodynamic therapy of a wide variety of human tumors[12]. The progress in the use of PDT for brain tumors has been extensively reviewed by Kaye and Hill[25].

More recently, an enriched fraction of HpD called Photofrin® has been prepared by isolating a mixture of dihematoporphyrin ethers and esters that are responsible for HpD's photosensitizing properties[6]. Photofrin has currently been approved for use in the U.S. in the photodynamic therapy of esophageal cancer. Photofrin is not without its limitations; weak light absorbtion at long light wavelengths (wavelengths above 600 nm allow better tissue penetration) and prolonged photosensitivity are among them. Thus, new compounds with better light absorbance properties and fewer toxicities have been developed. These compounds are highly varied and include porphyrins, purpurins, chlorins and phthalocyanines[6]. Most of these compounds have been classified as the second-generation of photosensitizers[6]. Several porphyrin-based compounds have been studied in animals. Benzoporphyrin derivative mono-acid ring A (BPD-MA) has been the subject of extensive study for use in the treatment of cancer[26-28], atherosclerosis[29, 30] and macular degeneration[3]. *Meso*-tetra(hydroxyphenyl)porphyrin (m-THPP) has shown promise in the treatment of mammary carcinoma[31], glioma[32] and plasma cell tumors[33]

in mouse models. Tetraphenylporphine sulfonates (TPPS) have also received attention due to their water solutility and tumor localization[34]; however, these compounds demonstrated neurotoxicity[35, 36]. Success in the use of 3,1-*meso*-tetrakis(*o*-propionamidophenyl)porphyrin (3,1-TPro)[37], and meso-tetra(4N-methylpyridyl)porphine[38] in animal tumor models were also reported. The naphthalocyanine bis(di-isobutyl ocatadecylsiloxy)silicon 2,3-naphthalocyanine has been used to treat urothelial-based tumors in rats[39]. Success in veterinary clinical studies has been reported using the chloroaluminum phthalocyanine tetrasulfonate ($AlPcS_4$) to treat tumors in several species[40, 41]. And mono-L-aspartyl chlorin e6 (NPe6) has demonstrated efficacy in mammary carcinomas in mice[42, 43].

BNCT has a shorter history than PDT. Following the discovery of neutrons by Chadwick in 1932, Fermi observed the ability of certain nuclei to capture slow neutrons[44]. This capture can be briefly defined as the ability of the nucleus to absorb a neutron that passes through it[45]. Subsequent work showed that the ability of nuclei to capture neutrons was related to their structure and that ^{10}B possessed an exceptionally large capture cross-section[45]. This capture cross-section is related to the nuclear structure of the element, and indicates the ability of this element to capture neutrons[45]. Taylor, in 1935[46], demonstrated the capture of neutrons by ^{10}B nuclei followed by the fission reaction shown in Figure 1.1.

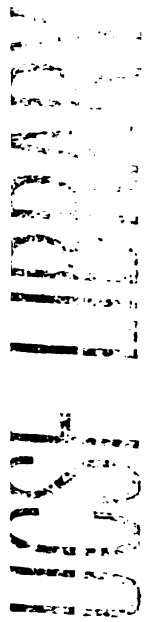
The medical applications of this phenomenon were defined by Locher[45], who outlined the method for binary therapy using boron as the sensitizing agent. Kruger[47] and Zahl[48] followed the work of Locher with studies on mouse tumors using boric acid and other boron compounds injected directly into the tumors. Those studies demonstrated the ability of neutron capture to hinder tumor growth and progression, but were severely limited by poor neutron sources and the need for a sensitizer that could be administered systemically.

The construction of the Brookhaven Graphite Research Reactor in 1950[44] and the work of Sweet, Farr and colleagues in the 1950's rekindled interest in BNCT. Using thermal neutrons produced at Brookhaven and ^{10}B enriched borax as the sensitizing agent, those investigators undertook a number of studies in human volunteers with cerebral gliomas but the results of their treatment were no improvement in survival over those patients that were untreated; but some improvement in clinical condition was noted along with histopathologic evidence of damage to the tumor[49, 50]. In reviewing these studies, Slatkin[44] suggests that the major factors limiting the success of these early clinical trials were the lack of a neutron source that could provide enough neutrons for therapy, the inability to accurately and rapidly determine blood and tissue boron concentrations (which limited treatment planning), and the lack of boron-containing sensitizers that localize specifically to brain tumors. In the late 1950s and into the 1960's, Soloway and colleagues addressed one of these limitations by investigating 125 boron containing compounds for toxicity and uptake into tumors[51, 52]. Clinical studies of the most promising of these compounds, $Na_2B_{10}H_{10}$, proved unsuccessful[53] and the failure was thought to be the result of high blood levels of boron at the time of neutron treatment resulting in vascular injury. In 1967, Soloway et al.[54] reported the use of a boron compound containing a sulfhydryl group, $Na_2B_{12}H_{11}SH$, in mice bearing subcutaneous ependymoblastomas. This compound exhibited improved tumor binding, promising tumor-to-blood and tumor-to-normal brain differentials and low toxicity in comparison with previously studied boron compounds. This compound was named BSH and became the benchmark sensitizing agent for many BNCT studies.

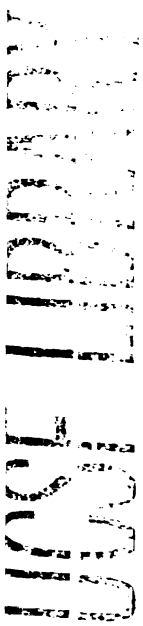
The discovery of BSH and its potential as a sensitizing agent led to its use in human clinical trials. Much of this work was performed beginning in 1968 by Hiroshi Hatanaka and colleagues in Japan. Over the next 20 plus years, Hatanaka and colleagues treated nearly 150 human patients with malignant glioma using a combination of BSH and a thermal neutron beam[55]. Those results were very promising, but controversial due to the

lack of adequate documentation and controls. Nakagawa[55] reported 2+ year survival rates following BNCT of approximately 9 and 55% for glioblastoma and anaplastic astrocytoma, respectively. The patients in these studies were treated with BNCT 1-2 weeks following surgical debulking of the tumor, and no other ancillary therapy was used[55]. The survival rate seen by Nakagawa is similar to the overall survival rate for these tumors reported by CBTRUS[1] (8.5 and 44%). A recent review of some of the patients treated by the Hatanaka/Nakagawa group from 1987-1994 reported no improvement in survival when compared to conventionally treated patients[56]. However, these studies do not take into consideration more recent advances in BNCT such as improved neutron beams and sensitizer administration strategies. Regardless, the success of Hatanaka and his colleagues led to an increased interest in BNCT, and in BSH in particular[11].

More recently, clinical studies have begun using another sensitizing agent, boronophenylalanine (BPA), in the treatment of glioblastoma multiforme[57, 58]. These studies are still in the preliminary stages, but BPA has demonstrated a low toxicity at clinically relevant doses and the response to therapy has been found to be related to dose delivered to tumor. While these studies have shown the promise of BNCT, limitations have been elucidated with respect to perfecting the BNCT technique. These limitations have had two main foci; the need for improvements in neutron beam penetration and purity, and the improvement in tumor localization of boron-containing sensitizing agents. Currently, the best sources of neutrons are nuclear reactors which makes the widespread use of BNCT problematic due to the expense and scarcity of appropriate sources. Advances in beam technology have led not only to improvements in the purity, power and understanding of neutron beams produced by conventional methods, but also to other methods of beam production (e.g. proton accelerators). In addition, great strides have been made in the field of neutron dosimetry, allowing for better predictions of the mechanisms of, and treatment planning for, BNCT[59]. The other focus of attention in BNCT has been the development of new and more tumor-specific sensitizing agents.



Central to the discussion of PDT-mediated tumor destruction is the mechanism of tissue uptake of PDT sensitizing agents. Since selective uptake into tumor tissues is highly desirable in PDT (and BNCT) sensitizing agents, understanding the details of this uptake is important in predicting the potential for use of many sensitizing agents. There are several theories regarding cellular or tissue uptake of such agents. One of the most interesting is the selective uptake of porphyrins bound to lipoproteins. Porphyrins are the most commonly used PDT sensitizers, and when administered systemically in animals, they are found to be highly plasma-protein bound[60, 61]. The proteins to which porphyrins bind are dependent on the structure of the porphyrin[61-64]. In general, more hydrophilic porphyrins are thought to associate with albumin while more lipophilic porphyrins associate to a higher degree with lipoproteins[62, 65]. Other factors such as charge distribution[62] and the aggregation state of the porphyrin[66] may also be important. The binding of porphyrins to lipoproteins has led to the theory that lipoprotein-mediated uptake into tumor cells may be a mechanism by which porphyrins selectively accumulate in cancerous tissue. Tumor cells are known to express increased levels of lipoprotein receptors on their surface, in particular low density lipoproteins (LDLs) and have an elevated uptake of lipoproteins when compared to normal cells[67, 68]. This theory is also supported by evidence that the normal tissue distribution of administered porphyrins tends to be concentrated in those tissues which normally express high levels of LDL receptor (liver, kidney, adrenal)[68-71]. Another mechanism for tissue uptake of porphyrins involves the binding of porphyrins to stromal elements in the tissues. Porphyrins have been found to bind to elastin, collagen and fibrin which are present in tumorous tissue[72]. Many tumors contain a large amount of interstitial fibrin and collagen which may allow for the binding of significant quantities of porphyrins. The question whether this binding causes enhanced efficacy due to uptake into cells from this depot or decreased efficacy due to sequestration of sensitizer in the interstitium has not been answered. Finally, a more recent proposal has suggested that the uptake of porphyrins by normal and tumorous



tissues may be related to the uptake of lipoprotein-porphyrin conjugates by phagocytic scavenger cells[73]. This may have advantageous or detrimental effects on tumor damage, depending on the effects of photoactivation on the phagocytic cells.

An important part of the knowledge base for any therapeutic compound is its toxicity in experimental subjects. While data are available regarding the therapeutic utility of porphyrin-based sensitizing agents, little is published concerning their toxicity. However, porphyrias, the diseases associated with abnormal accumulations of endogenous porphyrins, have been the subject of an extensive literature. These diseases are the result of a range of metabolic disorders in the heme biosynthetic pathway[74]. The hallmark clinical manifestation of porphyrias is cutaneous photosensitivity, but effects on the hematologic, hepatic and neurologic effects are also seen[75]. The type and degree of toxic effects are dependent on the species of accumulating porphyrin, which is in turn dependent on the step in the heme biosynthetic pathway that is affected. Reviews by Pimstone[75], Werman[74] and Moore[76] provide greater detail on these diseases.

Therapeutic porphyrins can be expected to share some of the toxicities seen with porphyrias, but may have other toxicities not commonly seen with endogenous porphyrins. Therapeutic porphyrins are commonly found to cause cutaneous photosensitivity[77-79], similar to porphyric diseases. Additionally, other types of toxicities have also been observed, specifically hepatic and hematologic effects. Kahl et al.[80]found that a boronated tetraphenylporphyrin containing open (nido) carborane cages (BTPP) administered to mice caused moderate hepatotoxicity as reflected by plasma enzyme level changes and histopathologic evidence of liver damage. In addition, BTPP increased the risk of thrombocytopenia in these mice. Miura et al.[81] administered a nidocarboranyl deuteroporphyrin (VCDP) to mice and demonstrated thrombocytopenia with some suggestion of hepatic injury. In another study by Miura[82], mice were treated with a nickel-containing porphyrin (NiTCP) and showed little or no toxicity at times up to 3 months. Hunt et al.[83] described an increase in circulating leukocytes and lymphocytes

following treatment of mice with Photofrin. Sima et al. also found that $TPPS_4$ administration at high doses (150 mg/kg) caused immediate motor nerve conduction abnormalities followed by pathologic changes in peripheral nerves at >30 days post-treatment. Winkelman and Collins[35] reported histologic evidence of neurotoxicity with $TPPS_4$ doses as low as 5 mg/kg. Neither investigator reported clinical evidence of neurotoxicity.

Severely toxic doses have been reported for some PDT sensitizing agents. Dougherty[78] reported LD_{50} levels (in darkness) of 275 mg/kg at 24 hours for HpD administered intraperitoneally (IP) to mice. At 14 days, the LD_{50} was 230 and 180 mg/kg for male and female mice respectively. He also reported that dihematoporphyrin ester (DHE) had an LD_{50} of 130 mg/kg. Death in all cases was found to be due to liver and kidney necrosis with accompanying splenic and thymic lymphocyte necrosis. With exposure to light, the LD_{50} values for HpD were much lower (7.5 mg/kg at 24 hrs and 14 days). Cause of death in this study could not be determined. Sima et al.[36] found that 150 mg/kg HpD administered IV caused death in 6/8 mice in the absence of light and that > 400 mg/kg of tetraphenylporphine tetrasulfonate ($TPPS_4$) (IV) caused death in all mice treated. Bianchi et al.[84] reported an acute LD_{50} of 119 mg/kg IP for Photofrin in mice. While these data are limited to those doses causing extreme toxicity, it is likely that porphyrin doses below 100 mg/kg given to rodents in the absence of light are not likely to cause such effects. The exposure to light following porphyrin administration may drastically increase the incidence of toxicity.

Most other studies using porphyrin-based compounds have not reported evidence of toxic effects at the dose and time the animals were studied. It is unclear whether this is reflective of use of sub-toxic doses in these studies, or the failure to adequately monitor for sublethal toxic effects. More extensive studies of clinical toxicity have been performed with hematin, the oxidative metabolite of heme. Hematin has been used in humans to treat porphyrias and has shown numerous toxic effects. Phlebitis, thrombocytopenia, and

coagulopathies have been noted following hematin treatment[85]. In summary, due to the tremendous diversity in the physical and chemical characteristics of porphyrin-based compounds, it is logical to predict that the toxic effects associated with each compound may be unique both in degree and type. Therefore, as each compound approaches clinical use, extensive study will be necessary to determine each compounds distinct toxicologic profile.

A knowledge of the pharmacokinetics and tissue distribution of sensitizing agents is important in treatment planning for both PDT and BNCT. For BNCT, it is of great importance to know which normal tissues contain significant quantities of boron so that neutron activation can avoid those tissues. The concentration of the sensitizing agent in the tumor and its periphery is of equal importance in PDT. Pharmacokinetic data can help in planning the timing of the light or neutron activation by allowing some prediction of the plasma concentration of the sensitizing agent. The pharmacokinetics of sensitizing agents, and in particular porphyrins, have not been extensively studied. Most of the published work has been carried out using mice. Bellnier et al.[69] found serum elimination of Photofrin II in mice to be tri-exponential with the terminal half-life ($t_{1/2}$) of 220 hours following IV injection. (A brief explanation of pharmacokinetic parameters is available in Appendix I). Pantelides et al.[71] reported a bi-exponential elimination of Photofrin II in mice following IV injection with a terminal $t_{1/2}$ of 27 hours. Differences in mouse strain and Photofrin analysis method may explain the disagreement in results. Lindsay et al.[32] used the tumor sensitizer m-tetra hydroxyphenyl porphyrin (m-THPP) in mice to find a terminal $t_{1/2}$ of 96 hours. Richter et al.[86] examined the pharmacokinetics of benzoporphyrin derivative administered IV to mice and found a terminal $t_{1/2}$ of 8 hours. Using rats and hematoporphyrin derivative, Gervais et al.[87] found a terminal $t_{1/2}$ of 25 minutes with a volume of distribution (V_d, Appendix I) of 53.7 ml/rat. Brown et al.[88] administered Photofrin to human patients IV and found a terminal $t_{1/2}$ of 452 hours and a V_d of 0.4 L/kg. Due to the wide variation in analytical methods, compounds administered,

and strains and species used in these studies, it is difficult to predict the pharmacokinetic behavior of a new porphyrin.

The tissue distribution of porphyrins has been studied more thoroughly than their pharmacokinetics. Reports of tissue distribution of porphyrins has included the study of endogenously produced porphyrins, such as coproporphyrin, uroporphyrin and protoporphyrin, and several porphyrin-based therapeutic agents. Zawirska[88-93] studied the distribution of endogenously produced porphyrins in humans with various disease states and found that they tend to deposit in tissues such as the liver, adrenals, kidney (especially the cortex) and occasionally the bone marrow. Evensen et al.[94] and Gervais et al.[87] found spleen, kidney, liver and lung contained the highest ^{3}H levels following intravenous administration of tritiated HpD to mice and rats, respectively. Richter et al.[86] found similar results using ^{3}H benzoporphyrin derivative-monoacid ring A (BPD-MA) in mice. The administration of the relatively hydrophilic tetraphenylporphinesulfonate ($TPPS_4$), resulted in high levels in the kidney and liver[95]. A study of tissue levels of HpD, hematoporphyrin (Hp), and several pure, monomeric porphyrins of varying saline/2-octanol partition coefficients 24 hours after administration to mice found that liver, spleen, and kidney had the highest porphyrin concentrations in 10/13 tested compounds[96]. Using ^{14}C Photofrin II, Bellnier et al.[69] found that the label distributed heavily into liver, spleen, adrenal, pancreas and bladder prolonged accumulation of ^{14}C in liver, spleen and adrenal for periods of 15 days. Pantelides[71] reported similar results. A small number of studies have looked at boronated porphyrin compounds. VCDP (a nidocarboranyl containing porphyrin)[81] and BTPP (a boronated tetraphenylporphyrin)[80]were found to localize at high levels in liver, spleen and kidney and similar findings were reported for BOPP (a boronated protoporphyrin) in rats[97].

Information on the pharmacokinetics and tissue distribution of therapeutic pophyrins in large animal models is particularly scarce. Only Smetana[98], in 1938, has published data on the plasma concentrations of a porphyrin-based compound in large

animals. That study found that hematoporphyrin exhibited multiexponential plasma kinetics in the rabbit and dog. Also, hematoporphyrin was eliminated largely in the bile. Steichen[99] reported some data on the tissue distribution of ^{3}H-HpD in neonatal beagles, finding high levels in the liver and low levels in normal brain tissues at 48 hours after administration. An important step toward the clinical use of porphyrins is the examination of the pharmacokinetics and tissue distribution of potentially therapeutic compounds in a large animal model. As can be seen from the above small animal studies, each porphyrin compound has a unique pharmacokinetic profile and extrapolations from one compound to another with a similar chemical structure are difficult. And there are even significant differences in the pharmacokinetic parameters obtained using the same compound in the same species. Clearly, more work on the pharmacokinetics and tissue distribution of porphyrin compounds is necessary to allow better predictions of their behavior in humans and possibly establish a representative model species for testing these compounds.

A compound currently under study for both PDT and BNCT is the boronated protoporphyrin BOPP[100] Figure 1.2.

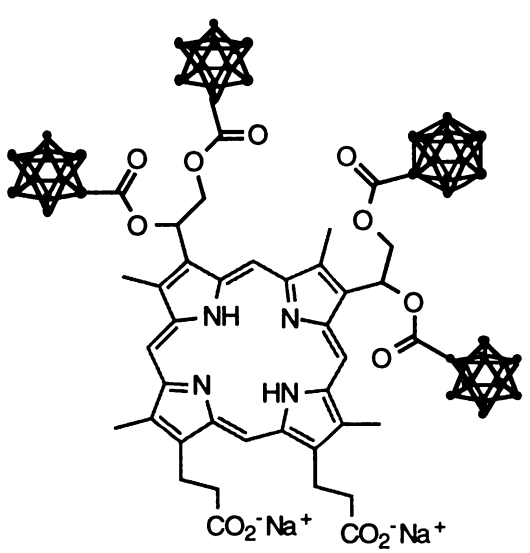
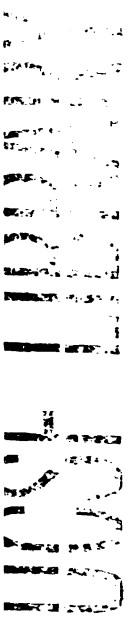


Figure 1.2. BOPP disodium salt. Carborane molecules indicated by 

BOPP has spectral absorbance properties comparable to HpD[101] and has potential use in PDT. BOPP is ~30% by weight boron and is of great interest as a BNCT sensitizer. Small animal studies have shown that this compound exhibits excellent uptake into brain tumors with little uptake into surrounding tissue[101]. In that study, BOPP showed no apparent toxic effects in mice at doses ≤200 mg/kg and more extensive toxicologic studies of BOPP in small animals are currently being undertaken. Two papers have reported pharmacokinetic data for BOPP. Hill et al.[101] found an initial $t_{1/2}$ of approximately 10 hours using 10 or 100 mg/kg doses of BOPP in mice. Using rats, Ceberg[97] found biexponential pharmacokinetics associated with blood boron levels and calculated an initial $t_{1/2}$ of 1.6 hours and a terminal $t_{1/2}$ of 17.3 hours. This thesis project has concentrated on the effects of BOPP in a large animal model, the dog. Very little is known about the effects of therapeutic porphyrins in large animal models. Few studies have been published and most of these studies reported no evidence of toxicity[77, 99, 102-106]. Tochner et al.[107] found that dogs treated with Photofrin and intraperitoneal light treatment showed transient elevations in liver transaminases and decreased lymphocyte counts. In addition, those animals also exhibited some mild hepatic pathology. Peaston et al.[40]treated cats bearing nasal or aural squamous cell carcinomas with aluminum phthalocyanine tetrasulfonate ($AlPcS_4$) and found that some cats vomited and one cat suffered a severe hepatic crisis following treatment. This limited number of studies has suggested some toxicities that may be expected following photosensitizer administration, but compound-specific experiments using large animals appear to be vital.

In the present study we evaluate the clinical and pathologic effects of a 35 mg/kg IV dose of BOPP at times ranging from 1 to 28 days following intravenous (IV) administration. We have selected the dog as a model to study the pharmacokinetics and tissue distribution of BOPP. The dog is a good candidate for these studies due to its large size and similar physiology to humans. The dog is also an excellent candidate for studies involving tumor-bearing subjects due to its size which provides ample distance between

peripheral tumors and other body parts during activating agent administration. Unfortunately, a reproducible, technically practical brain tumor model is not available in the dog. Nevertheless, dogs with spontaneous brain tumors have been used extensively[108-111] in BNCT studies and are currently the preferred large animal model in those types of studies. The dog is a commonly used model in pharmacokinetic and toxicologic studies of therapeutic agents, and thus has a well-characterized physiology.



1.2 MATERIALS AND METHODS:

Chemicals

The disodium salt of BOPP (Na_2BOPP) was synthesized, as described previously[112], by M.S. Koo in the Kahl laboratory. BOPP solutions were prepared by dissolving the required dose (35 mg BOPP/kg body weight) in 490 ml of 0.45% NaCl (Baxter, Deerfield, IL) and 10 ml 8.4% $NaHCO_3$ (Abbott Labs, North Chicago, IL). The final pH of the solution was 7.6. BOPP solutions were protected from light by wrapping fluid bags in aluminum foil and were used within 2 hours of preparation. Control solution consisted of 490 ml of 0.45% NaCl and 10 ml 8.4% $NaHCO_3$.

Animals

Seventeen adult male beagle dogs, 1-2 years of age were used. Seven of the dogs had received focal cerebral ^{125}I irradiation 8-10 weeks previously[113]. Dogs were housed and cared for in accordance with the United States Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and institutional guidelines for the care and handling of laboratory animals.

Treatment Protocol

Subjects were fasted for 12 hours prior to the administration of BOPP. Groups of 4 dogs were studied for either 25 hours, 79 hours, 240 hours or 672 hours after intravenous (i.v.) infusion of BOPP (Table 1.1b). One subject was administered control solution and studied for 240 hours after infusion. The BOPP disodium salt solution was administered over 1-2 hours via a 20 ga catheter placed in the right cephalic vein. BOPP solutions administered to dogs 4469, 4470, 4480, 4481, 4501 and 4502 were filtered via an inline 0.22 μ methylcellulose filter. This group constituted 240-hour dogs (2 of 4) and 672-hour dogs (4 of 4). All other dogs were administered BOPP solution unfiltered.

Following administration, the catheter was removed. Feeding and watering were normal following the administration of drug. The dogs were examined (weight, temperature, pulse, respiration, physical exam) daily or at the time of each blood sampling. Blood samples were obtained before and at several time points during the study (Table 1.1a). Urine samples were obtained prior to and at the end of the study in all dogs. 25 hour subjects had urine collected at each blood sampling time via urethral catheterization. 79 and 240 hour subjects were housed in metabolic cages and urine samples collected in foil-wrapped glass bottles twice daily. Urine volumes were quantitated and samples for urinalysis and boron analysis were stored at 4° C until use.

Table 1.1a . Approximate blood sampling times (hours)

25 hour study	79 hour study	240 hour study	28 day study
0	0	0	0
1	2	1	2
2	4	4	4
4	8	12	24
8	12	24	48
12	24	48	96
16	36	72	144
20	48	96	192
25	60	120	240
	72	144	288
	79	168	336
		192	384
		216	432
		240	480
			528
			576
			624
			672

Table 1.1b Dog numbers and study duration

25 hour study	79 hour study	240 hour study	28 day study
43535	43826	4419	4480
43643	43891	4420	4481
43769	43892	4469	4501
43825	43966	4470	4502

Sample Preparation

Samples for clinical chemistry were initially placed in tubes containing no additive. After clotting at 4° C, samples were spun at 2000 rpm for 12 minutes. The serum was decanted via plastic pipet into polypropylene tubes and stored at 4° C until evaluation. Samples for hematology were collected in tubes containing K_3EDTA (7.5%) and stored at 4° C until evaluation. All urine samples used for urinalysis were obtained via urinary cathterization. Clinical chemistry, hematology and urinalysis were performed by Consolidated Veterinary Diagnostics, West Sacramento CA. Table 1.2 lists the chemical and hematologic tests performed. Samples for boron analysis were obtained via jugular venipuncture into plastic syringes and placed in sterile vacutainer tubes containing heparin. Vacutainer tubes were centrifuged at 2000 rpm for 12 minutes to separate plasma from cells. Plasma was decanted into polyethylene tubes and frozen at -4° C until analysis.

Necropsy and Histopathology

At the conclusion of the study, dogs were humanely euthanatized by barbiturate overdose and a necropsy was performed. Tissue samples were obtained (Table 1.3) and immersed in 10% buffered formalin until processing for histopathology. These samples were then processed using alcohol/xylene fixation followed by paraffin embedding. Five μ thick sections were cut and placed on clean glass slides. After overnight air drying,

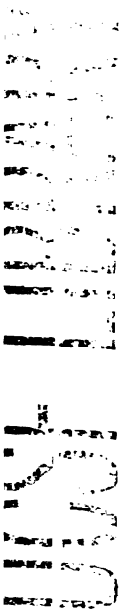
sections were stained with hematoxylin and eosin and examined for evidence of histopathologic damage. Histopathologic evaluations were performed by Dr. Patrick Gavin at Washington State University (Dogs 43535, 43643) or by Jay Tibbitts and Dr. Andrew Bollen at UCSF. Other tissues samples were obtained for boron analysis. Those samples were weighed and placed in polypropylene tubes, and stored at -4° C until analysis. A list of tissue obtained for histopathology and boron analysis is shown in Table 1.3.

Table 1.2 Clinical chemistry and hematology tests performed

Chemistry			Hematology		
Alkaline Phosphatase	Total Bilirubin	Calcium	White Blood Cells (WBC)	Mean Corpuscular Hemoglobin Content (MCHC)	Lymphocytes
Alanine Aminotransferase (ALT)	Direct Bilirubin	Phosphorus	Red Blood Cells (RBC)	nucleated RBC	Monocytes
Aspartate Aminotransferase (AST)	Indirect Bilirubin	Bicarbonate	Hemoglobin	Segmented Neutrophils	Eosinophils
Creatine Phosphokinase (CPK)	Blood Urea Nitrogen (BUN)	Chloride	Hematocrit	Band Neutrophils	Basophils
Albumin	Creatinine	Sodium	Mean Corpuscular Volume (MCV)		Platelets
Total Protein	Cholesterol	Glucose	Mean Cellular Hemoglobin (MCH)		Reticulocytes
Globulin					

Table 1.3 Tissues collected for analysis following BOPP administration

Temporal muscle	Lymph node (intestinal)
Lymph node (submandibular)	Bile
Cerebellum	Colon
Cerebrum	Adrenal (cortex/medulla)
Dura mater	Fat (abdominal)
Intraocular fluid	Testicle
Cerebrospinal fluid	
Pituitary gland	Lung
Tongue	Cardiac muscle
Salivary gland (parietal)	
Mucous membrane (lip)	Bone marrow (femur)
Skin (nasal planum)	Bone marrow aspirate
Posterior globe (eye)	Bone
	Cephalic vein
Kidney (cortex, medulla)	Thyroid
Spleen	Carotid art
Liver	
Duodenum	
Pancreas	
Stomach	



Fluorescence Microscopy

Carotid artery samples from dogs 4480 and 4481 were imbedded in gum tragacanth and immersed in liquid nitrogen until frozen. Samples were stored at -80° C until use. 5 μ thick sections were cut and placed on clean glass slides. Two consecutive sections were cut from each sample, the first unstained and the second stained with hematoxylin and eosin. Slides were examined using a Zeiss Axioplan fluorescence microscope with a G365 filter set. Excitation wavelength was 365 nm, beam splitter was at 395 nm and emission barrier filter was at 420 nm. Photographs were taken using a microscope mounted Zeiss MC100 camera and Kodak Elite 400ASA film.

Statistics

Some clinical, blood chemistry and hematologic parameters found to show changes from baseline levels were evaluated using the t-test or paired t-test. Comparisons were made between the baseline (time 0) value and the highest or lowest abnormal value to determine if the change in the parameter values was statistically significant. In some cases, one or more subjects had clinical pathologic findings that were outside the normal range during the course of the study but the mean value for this parameter (for all dogs studied) was not outside the normal range. These outliers are summarized in Table 1.5. The maximum (or minimum) value is the highest (or lowest) value for that parameter seen for any dog at any time during the entire study. The "mean of the maximum" or "mean of the minimum" was calculated as the mean of the maximum or minimum values of that parameter measured for each dog during the duration of the study.

Ex Vivo Tests

To determine the potential for BOPP to interfere with the clinical chemistry tests (Table 1.2) described above, BOPP was dissolved in sterile 0.45% saline and added to dog plasma at varying concentrations. These samples were also submitted to Consolidated Veterinary Diagnostics for analysis.

Boron Analysis

Boron analysis was via Inductively Coupled Plasma-Atomic Emission Spectrometry and was performed at the Idaho Nuclear Engineering Laboratory, Idaho Falls, ID[114]. The limit of detection of B with this assay was approximately 1 μg B/g sample.

Pharmacokinetic Analysis

Boron plasma concentration data from all subjects were pooled and the fits of 2- and 3-compartment models, both with zero-order input, were compared using the AKAIKE criteria (Program Minim 3.0.8, R. D. Purves, Dunedin, New Zealand). The comparison was confirmed using the Likelihood Ratio Test with a mixed effects (population) model (*NONMEM, Version V*, Beal SL, Sheiner LB [eds.], *NONMEM Users Guides*, NONMEM Project Group, University of California San Francisco).

Because individual animals did not have sufficient information to obtain fits to their data independently, maximum likelihood estimation with mixed effects modeling was used to obtain the reported parameter estimates. Unlike naive pooling and ordinary regression analysis, a mixed effects model tracks which data belong to which subject and thereby recognizes and distinguishes interanimal and intraanimal variabilities. It also handles imbalance in data (i.e., varying selection of time points among animals and varying study lengths). Intraanimal variability was modeled as being proportional to the predicted value. Interanimal variability was modeled for as many parameters as the data was able to support and was proportional to the population predictions. However, when population predictions do not vary, as was the case for all parameters except clearance, a proportional (constant coefficient of variation) model is the same as an additive (constant standard deviation) model. The pharmacokinetic parameters were allowed to covary if the addition of the covariance parameters improved the goodness of fit significantly. Standard errors for the primary parameters were determined with the usual asymptomic covariance matrix. Standard errors for the secondary parameters were obtained with error propogation.

Endotoxin Assay

BOPP disodium samples, made by M.S. Koo[100] on 5/8/1996 and 7/20/1996 were obtained and 40 mg of BOPP was weighed on weigh paper and placed in sterile

polypropylene 60cc syringes. 50 ml of 0.45% NaCl solution (containing 10 ml of 8.4% Na HCO_3 in 490 ml NaCl solution) was drawn into the syringe through a new 20 ga needle. Na_2BOPP-NaCl solution mixture was mixed in the syringe until Na_2BOPP was dissolved. Approximately 2.5 ml of this mixture was filtered via a 0.2 μ methylcellulose filter into sterile polypropylene tubes (Corning Inc., Corning, NY). Tubes were wrapped in foil and stored at 4° C until analysis. Endotoxin assay was the Limulus Amoebocyte Lysate Gel-Clot Assay (Pyrotell, UCSF Cell Culture Facility). Sample dilutions of BOPP were made and tested for endotoxin at levels as low as 0.015 ng/ml.

1.3 RESULTS:

The clinically apparent adverse effects of the i.v. administration of 35 mg/kg of BOPP are summarized in Table 1.4. Nausea was apparent, and both nausea and vomiting had resolved by 24 hours post-administration. Vomiting was not severe and was limited to one to two vomitions. Photosensitivity reactions were mild and were limited to some evidence of reddening of the skin of the nasal planum, episodes of ocular photosensitivity (blepharospasm, conjunctivitis, tearing, photophobia) that lasted 1-2 days following exposure to bright sunlight during daily exercise periods. Ocular photosensitivity reactions were seen as late as 17 days following BOPP administration. If photosensitivity reaction occurred, sunlight exposure was avoided with no further reactions noted. Cephalic vein thrombosis was present (10 of 14 observed subjects) in the vein into which the BOPP was administered. Palpation of the thrombosis did not elicit pain, nor was there any evidence of distal edema or redness in the area of the thrombus. In dogs studied for 28 days, the thrombus was evaluated daily by palpation and was found to have resolved by 17-18 days after BOPP administration. Histopathologic examination of four dogs at 28 days found little or no evidence of cephalic vein damage.

Figure 1.3 shows the range in body weight change after BOPP administration. Maximum average body weight decreased approximately 6-7% early in the study, stabilized by 8-10 days and appeared to rebound slightly by 28 days. This change was found to be statistically significant when compared to the baseline weight using the paired t-test ($p \leq 0.01$). One dog exhibited inappetance; all other dogs appeared to maintain a normal appetite although food consumption was not quantified. One dog was found to have a net increase in weight during the course of the study.

Most dogs exhibited a mild febrile response following BOPP administration (Figure 1.4). The mean temperature peaked at approximately 1-2 days and returned to baseline levels by 4-5 days. The difference between the baseline temperature and the maximum temperature was evaluated using the paired t-test and found to differ significantly ($p \leq 0.01$).

Clinical pathologic findings are summarized in Figures 1.5-1.11 and Table 1.4. Clinical pathologic parameters found to have mean values that were outside the reference range (as defined by the clinical pathology lab) were reported graphically (Figures 1.5-1.11). Due to unequal sample size at most time points, Figures 1.5-1.8 are reported as the mean of the parameter with error bars indicating the range. In some cases, one or more subjects had clinical pathologic findings that were outside the normal range during the course of the study but the mean value for this parameter (for all dogs studied) was not outside the normal range. These outliers are summarized in Table 1.5. Two dogs had increased phosphorus measurements at one time point. One dog had an increase in alkaline phosphatase at two time points. This subject showed no increase in liver enzymes during the study. Several dogs exhibited mild decreases in sodium, but no subject had two consecutive decreased sodium levels. Three dogs had decreased albumin levels within the first 24 hours of the study, with two of those subjects having mildly decreased albumin (2.4-2.5 g/dl) for 4-6 days following BOPP administration. None of these changes in clinical chemistry parameters were clinically significant. Figures 1.9-1.11 provide a

graphical representation of the ALT, AST and potassium results of the subjects whose serum levels of these parameters were out of the normal range.

Table 1.6 shows the histopathologic abnormalities.

Table 1.6 Histopathology findings following BOPP administration

Finding	Number affected
Cerebral cortical necrosis and astrogliosis	7/16
Liver-mild periportal acute and chronic inflammation	2/16
Cephalic vein-organizing thrombus	5/14

There were few abnormalities found in these subjects as a result of BOPP administration. Most tissues were found to be normal on both a gross and microscopic level. 7 of 16 dogs had a small (2-4 mm) cerebral lesion as a result of previous implantation of a ^{125}I seed. Cerebral lesions contained macrophage infiltrates, astrogliosis and resolving necrosis. It is important to note that the cerebral lesions were not caused by the administration of BOPP. Hepatic inflammation was noted in two 10 day subjects. These changes were characterized as diffuse cytoplasmic swelling of the hepatocytes and mixed inflammatory infiltrate in the periportal region. Cephalic vein lesions showed evidence of organizing thrombi with inflammation of the vessel wall. The histopathologic severity of these lesions were seen to decrease with increasing study duration.

One control dog, administered saline solution only, and monitored for 10 days showed no clinical or clinical pathologic abnormalities. A necropsy was not performed on this subject.

Ex vivo tests of BOPP's effect on clinical chemistry parameters are reported in Table 1.7 for those tests where there appeared to be an effect. BOPP caused an increase in bilirubin at BOPP concentrations ≥ 300 µg/ml, a concentration of BOPP that is seen in the

dogs during the first 4-6 hours following BOPP administration. In addition, in the presence of BOPP a mild decrease in alkaline phosphatase and AST was seen. Endotoxin assay was negative at all dilutions.

Table 1.7 Effect of BOPP added to normal plasma *ex vivo*

BOPP (µg/ml)	Alkaline phosphatase (IU/l)	AST (IU/l)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)
0	67	21	0.2	0.1
100	57	15	0.2	0.1
300	43	5	0.3	0.3
600	37	2	0.5	0.5

Pharmacokinetics

To determine the plasma pharmacokinetics of boron, we used the non-linear regression program Minim 3.0.8 to do a preliminary fitting of the pooled data to obtain the optimal compartmental model that described the data. Using AKAIKE criteria we found that a three compartment model with a zero order input provided the most appropriate fit (Appendix I). We confirmed this using the likelihood ratio test. Because of variations in study length and sample collection times, we opted to analyze the data using a population approach-mixed effect model. Using this method allowed us to pool the data while still recognizing which data was from each individual. We chose 6 parameters for the model to estimate: V_c, the three compartmental exponential values (k_{alpha}, k_{beta}, k_{gamma}) and the microconstants k_{21} and k_{31}. These parameters would allow us to calculate clearance (CL) and to calculate the errors associated with these parameters. We allowed for interindividual variability for four parameters (k_{alpha}, V_c, k_{21} and k_{gamma}). There was insufficient data to allow for the addition of interindividual variability terms for all parameters. Three

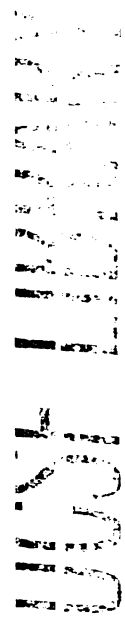
parameters were allowed to covary (k_{alpha} V_c and k_{21}). Increasing the number of covariate parameters did not improve the fit of the data.

Figure 1.12 shows the observed plasma boron concentrations vs. time for all dogs and Table 1.8 lists the pharmacokinetic parameters of the 3-compartment model. Half-lives were calculated using the formula $t_{1/2}$=0.693/k. Clearance was calculated based on the following relationships:

$$CL=k_{10}*V_c$$

$$k_{10}=k_{alpha}*k_{beta}*k_{gamma}/k_{21}*k_{31}$$

High levels of boron were found in several tissues following BOPP administration. These tissues included liver, adrenal gland, lymph nodes, and kidney (Figure 1.13). There appears to be a persistence of these high levels of boron in the liver and lymph nodes. Figure 1.14 shows the boron levels in tissues of the head plotted with the plasma concentration as a reference value. Most of the important tissues of the head, in particular the brain tissues, exhibited very low concentrations of boron. The notable exceptions are the carotid artery and the peripheral (submandibular) lymph node. Table 1.9 shows a more complete listing of tissues and their boron concentrations.



Fluorescence microscopic images of carotid artery from dogs 4480 and 4481 are shown in Plates 1.1, 1.2. Lower plates are hematoxylin and eosin stained sections of the carotid artery while upper plates are UV excited fluorescence images of same artery in a consecutive 5 μ section. The blue color in the fluorescence images is tissue autofluorescence, while the reddish color is most likely due to the fluorescence of a porphyrin compound which has a fluorescence emission in the 620 nm (red) range.

1.4 DISCUSSION:

1.4.1 Clinical toxicity

Important to the utility of any therapeutic compound is the demonstration of an acceptable level of toxicity in animals. This is true for binary therapy sensitizing agents as well. The toxicity data obtained in animal studies is important in determining the potential for use of a compound in humans. In this study we administered 35 mg/kg of BOPP to dogs and found that, at this dose, BOPP is well-tolerated and toxic effects are minimal. It is significant to note that the dose administered in this study was considered to be quite high for a porphyrin-based compound. Body surface area scaling of this dose would extrapolate to a dose of 200 mg/kg and 140 mg/kg in mice and rats, respectively. Doses of HpD and DHE at this level were found to be lethal in mice[4]. Thus, the relatively mild toxicity noted in this study gives support for further studies with this compound.

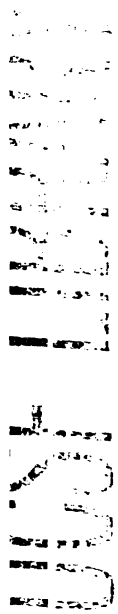
Clinically apparent toxic effects were mild and transient, limited mostly to some nausea, vomiting and photosensitivity (Table 1.4).

Table 1.4 Clinical abnormalities noted after BOPP administration

Abnormality	Number of Subjects	Onset	Duration
Vomiting	8/16	4 hours	24 hours
Photosensitivity	4/16	variable	48 hours
Cephalic thrombosis	10/14	2 hours	18 days

Nausea and vomiting are common findings in human patients with variegate porphyria and hereditary coproporphyria. Vomiting was seen by Peaston[40] using aluminum phthalocyanine tetrasulfonate ($AlPcS_4$) in cats. Unlike the present study, vomition occurred during administration of the drug and only 2 of 18 cats showed this effect. Other studies using porphyrin-based therapeutic compounds in animals capable of vomiting did

not note any evidence of this effect. Vomiting and nausea were most evident at the beginning of the study and may be due to high levels of BOPP in the plasma following administration. Weight loss by the dogs in this study was mild (Figure 1.3), and overt inappetance was uncommon. Weight loss has been reported in mice following administration of a nickel carboranyl porphyrin (NiTCP)[82] and by Kahl et al.[80] using a nidocarboranylporphyrin (BTPP). Mice lost weight by 48



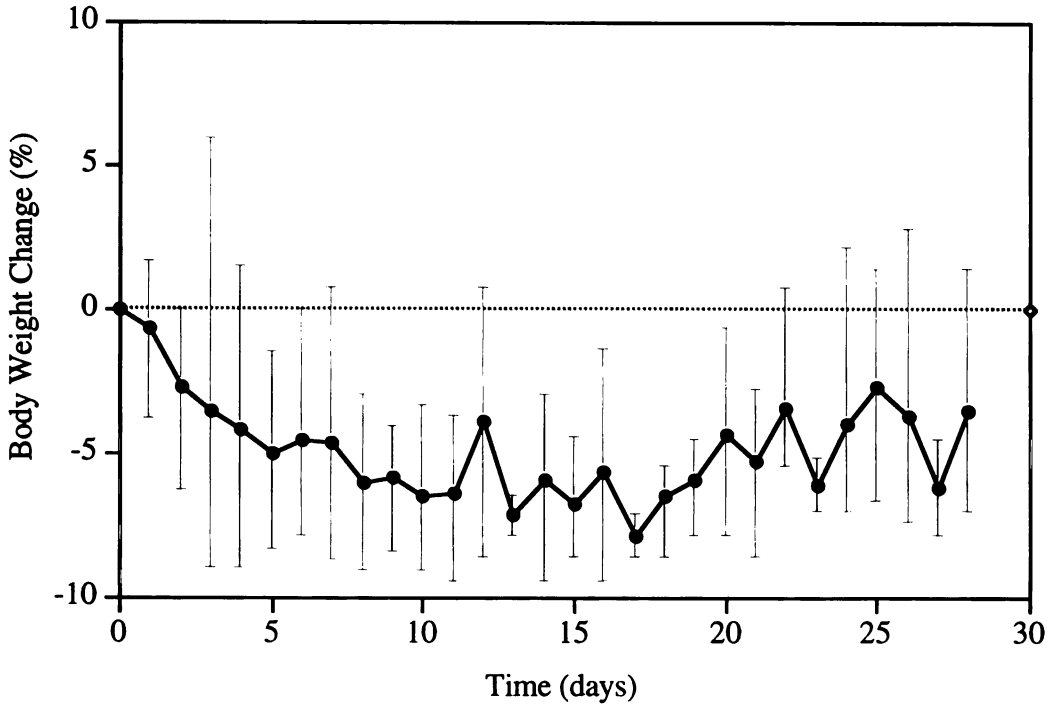


Figure 1.3. Mean body weight change following BOPP administration. Bars indicate range of maximum and minimum values.

hours after NiTCP administration was started and did not have a weight gain equal to the control subjects at 1 week following treatment[82]. Three months after treatment, subjects given NiTCP outgained control subjects. Kahl et al. noted significant weight loss in mice at 4 days following commencement of an infusion of BTPP. Comparisons between those two studies and the present study are tenuous due to the species and compound differences and based on the fact that the two previous studies used multiple dosing or constant infusions. The cause of the weight loss in the present study
and previous studies may be related to the above-mentioned nausea, although nausea in mice is difficult to assess. In future studies, careful monitoring of food intake would also detect any inappetance subsequent to compound administration.

A mild increase in body temperature was seen (Figure 1.4) which may have contributed to a decreased appetite and the observed weight loss. This temperature change may be the result of an inflammatory response elicited by BOPP as is evidenced by an increase in circulating white blood cells (Figure 1.5).

A clinical finding of greater concern was that of cephalic vein thrombosis in several of the subjects. Similar findings have not been reported in studies using porphyrins in animals, but Simionatto[115] and others[85, 116-118] have reported local thrombosis in humans following the intravenous administration to humans of hematin, an oxidative metabolite of heme. In addition, hematin has also been shown to cause severe thrombotic effects in dogs[119]. The clinical picture of the thrombosis in human cephalic veins was more severe than seen in the present study, with the human subjects exhibiting local discoloration and induration. The time required for resolution of the injury was shorter in humans treated with hematin (2-3 days) than the dogs treated with BOPP (17-18 days). Also, it was noted that a longer duration of hematin infusion resulted in a decreased incidence of thrombosis. There are three possible mechanisms for BOPP-induced thrombosis. (i.) Damage to the endothelium may cause the expression of pro-

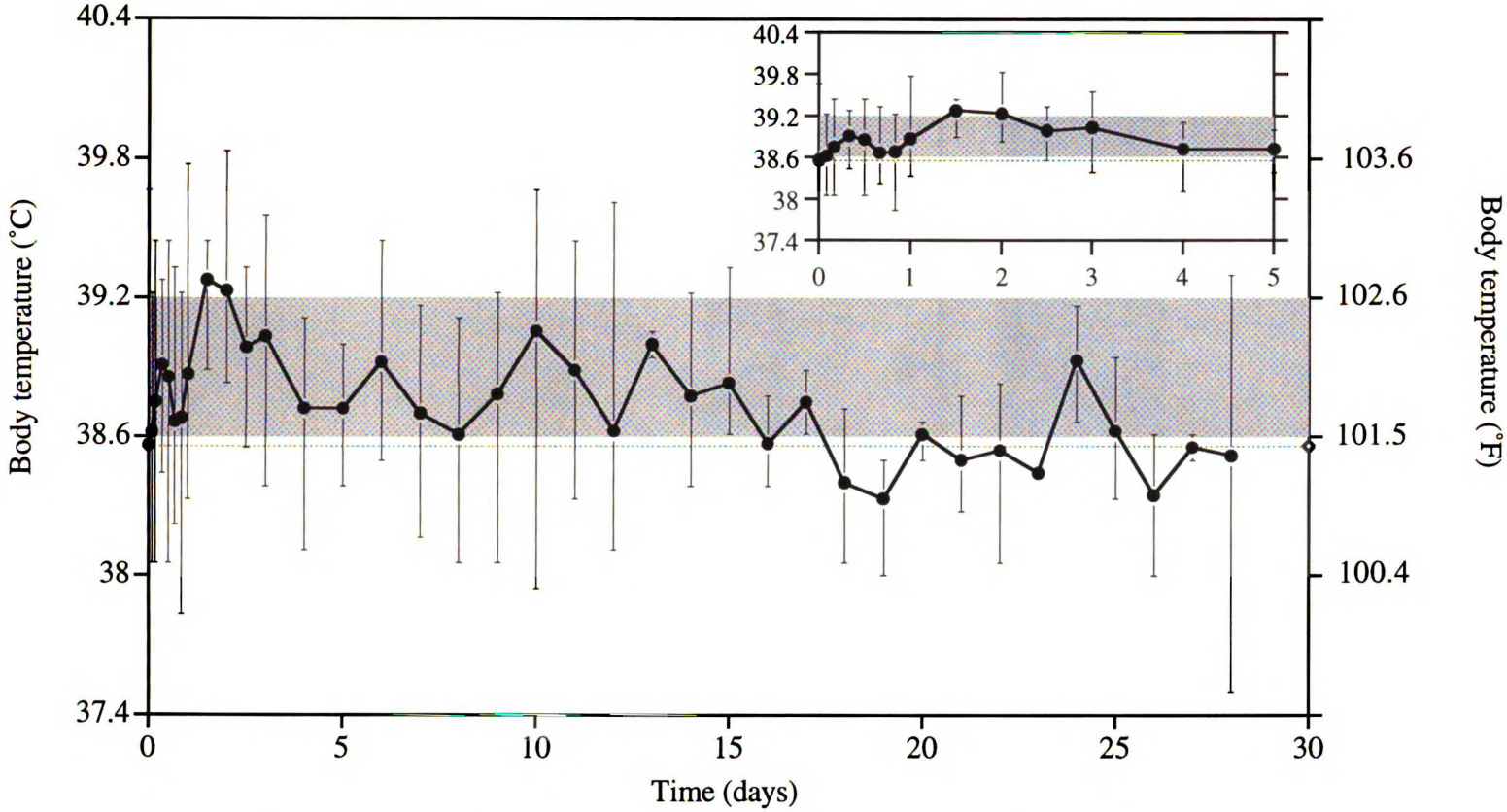


Figure 1.4. Range in body temperature following BOPP administration. Symbols indicate mean values. Shaded area represents normal body temperature range. Dotted line is mean body temperature of subjects at time zero. Inset is expansion of data from days 0-5.

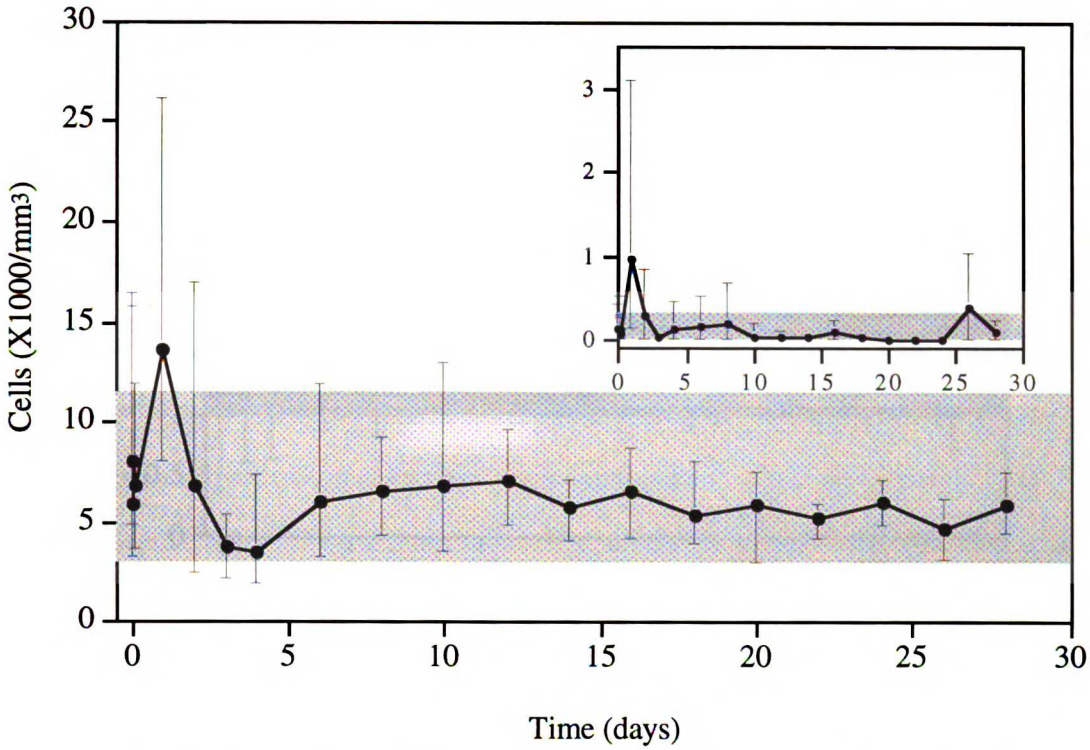


Figure 1.5. Mean segmented neutrophil counts after BOPP administration. Inset shows band neutrophil counts. Shaded areas indicate reference range for represented cells. Bars indicate range of maximum and minimum values.

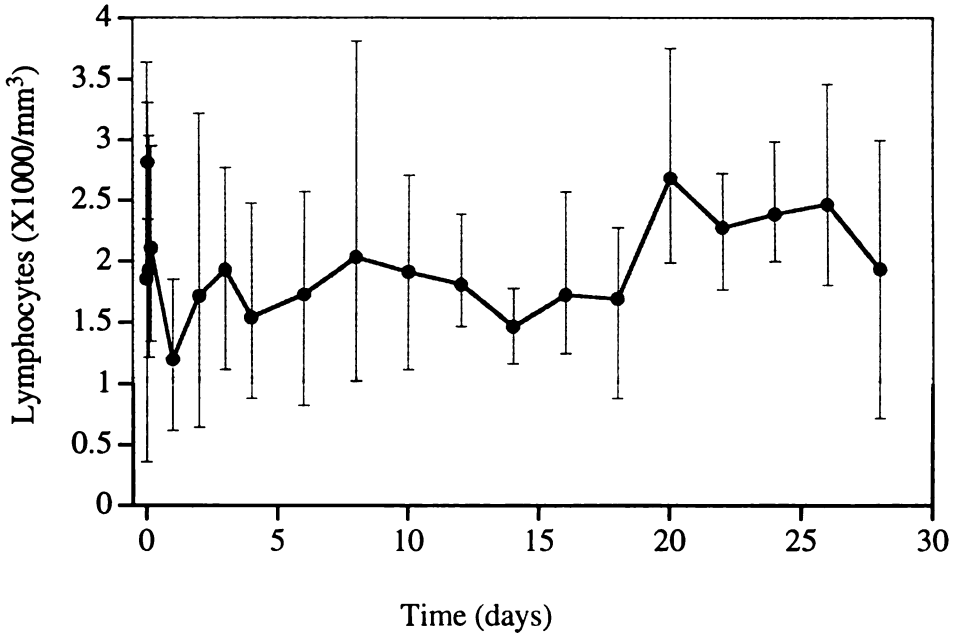


Figure 1.6. Mean lymphocyte counts (circles) after administration of BOPP Shaded areas represent reference range for lymphocytes. Error bars represent maximum and minimum values at each time point.

coagulant factors on endothelial cells or the exposure of the subendothelium. This could stimulate clotting mechanisms and resulting in subsequent thrombus formation. Neely et al.[120] found marked morphologic changes in endothelial cells treated with 40 μg/ml of hematin *in vitro*, with cell detachment at higher concentrations. These effects by hematin on endothelial cells may precipitate local thrombosis. (ii.) Stimulation of the coagulation cascade or inhibition of fibrinolytic mechanisms may alter the delicate balance that prevents the formation of thrombi under normal conditions. Protoporphyrin-induced activation of factor XII-dependent pathways has been seen in human plasma[121], suggesting that porphyrins may be able to affect the coagulation cascade and trigger thrombosis. (iii.) BOPP may be causing local platelet aggregation or adhesion to vessel walls, but a study of platelets and endothelial cells[122] *in vitro* found that aggregation was inhibited in the presence of Photofrin® at concentrations (25 μM) less than that of BOPP at its highest plasma concentration (~100 μM). Conversely, hematin has been found to cause increased platelet adherence to endothelial cells[120] and to induce platelet aggregation[85]. Foster et al.[123] reported the increased release of the platelet adhesion factor vWF from endothelial cells following Photofrin®-mediated PDT. This release could stimulate local platelet aggregation resulting in thrombosis. The presence of light in the formation of thrombi in capillaries has been studied and found to be of importance. Both Reed et al.[124] and Star et al.[125] showed platelet-induced thrombi formation in small vessels as a result of light activation of Photofrin® or HpD in rats. Animals not exposed to light showed no thrombi. The amount of light necessary to elicit this response was not studied, and is likely to vary depending on the photosensitizing agent. The effect of light on the formation of thrombi in the present study is not known, but shielding the administration apparatus and the administration site using an opaque wrap had no effect on the incidence of thrombosis.

BOPP administration may cause one or a combination of the above effects, although it appears that the effect is concentration-dependent due to the lack of evidence of increased thrombosis elsewhere in the tissues of these dogs. Histopathologic studies of the

lungs, kidneys and other tissues revealed no thrombi present in these tissues at times as early as 25 hours after BOPP administration suggesting that the thrombogenic action of BOPP is not systemic in nature. In conclusion, it appears that the thrombosis caused by BOPP is a local phenomenon and occurs only at the site of injection.

Hematology and blood chemistry results revealed few serious abnormalities. Platelet levels (Figure 1.8) did not become low enough to raise concern of bleeding risks, and no dogs had any evidence of clinical clotting abnormalities (petechiae, purpura). Decreased platelet counts have been reported by Kahl et al.[80] in mice treated with BTPP and by Miura[126] who found thrombocytopenia after administration of both $TPPS_4$ and VCDP to mice. As the Kahl study measured platelets only after 7 days of BTPP administration, it is difficult to determine if the effect was temporary and how soon after administration it occured. Like the present study, Miura et al. found that in VCDP-treated mice platelet levels returned to normal within 6 days after cessation of drug treatment, but their study did not examine the onset of thrombocytopenia since their first reported platelet count was taken 3 days following initiation of treatment. The decrease in platelets as a result of the administration of these compounds is not likely to be due to aggregation or platelet adhesion to blood vessel walls. As discussed above[122, 123], the study of the effect of porphyrin sensitizing agents on platelets and endothelial cells has resulted in differences in opinion, but there is agreement that these compounds do not cause platelet aggregation in the absence of light activation; a scenario not present in the dogs used in this study. While hematin has been shown to promote platelet aggregation[85], this effect has not been reported with other porphyrin-based compounds. Widespread aggregation of platelets or adhesion of platelets to vessel walls would be expected to cause a deposition of these aggregates in capillary beds and might lead to disseminated intravascular coagulation. No evidence of this phenomenon was found. It is also possible that platelets are taken up by the spleen. The spleen contains approximately 1/3 of the mature platelets and the

interaction between the spleen and peripheral circulation is very dynamic[127]. It was not determined if platelets were being sequestered in the spleen.

An increase in the white blood cell (WBC) count occurred approximately 24 hours after BOPP administration and was characterized by an increase in segmented neutrophils and band neutrophils (Figure 1.5). The maximum increase in segmented cells was significantly different from baseline levels ($p \leq 0.01$). This increase may be the result of an inflammatory response elicited by BOPP or the release of white blood cells from peripheral stores, and may be associated with the concomitant increase in body temperature (Figure 1.4). Alternatively, BOPP may be stimulating the bone marrow to release neutrophils and bands from the marrow storage pools. Stress may also result in an increase in circulating neutrophils, but this response is usually immediate and is often accompanied by a concurrent lymphopenia and monocytosis which were not evident in these studies (Figure 1.6, data not shown). Hunt et al.[83] reported an increase in circulating leukocytes and lymphocytes in mice 7 days following Photofrin® administration. This finding was in conjunction with an earlier (48-72 hours) stimulatory effect on hematopoiesis in Photofrin®-treated mice as measured by an *in vitro* assay. A BOPP-induced stimulation of hematopoiesis is unlikely to explain this increase in circulating neutrophils due to the time required for the production of these cells by the bone marrow (~100hrs[127]) and the fact that only neutrophils and band cells showed an increase in numbers. The presence of endotoxin has the potential to cause an inflammatory response resulting in the observed changes in circulating neutrophils. Endotoxin was not found in samples of BOPP tested with the Limulus Amoebocyte Lysate Assay. The subsequent decrease in neutrophils in BOPP-treated dogs is more difficult to explain. Schalm[127] suggested that a decrease in neutrophils can be explained by

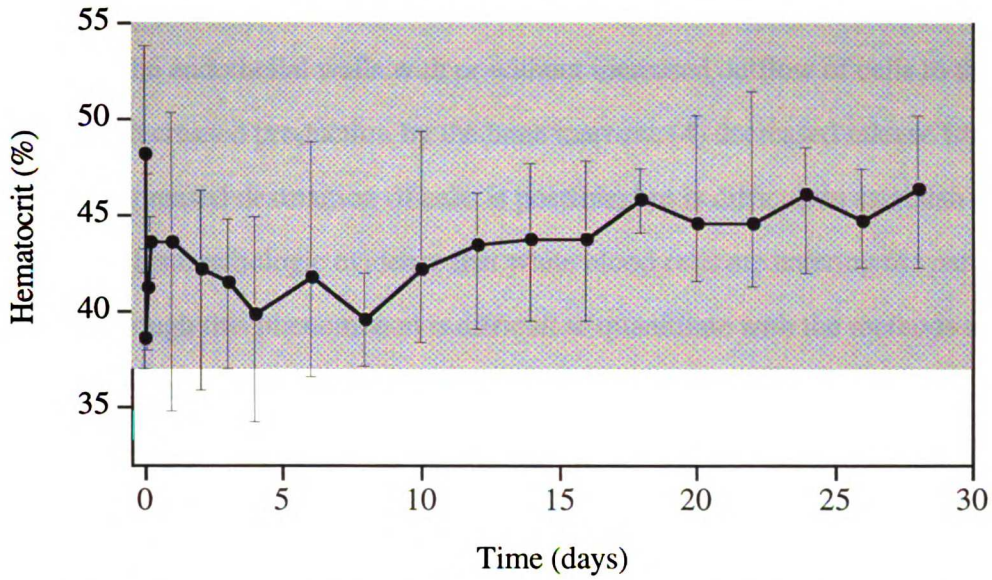


Figure 1.7. Range in hematocrit levels after BOPP administration. Symbols indicate mean values. Shaded area represents reference range for HCT. Error bars indicate maximum and minimum values.

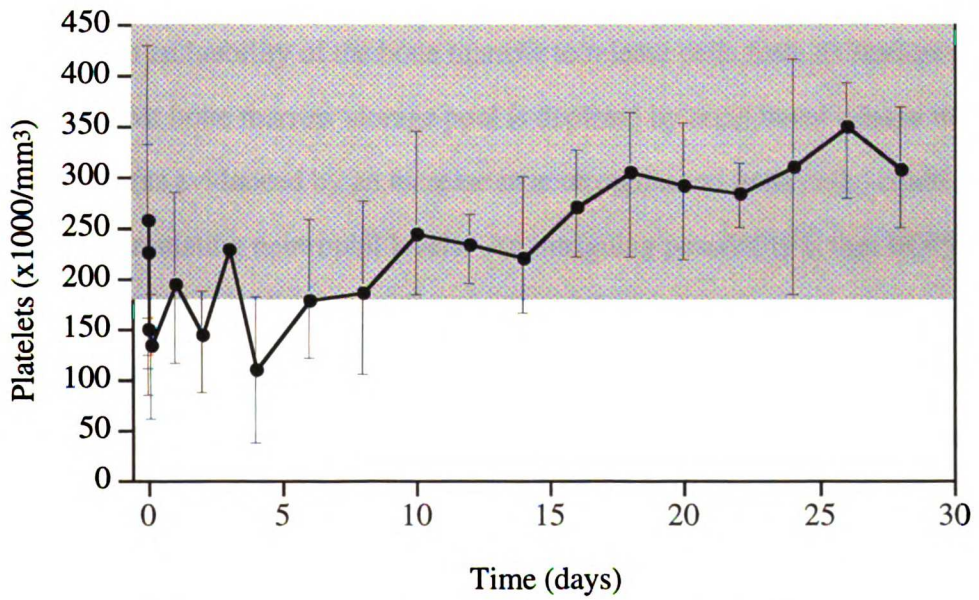


Figure 1.8. Mean platelet counts after BOPP administration. Shaded area represents reference range for platelets. Bars indicate range of maximum and minimum values.

four general mechanisms: (1) increased destruction of cells in circulation, (2) margination of cells on the endothelial walls with or without increased outflow of cells to the peripheral tissues (3) decreased production by the bone marrow, (4) decreased release from the bone marrow. Increased destruction of cells is possible, but is difficult to establish directly. There is no histopathologic evidence that white blood cells are entering the peripheral tissues, although this phenomenon is difficult to quanititate with the methods used in this study. A decrease in production is possible, since the life span of neutrophils is such (neutrophil half-life in circulation is approximately 6 hrs[127]) that even with normal loss of cells a rapid depletion in circulating levels could occur. However, the bone marrow is known to have a large storage capacity for mature neutrophils which may be rapidly released into the peripheral circulation in response to depletion in that compartment. Alternatively, a decreased release of neutophils is possible when one considers the rate of neutrophil turnover in the peripheral circulation causing depletion of circulating cells. This, coupled with an inability of the bone marrow to release cells from its storage pool and the possibility that bone marrow storage pool is depleted by accelerated release of cells a few days earlier (as evidenced by an increase in neutrophils and band cells) could result in decreased circulating neutrophil levels. An intriguing possibility is that BOPP is causing a mild toxic effect on endothelial cells or neutrophils resulting in the temporary adherence of neutrophils to the endothelium and thus decreasing the number of cells that are available for counting. The marginal pool (those neutrophils attached to the endothelium) of neutrophils in the dog is approximately equal to the circulating pool[127]. There are many mechanisms by which neutrophils can be stimulated to adhere to endothelium. Most of these mechanisms occur soon (within 4-6 hours) after a stimulus, such as endotoxin or chemotactic factors, and are accompanied by a myriad of concurrent events associated with inflammation and emigration of the neutrophil into the extravascular tissue[128, 129]. Thus, stimulation of neutrophils or endothelial cells that results in adherence without subsequent migration or the release of inflammatory mediators is not likely. Sandberg et al.[130]

reported that neutrophils *in vitro* are sensitive to the effects of low concentrations of protoporphyrin. Neutrophils were found to lose their migration ability, to experience photodamage to cellular enzymes, and to suffer damage to mitochondria. These effects may cause neutrophils to marginate or be taken up by the spleen *in vivo*, thus decreasing the circulating neutrophil pool. The mechanism of photodamage to neutrophils is not known, nor is the effect of photodamage on other leukocytes.

A decrease in hematocrit (HCT, Figure 1.7) was also found, characterized by an initial profound decrease followed by a rebound and a more persistent mild diminution (as compared to the pre-treatment HCT) lasting for several days. This delayed diminition in HCT showed a significant difference from baseline as determined by paired t-test ($p \leq 0.05$). The initial decrease is likely caused by hemodilution as a result of the administration of a quantity of BOPP solution that was approximately 60% of the blood volume of the subjects. The persistent decrease in HCT was not accompanied by any evidence of a regenerative response by the bone marrow (nucleated RBCs, increased reticulocytes, polychromasia, Howell-Jolly bodies). No evidence of blood loss was seen. Another possibility for the decrease in the HCT is hemolysis. The occurrence of hemolysis would probably result in changes in plasma bilirubin. An increase in total bilirubin was seen (data not shown) following BOPP administration, with a slightly larger proportion of indirect reacting (unconjugated) than direct reacting (conjugated). But, as will be discussed later, BOPP was found to exhibit interference with the assay used to determine bilirubin. This increase in bilirubin was not clinically significant and there was no evidence of icterus. No increase in urine bilirubin was found (results not shown).

Changes in liver enzyme levels, especially ALT, are sensitive indicators of liver damage. Increases in liver enzymes have been reported in dogs following Photofrin® treatment[107] and in mice after BTPP treatment[80]. Figures 1.9 and 1.10 show liver enzyme results for those subjects found to have levels above the

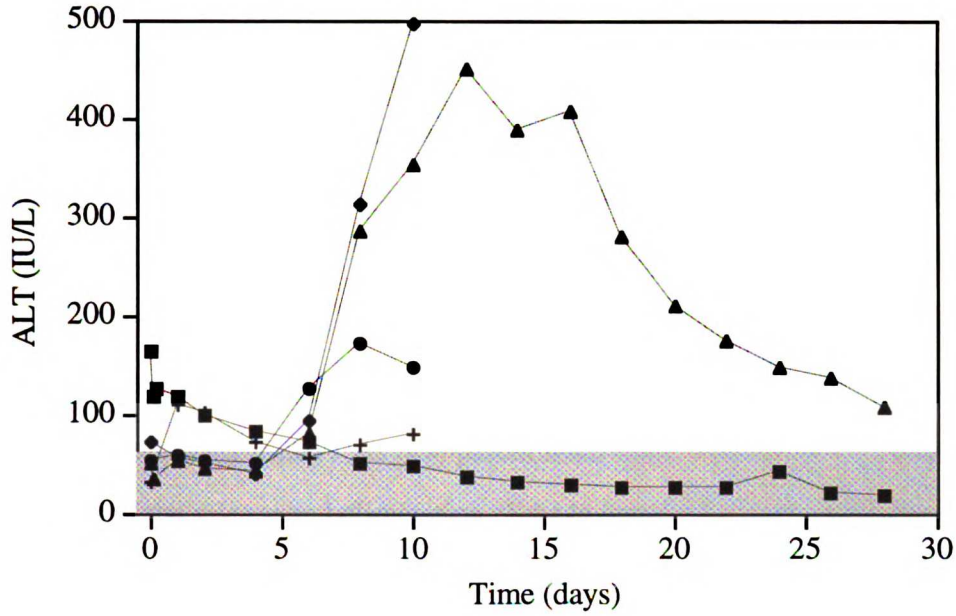


Figure 1.9. Serum ALT levels following BOPP administration. Shaded area represents reference range for ALT. Dog 4419 (circle), 4420 (diamond), 4469 (cross), 4481 (triangle), 4501 (square).

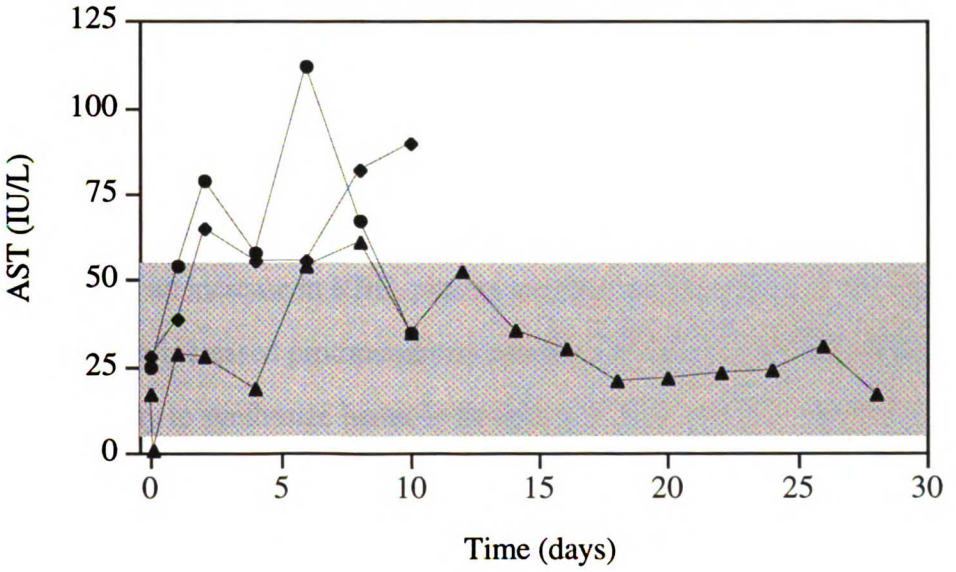


Figure 1.10. Serum AST levels following BOPP administration. Shaded area represents reference range for AST. Dogs 4419 (circle), 4420 (diamond), 4481 (triangle) Note: values not adjusted for interference by BOPP (see Table 1.7)

established reference range. Both these enzymes increase in response to cellular injury with or without changes in cell membrane permeability. ALT is found predominantly in the hepatocytes and increases in plasma levels are strongly suggestive of active liver injury. ALT is known to increase almost immediately after liver damage[131] and has a reported half-life of 3 hours to 4 days[132]. AST is found not only in hepatocytes but in other tissues as well and its increase is not as specific for liver injury. AST increases following liver injury are usually slightly delayed (within 3 days of injury) and tend to decline about 2-3 weeks following injury. As is apparent from these reports[131, 132], the temporal properties of the release and elimination of these enzymes in dogs in response to hepatic injury is not completely characterized. Finally, alkaline phosphatase levels were not found to be increased in these subjects indicating that there was little or no biliary system injury.

The increase in the ALT and AST seen in subjects 4419 and 4420 (Figures 1.9 and 1.10) correlated with the histopathologic changes seen in the livers of these dogs (Table 1.6). This suggests that the administration of BOPP may result in increases in liver enzyme levels that are reflective of damage to the liver. The changes seen in the livers of these BOPP treated dogs is similar to that reported in studies of humans with erythropoietic protoporphyria, a disease caused by a defect in heme synthase[76, 133, 134]. The result of this defect is an increase in RBC, plasma and liver protoporphyrin[134]. The primary source for the increased protoporphyrin appears to be the erythropoietic system; the liver, with its ability to synthesize heme, is thought to be only a minor player in the excess sythesis of protoporphyrin[76]. Patient to patient variation in the location of this overproduction is thought to occur and may be an important factor in the susceptibility to hepatic injury occasionally seen with this disease[76]. The excess protoporphyrin is transported by the plasma proteins to the liver and excreted in the bile. The damage resulting from protoporphyria is characterized by portal inflammation, hepatocellular necrosis and portal fibrosis[75]. In addition, large amounts of pigment are deposited in hepatocytes and Kupffer cells, something not seen in the BOPP study. This pigment is

thought to be porphyrin crystals[135], which may be causing damage to the hepatocytes and obstructing the biliary system. The mechanism for the hepatic damage is thought to be due to the limited solubility of protoporphyrin and the deposition of crystals in the hepatocytes and portal system[135]. Lee et al.[136] administered protoporphyrin to isolated, perfused rat livers for a short time and found ultrastructural evidence that protoporphyrin is a hepatotoxin. In the present study no crystals were found in the hepatocytes or Kupffer cells. It is likely that crystal formation is a result of chronic exposure to abnormally high amounts of porphyrin, something not anticipated for BOPP therapy. BOPP administration in this study resulted in BOPP concentrations that were initially much higher than the protoporphyrin concentrations typically associated with protoporphyria, but this high BOPP concentration is transient and BOPP is more water soluble (and less likely to form insoluble crystals) than protoporphyrin. Thus it is less likely that BOPP will cause the degree of damage to hepatocytes that is seen in protoporphyria. Patients with porphyria as a result of decreased uroporphyrinogen decarboxylase (UROD) activity ("familial porphyria cutanea tarda", "cutaneous hepatic porphyria", "hepatoerythropoietic porphyria") are commonly found to have a hepatic component to their disease[137, 138]. The damage is usually mild and is characterized by fatty change[138, 139], needle-like inclusion bodies in hepatocytes[138] and portal inflammatory cell infiltrates and periportal lymphoid aggregates[138]. Decreased UROD activity results in the increase in plasma levels of 7- and 8-carboxylate porphyrins[137] which are highly water soluble, very different than protoporphyrin (the zinc-containing form of which is found to accumulate in the red blood cells of these patients)[139]. The mechanisms of this hepatotoxicity are not well understood, but may be the result of hepatocellular production of elevated levels of uroporphyrin and related compounds and subsequent accumulation of these porphyrins.

In studies of therapeutic porphyrins, Tochner et al.[107] reported hemosiderin-like deposits in the periportal parenchymal cells and a very mild non-specific reactive hepatitis in dogs treated with both IV and IP Photofrin® and intraperitoneal light exposure. It is

unclear from Tochner et al. whether these findings were also present in animals given Photofrin® without light treatment. Subjects were examined 4 days and 2 months after Photofrin®/light treatment, but it is not stated if these pathologic findings were present at both times. Kahl et al.[80] found that mice administered BTPP exhibited diffuse fatty change in their livers but the details of the incidence and duration of this injury were not reported. An interesting phenomenon in the present study was the delay in the increase of liver enzyme levels, occurring several days following BOPP administration. As discussed above, serum levels of liver enzymes (especially AST) become elevated soon after cellular damage. The cause of this phenomenon may be related to the formation of a hepatotoxic metabolite, or the slow accumulation of BOPP or a metabolite in the hepatocytes that resulted in toxicity. A similar time delay was found by Peaston et al., who reported a case of severe acute hepatic necrosis in a cat 5 days after $AlPcS_4$ administration[40]. There is evidence that the hepatic injury following BOPP administration may be a transient effect. Dog 4481 (Figures 1.9, 1.10) showed an increase in liver enzymes that had returned to normal or near normal levels by 28 days. This subject had no histopathologic evidence of liver damage. An acute, non-sustained injury would cause a rapid increase in ALT and AST and would show the slow steady decline in these enzymes as seen in this case. Also of interest is the finding that less than half the subjects (3 of 8) studied for 10 days or greater exhibited evidence of hepatic injury. It is difficult to speculate on the reasons for this, except to suggest that differences in ability to metabolize and eliminate BOPP may be responsible for a greater susceptibility to the hepatotoxic effects of BOPP. The ability of BOPP to interfere with the test for AST may be responsible for the delay between BOPP administration and AST elevation, but BOPP did not interfere with ALT which did not become elevated until AST was increased. Furthermore, the concentration of boron (and possibly BOPP) in the plasma had decreased to levels below the 30 μg B/ml (approximately 100 μg BOPP/ml, data not shown) by 50 hours after administration. At these BOPP concentrations the interference of BOPP with AST is minimal. In summary,

there is evidence that BOPP may have toxic effects on the liver. The effects seen in this study were mild and may be transient. However, caution is needed in evaluating the effects of BOPP on the liver. A better understanding of the mechanisms of BOPP hepatotoxicity may help predict those at risk for this effect and also help in devising methods of avoiding it.

Four of sixteen subjects showed a decrease in serum potassium levels (Figure 1.11) soon after BOPP administration. These decreases were transient and caused no clinical effect. There is no obvious explanation for this finding. Hypokalemia are usually associated with alkalosis, but the serum bicarbonate levels of these subjects were within the reference range. Sodium bicarbonate (10mEq) was administered with BOPP as a buffering agent, which might have caused a transient alkalosis, but serum bicarbonate levels were within the reference range in all animals immediately following BOPP administration. An increase in the mineralocorticoid aldosterone can also lead to hypokalemia, but increases in aldosterone levels are usually associated with other metabolic disturbances (alkalosis, hypernatremia) which were not seen in these subjects[140].

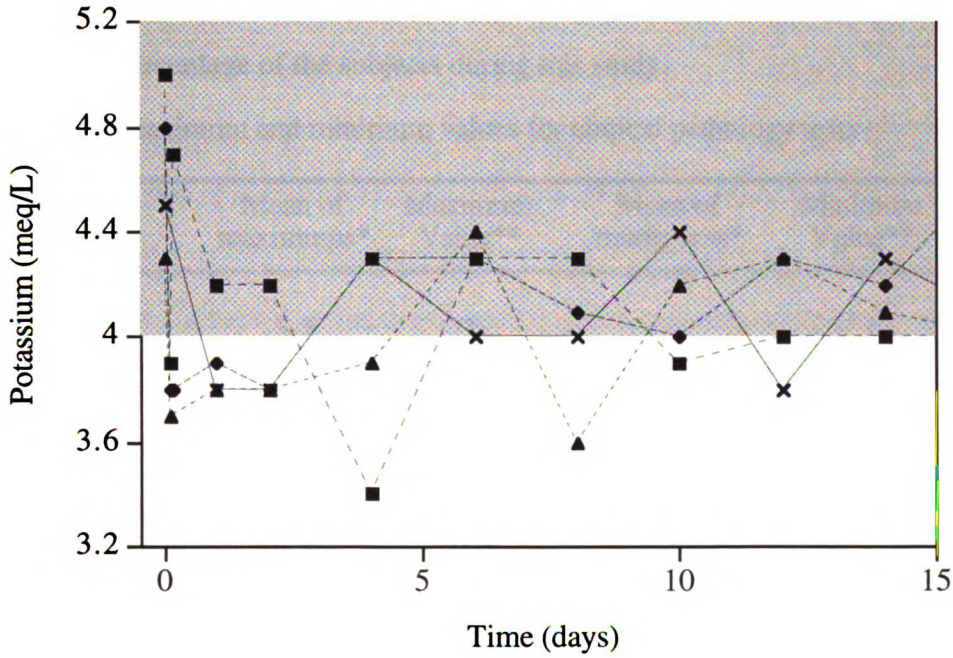


Figure 1.11. Serum potassium levels following BOPP administration. Shaded area represents reference range for K levels. Dogs 4480 (x), 4481 (triangle), 4501 (square), 4502 (diamond).

Table 1.5 shows the clinical chemistry parameters that were found to be abnormal in a small percentage of the subjects during this study.

Table 1.5 Maximum and minimum values for clinical pathology tests

Parameter	Mean of maximums*	Maximum Value**	Mean of minimums*	Minimum Value**	Reference Range
Phosphorus (meq/l)	5.2	7			2.1-6.3
Chloride (meq/l)	113.25	118			105-115
Alkaline Phosphatase (IU/L)	103.9	301			10-180
AST (IU/L)	50.56	112			5-55
ALT (IU/L)	116.75	499			5-60
Albumin (g/dl)			2.8	2.4	2.6-4.3
Potassium (meq/l)			4.0	3.4	4.0-5.6
Sodium (meq/l)			140.4	133	141-156

Data for subjects found to have values outside the reference range, but not to have a mean value outside the reference range at any time.

*Mean of maximums and Mean of the minimums = mean of the maximum or minimum reported value for all subjects

**Maximum value or Minimum value = the highest or lowest value reported for all subjects.

As can be seen, most deviations were mild and none caused clinical effect. Albumin levels in several animals were found to decrease after administration of BOPP due to hemodilution. The finding in two animals of a decreased albumin at 4-10 days after BOPP administration is more difficult to understand. One animal (4481) had concurrent liver injury, but the severity of liver injury in this animal was not sufficient to cause decreased albumin. The other dog (4501) had no evidence of hepatic injury. Other

possible causes include severe inappetance, diarrhea and kidney disease, none of which were noted during this study.

These results provide very valuable information in the assessment of BOPP as a sensitizing agent for binary therapy. As stated earlier, a sensitizing agent should be of low toxicity and the results of this study suggest that BOPP meets that criteria. The administration of BOPP at this dose resulted in very few adverse effects. Some of the untoward effects of BOPP, such as cephalic thrombosis, may be avoided by changing the method and rate of administration. Vomiting and nausea may be palliated using anti-emetics. The hepatic injury seen in some subjects was found to be mild and may also be transient, but further studies are necessary. Of course, the toxic effects of any agent are dose dependent and the dose of BOPP required for therapeutic effect is not yet known. Thus, if much higher doses are required to provide adequate tumor boron or porphyrin levels then the toxic effects may be more pronounced and may limit the use of this compound. An equivalent dose based on body surface area in humans would be approximately 15 mg/kg. Phase I clinical trials with BOPP as a PDT sensitizer in patients with glioblastoma are scheduled to began in June, 1998 at the Royal Melbourne Hospital. These studies will began with an initial does of 0.25 mg/kg and, when complete, will provide detailed information about the toxicological profile of BOPP administration in humans. More extensive studies of this compound and its toxic effects are necessary in addition to examination of ways to avoid some of the more serious toxic effects (hepatic injury, cephalic thrombosis) seen in this study. Those studies certainly must include administering increasing doses to determine whether the toxic effects found in this study are exacerbated by higher doses, to ascertain which toxic effect is likely to limit the amount of BOPP that can be administered and to determine if other toxicities will become apparent with higher doses. In addition, a more detailed analysis of formulation issues must be undertaken to find an appropriate method of formulation and administration that will avoid problems with thrombosis. Finally, mechanistic studies into the pathogenesis of the

hepatotoxicity and the thrombosis seen with BOPP will provide valuable information not only about this compound but about porphyrin compounds in general.

1.4.2 Boron pharmacokinetics discussion

In evaluating the pharmacokinetics and tissue distribution of a BNCT sensitizing compound it is important to realize that the actual sensitizing agent is the boron , not the porphyrin per se. The amount of boron in the plasma and the tissues are the important factors in determining the utility of this compound for BNCT. Thus, we elected to determine the plasma and tissue boron levels present in dogs as a result of the administration of BOPP. It is therefore possible that the data that is reported in this study is not BOPP alone but also of any possible metabolites of BOPP. Currently, metabolites of the BOPP compound have not been measured or characterized due to the difficulty in obtaining a suitable method for their analysis, but work is ongoing towards the solution of this problem.

Plasma boron concentrations in this study were remarkably consistent from subject to subject (Figure 1.12) Plasma concentrations of boron decreased rapidly soon after administration of BOPP, but later had a much slower decline (Figure 1.12). This phenomenon was reflected in the short initial half-life of 2 hours (Table 1.8).

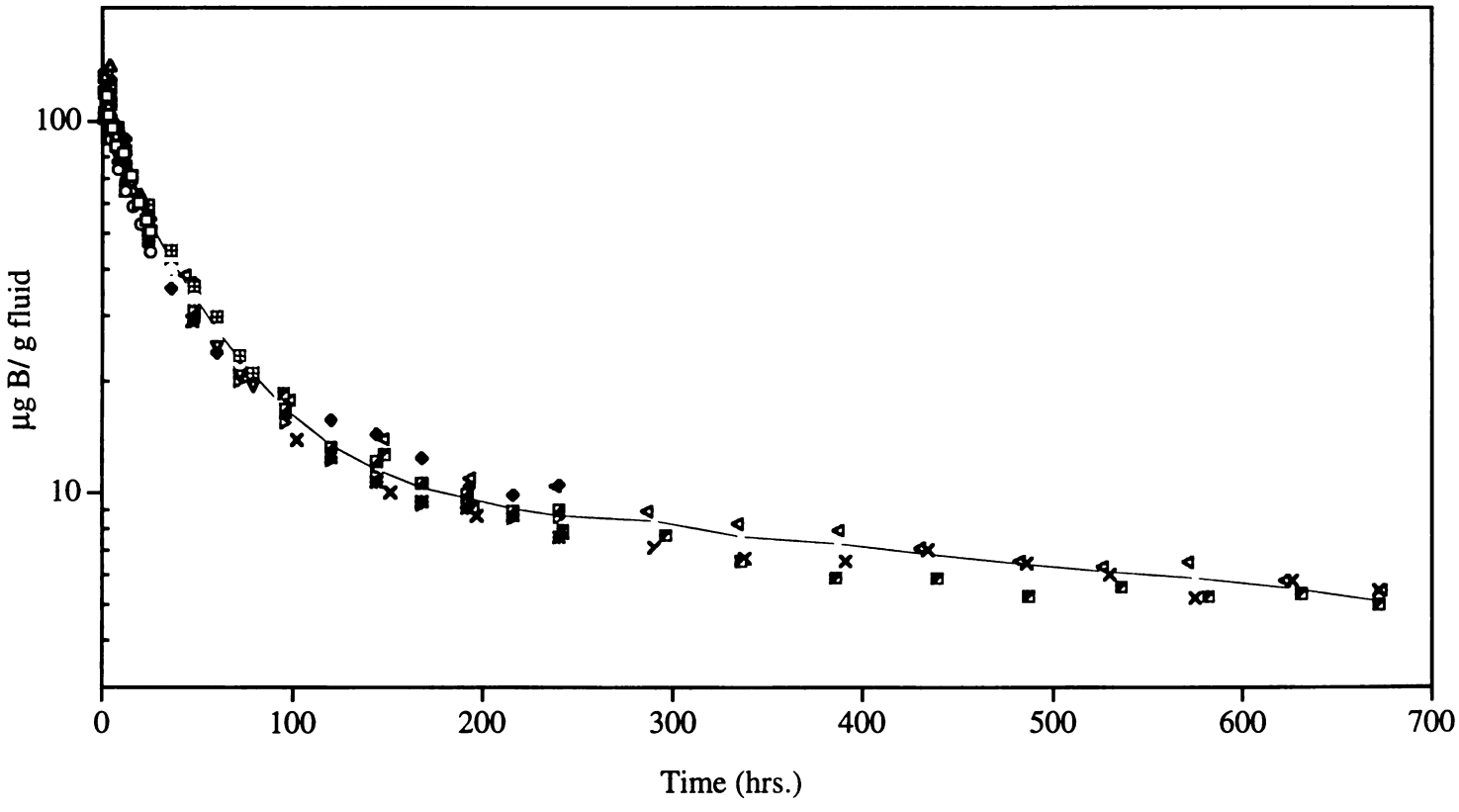


Figure 1.12. Plasma boron concentration following intravenous administration of 35 mg/kg BOPP. Line illustrates fit of data as predicted by NONMEM.

Table 1.8 Values of pharmacokinetic parameters for boron following BOPP administration (± S.E.)

Parameter	
$t_{1/2}$ alpha (hours)	2.0 (0.3)
$t_{1/2}$ beta (hours)	26.8 (1.2)
$t_{1/2}$ gamma (hours)	558.9 (65.8)
V_c (ml/kg)	74.2 (4.6)
CL (ml/hr/kg)	0.90 (0.25)
k_{21} ($hours^{-1}$)	0.00427 (0.000352)
k_{31} ($hours^{-1}$)	0.212 (0.0251)
k_{10} ($hours^{-1}$)	0.0121 (0.00327)

This suggests that boron is initially eliminated or distributed rapidly, followed by a much slower elimination. There are several explanations for this phenomenon. Assuming that BOPP is not rapidly metabolized, it may be undergoing tissue distribution resulting in a rapid decline in plasma concentration. It is also possible that BOPP is being rapidly eliminated causing the same apparent effect on plasma boron concentrations. If, on the other hand, BOPP is undergoing a biotransformation, such as the cleavage of the carborane cages or metabolism of the porphyrin moiety, we might be seeing the distribution and elimination of these products in addition to that of BOPP. Information on the pharmacokinetics of boron and the carborane moiety has not been published. The carborane portion of BOPP is extremely stable in physiologic conditions and is unlikely to be metabolized to smaller units. Any carborane that might be liberated by metabolism would probably be rapidly eliminated by the kidney.

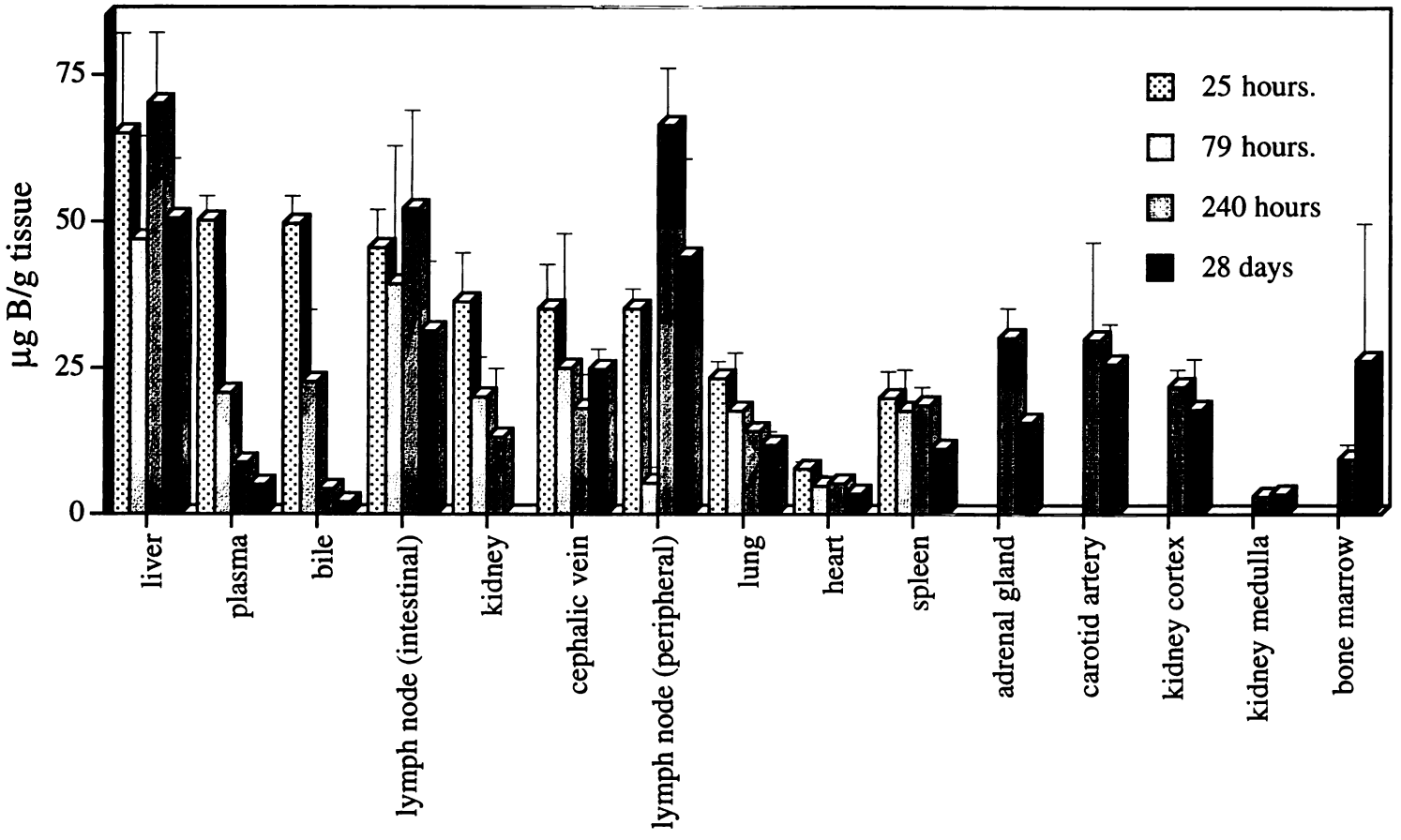


Figure 1.13 . Tissue boron concentrations following BOPP administration. Error bars indicate ± S.D.

The long terminal half-life (Table 1.8) may be the result of several factors. Boron is apparently entering a "depot compartment" and being released into plasma very slowly. Based on boron content (Figure 1.13) and liver mass, we calculate that 672 hours after BOPP administration approximately 30% of the administered dose was in the body with 60% of that in the liver. Thus, it is likely that the liver is acting as the boron "depot" which is consistent with the previously reported data using other porphyrin-based compounds[69, 86, 87, 141]. Binding of BOPP to plasma lipoproteins may also contribute to a long half-life. Porphyrins are known to associate with plasma lipoproteins. The association of porphyrins with high density lipoproteins (HDL), which are thought to have a plasma half-life of 4 days in humans[142], may contribute to an increased plasma half-life for porphyrins, including BOPP. With many conventional drugs, the long terminal half-life seen here might be a serious problem. Because BOPP is likely to be administered once, accumulation of boron that might result from chronic administration is not a concern.

From the standpoint of BNCT, the optimal blood or plasma boron levels are determined by a number of factors, the ratio of tumor to blood boron being most important. Ideally, this ratio should be high. High boron concentrations in tumor tissues allow minimization of the neutron dose. Low levels in the blood/plasma prevent unintentional damage to the vascular system in the local area. These ratios and concentrations are dependent on other factors as well, such as location of the boron within the cell or the blood vessel, or the characteristics of the neutron beam. Thus exact predictions of the desired blood or plasma concentration at the time of neutron treatment cannot be made from this study, but the information gathered will help in making those determinations.

Half-life values of other porphyrin-based compounds have been reported, and most studies also suggested that these compounds exhibit multicompartmental kinetics with a long terminal half-life. In mice treated with ^{14}C-Photofrin, both a two and three compartment model were proposed with terminal half-lives of 220 and 27 hours respectively[69]. In both of these studies, the duration of the study was less than 72 hours,

which may not have been enough time to completely characterize the existence of a third exponential term. Indeed, following serum Photofrin concentrations for 80 days following IP administration resulted in an 870 hour terminal half-life[69]. The initial half-life of approximately 5 hours reported by Pantelides is in contrast with the initial half life found by Bellnier et al. (0.75 hours). Differences in mouse strain may explain some of the discrepancy, but the modeling of the data are also a likely cause of this difference. Tritiated Benzoporphyrin derivative-monoacid, ring A (^{3}H-BPD-MA) was found to have an initial half-life of 10-30 minutes and a terminal half-life of 8 hours in blood in mice[86], although the determination of these values was not well described. The blood concentrations were assayed for 168 hours. There was some indication of metabolism of the parent compound, thus the reported blood values may represent parent and metabolite levels. Lindsay[32] suggested half-lives of 1 day and 4 days for m-THPP in rats, but did not report the half-life of an initial rapid decrease in serum levels. Thus it appears that m-THPP obeys three-exponential serum kinetics. Gervais[87] calculated half-lives of 7.6 and 25 minutes for HPD in rats during a study of approximately 140 minutes. In studies using non-porphyrin photosensitizers, Zuk et al.[39] tested a naphthalocyanine derivative, *iso*BOSINC, to find 2-exponential serum kinetics with half-lives of 2-3 hours and 16-18 hours in mice. And, using plasma data from mice published by Ferrario et al.[42] plasma half-lives for the chlorin NPe6 (mono-l-aspartyl chlorin e6) of approximately 2 and 35 hours were calculated. This is compared to a similar study of this compound by Gomer and Ferrario[43] that reported half-lives of 0.5 and 11.6 hours. Finally, Brown et al.[88] reported a terminal half-life of 452 hours for Photofrin in human patients. Thus, there are tremendous variations in half-lives calculated for these photosensitizing compounds. Factors such as the compound used, the study duration, and the strain and species of animal studied, and the formulation with which the compound was administered can affect the estimates of pharmacokinetic parameters.

For BOPP, two studies provide data that could be used for pharmacokinetic evaluation. Based on the data of Hill et al.[101], we fit the plasma BOPP concentrations (as measured by fluorescence) in mice to a two compartment model. Following a 10 mg/kg dose IV, we calculated half-lives of approximately 3 and 18 hours. For a dose of 100 mg/kg, the half-lives were 4.5 and 45 hours. Ceberg et al.[97] obtained blood boron data following a dose of approximately 35 mg/kg IV in rats. They calculated boron half-lives of 1.7 and 16 hours. (Like the present study, neither of these studies were designed to detect the presence of BOPP metabolites and thus pharmacokinetic parameters reported may represent BOPP and/or its metabolites.) In contrast to the long terminal half-life for boron in the dog (Table 1.8), a long terminal half-life was not seen in the rodents. The duration of the rodent studies may not, however, have been long enough for a third exponential term to be found. The plasma half-lives in the dog were comparable to those seen in rodents. All species exhibited a short initial half-life (2-5 hours) followed by a longer half-life. The limited duration of the rodent studies make comparisons between species of the second and potentially third half-lives difficult.

The initial volume of distribution (V_c) of boron was small (Table 1.8). The amphiphilic nature of the diacid BOPP compound (octanol/water partition coefficient = 4.2 at pH 7.4) probably contributes to this phenomenon. Like other porphyrins[143, 144], BOPP is thought to be extensively plasma protein bound which would also result in a small V_c. For BOPP, the V_{ss} of boron was estimated to be 600 ml/kg, suggesting that boron distributes into tissues (eg. liver) significantly after BOPP administration.

The plasma clearance for boron was found to be low (Table 1.8). A low clearance suggests that the boron is being eliminated from the body at a very slow overall rate. This finding, together with the large V_{ss}, is reflective of the long terminal half-life. Interestingly, the plasma clearance of Photofrin in humans, 0.91 ml/hr/kg[88], is also relatively low. The slow decrease in plasma boron concentration results in a large area under the plasma vs. time curve (AUC) which is directly related to the clearance

(CL=Dose/AUC). The total AUC for boron can be divided into portions that represent the AUC for each individual phase of the three exponential plasma kinetic profile. These partial AUCs provide some insight into the contribution to the entire AUC provided by each exponential term. The contribution to the total AUC of initial, middle and terminal elimination phases were estimated to be: 1.8%, 24.3% and 73.9%, respectively. Thus, the majority of the AUC can be attributed to the terminal phase of elimination. Approximately 30% of the total estimated AUC was extrapolated from the final plasma boron concentrations using the formula:

$AUC_{extrap.} = C_{last}/k_{gamma}$

where $AUC_{extrap.}$ is the extrapolated AUC, and C_{last} and k_{gamma} are the final plasma concentration and the terminal phase elimination rate constant, respectively. The terminal phase of the curve, with its large AUC, has a strong influence on the pharmacokinetic parameters that are dependent on AUC. One of these parameters is clearance (see Appendix I) As a result, clearance may not provide accurate reflection of the pharmacokinetic behavior of boron in this study except for its indication of a low overall rate of boron elimination.

Our study showed that the boron associated with the administration of BOPP was concentrated in the liver, lymph nodes, spleen, and adrenal glands. This is consistent with the results of numerous studies on porphyrin-based therapeutics and their tissue distribution[69, 80, 86, 87, 95, 98, 141, 145-148]. Often the accumulation of these compounds, and possibly their metabolites, is quite high and the duration of their persistence very long. Bellnier[69] found that 10% of administered radioactivity was present in mouse livers 75 days following ^{14}C-Photofrin administration. As early as 1938, Smetana[98] found that most of a dose of hematoporphyrin administered to dogs was taken up by the liver. Zawirska provided extensive data[89-93]on the distribution of porphyrins in the tissues of humans with porphyric diseases. Porphyrin concentrations were found to be elevated not only in the liver, kidney and spleen, but also in the adrenal glands. The mechanisms for

this distribution are not well understood. Liver uptake of protoporphyrin is thought to be via simple or facilitated diffusion[149], followed by metabolism or excretion into the bile. Studies of the mechanism of hepatic uptake of other porphyrins have not been published.

The tissue distribution and tumor uptake of porphyrins may be strongly related to their plasma protein binding behavior. Most porphyrin compounds are protein-bound in plasma[150]. While the importance of plasma protein binding in tumor uptake of porphyrins has been actively discussed[73, 151-154]; the role in normal tissue uptake of these compounds has been less well examined. The most important plasma proteins involved in transport and uptake of porphyrins are albumin and the lipoproteins. Studies of the binding of porphyrins to plasma proteins have suggested that increasing hydrophilicity of porphyrins leads to greater binding to albumin, while more lipophilic porphyrins tend to be associated with the lipoprotein fraction[34, 65]. The exception seems to be porphyrins with asymmetric charge distributions that allow a hydrophobic region of the molecule to associate with lipoproteins[65]. Also, aggregation of the porphyrin can have an effect on which plasma protein will bind that porphyrin. It is also an asymmetric diacid, likely to be in the di-anionic state at physiologic pH. At this pH, a significant amount of BOPP may be dimerized[155]. Thus, it is thought that BOPP may be associated primarily with the lipoprotein fraction of the serum[155].

Low density lipoproteins (LDLs) have been suggested as important carriers of photosensitizing agents[34, 62, 144, 151, 156]. Due to the increased number of LDL receptors present on many tumor cells, LDL-receptor mediated uptake of porphyrins has been postulated as a mechanism by which these compounds achieve selective uptake into cancerous tissue[28, 64, 67, 156] Indeed, both *in vitro* and *in vivo* studies of porphyrin-LDL complexes have shown enhanced uptake of the porphyrin-LDL complex when compared to non-complexed porphyrin[26-28, 62, 73, 157]. Other mechanisms for uptake of photosensitizers have been proposed[158], and this issue is still being resolved. For the purposes of this study, the binding of BOPP to LDL would help to explain its distribution

in normal tissues. The liver, spleen, kidney and adrenal glands are known to express high levels of receptors for apolipoprotein B (apo-B,E receptors), the predominant lipoprotein in LDLs,[159] and would be expected to accumulate compounds that are LDL-bound[151]. Although dogs have no apo-B,E receptors on the hepatic membrane, they do have HDL receptors (apo-E receptors)[160] on these membranes. Dogs are considered "HDL animals" meaning that the majority of their lipoproteins consist of apolipoprotein-E containing HDLs[161]. Like LDLs, HDLs may act as carriers of BOPP and other porphyrin-based photosensitizing agents to the liver and the kidney. It has been noted that LDLs that have been modified (methylation of the lysine residues on the apolipoprotein) resulted in rapid uptake of these LDLs by the liver of dogs[162]. Thus, LDLs recognized as abnormal may be cleared by the liver without receptor-mediated mechanisms. Porphyrins have been shown to bind to HDLs[34, 64, 65, 144, 156], thus a HDL-receptor mediated mechanism for uptake of porphyrins into the liver of dogs may exist. In addition to the liver, the kidney is reported to be another site of HDL uptake and catabolism[142]. Lipoprotein receptor populations in dog tissues other than liver are not known, so conclusions regarding uptake by these mechanisms are difficult to make. It appears that the distribution of boron in normal tissues may be related to the presence of lipoprotein receptors on those tissues. For example, uptake into the liver may involve the binding of BOPP bound HDLs to apo-E receptors on hepatocytes while accumulation in the spleen, adrenal and kidney may be related to BOPP binding to LDLs and subsequent uptake of this complex by cells expressing the apo-B,E receptor.

An alternative pathway for LDL degradation is a nonspecific "scavenger" pathway by which the LDLs are taken up by scavenger cells or macrophages in the reticuloendothelial system[142]. This pathway is thought to function only at times of elevated plasma levels of LDL. Hamblin and Newman[73] have advanced this theory in discussions regarding the mechanism for localization of porphyrin-based photosensitizing agents in tumors. This group hypothesized that the preferential uptake of porphyrins by

tumors is related to the increased uptake of porphyrins by the phagocytic cells present in those tissues. In studies by Hamblin[163] and Korbelik et al.[164], it was demostrated that phagocytic cells are capable of accumulating significant amounts of porphyrins. This phenomenon may also explain the uptake of porphyrin-based imaging agents by the lymphatic system[165] and the accumulation of HpD and Photofrin® in the lymph nodes of mice[166]. While this system has not been studied in the dog, its presence could help explain the high levels of boron in the lymph nodes, spleen and liver, tissues rich in phagocytic potential.

Albumin binding and uptake may play a role in the tissue distribution of BOPP as it does in numerous porphyrins, both endogenous[150, 167]and therapeutic[63, 65, 73]. Albumin-porphyrin conjugates are taken up by phagocytic cells[73]. Thus, albumin binding and subsequent uptake by phagocytic cells might explain some of the tissue distribution of BOPP, especially the high concentrations found in the macrophage-rich tissues of the liver, spleen and lymph nodes. This route may be important in the uptake of BOPP which has been shown to form dimers at concentrations seen in the plasma of the dogs used in this study[155].

Porphyrins binding to stromal elements in tissue is another variable in tissue distribution. Musser et al.[168] found substantial binding of porphyrins with collagen, especially immature acid-soluble collagen, and with elastin. El-Far and Pimstone[72] found that Photofrin and HpD bound best to collagen, with poor binding to fibrin and elastin. The binding of porphyrins to these proteins may influence their tissue localization properties, but the details of this phenomenon are not well-known and it is not possible to predict the tissue binding of BOPP based on this information.

An important finding in this study is the very low levels of boron found in cranial tissues. This finding is crucial in assessing the utility of BOPP as a BNCT sensitizing agent. One of the most important criteria for the suitability of a sensitizing agent for BNCT is the absence of high levels of boron in normal tissues surrounding the tumor. For brain

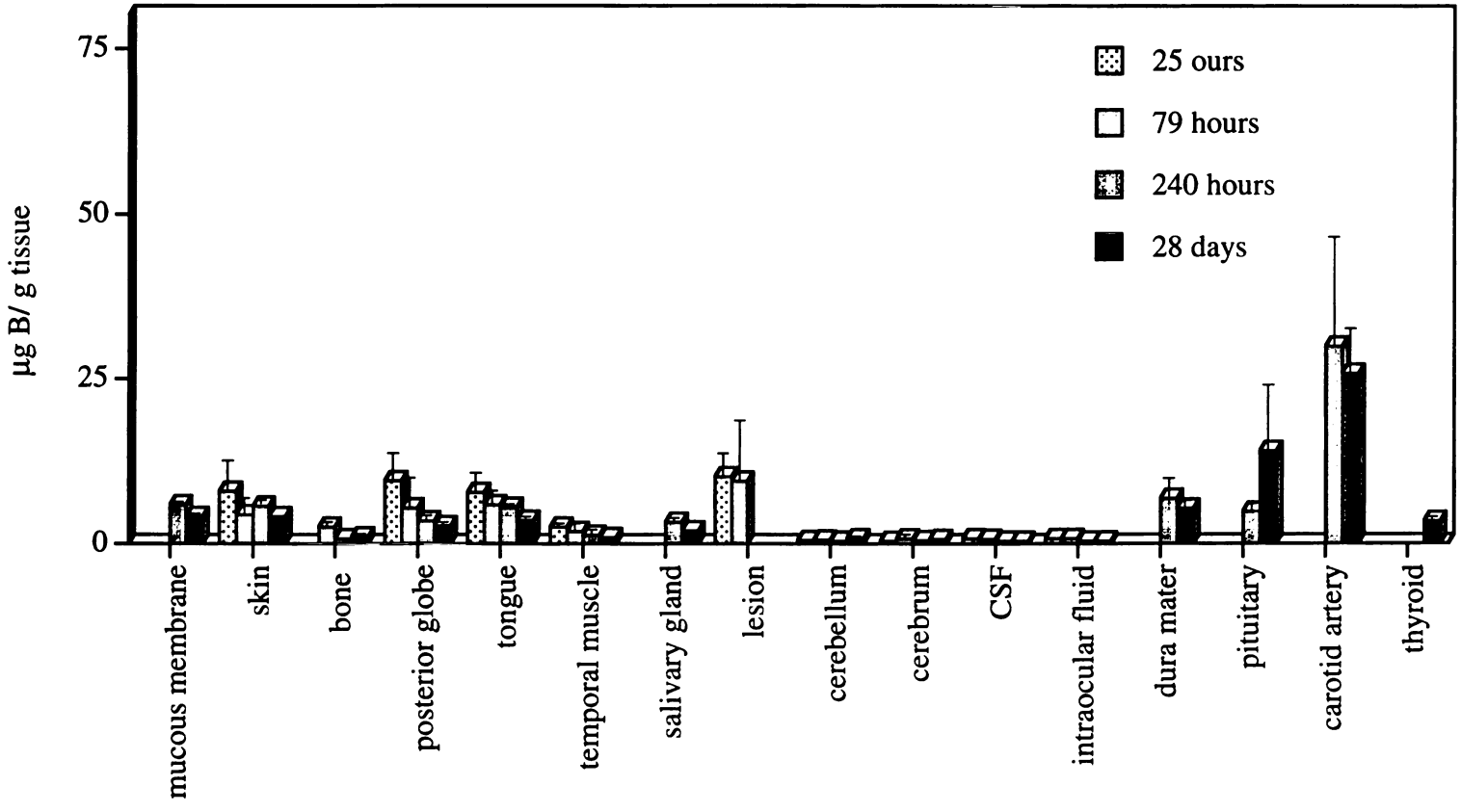


Figure 1.14. Cranial tissue boron levels following BOPP administration. Error bars indicate ±S.D.

tumors, the local normal tissues would include the brain, skin, bone, eye and others. As can be seen in Figure 1.14, most tissues of the head exhibited very low boron levels at all times following BOPP administration. While high levels were found in the lymph nodes, damage to these tissues would not preclude treatment of the head in BNCT. These data provide very encouraging results regarding the potential for the use of BOPP as a BNCT sensitizing agent for brain tumors.

The high levels of boron found in the carotid artery at 10 days and 28 days following BOPP administration were unanticipated and should be viewed with some concern. High levels of boron in blood vessels may lead to severe damage to these structures upon treatment with neutron beams. The mechanism of this accumulation of boron may be related to several properties of arterial wall tissue.

The accumulation of porphyrins in arterial tissue has been reported for one other compound. Uptake of benzoporphyrin derivative was seen in normal arteries following administration to rabbits[29] The authors suggest that the uptake was related to benzoporphyrin binding to LDL with subsequent uptake and retention in the vessel. The benzoporphyrin in this study was administered as a lipoprotein or liposome conjugate and absolute concentrations of BPD in normal arteries were not reported. Carotid artery uptake of benzoporphyrin derivative was reported in swine[30]. Normal swine vessels were found to contain ng/g levels of BPD following a 2 mg/kg dose, much less than the amounts (μg/g) seen in the dogs administered BOPP. While these studies suggest that porphyrins are capable of entering and accumulating in vessel walls, the details of this phenomenon have not been studied.

The endothelium lining the lumenal wall of the artery is permeable to both large and small molecules, including proteins as large as lipoproteins and albumin[169]. Entry into the vessel wall appears to be via size limited passive diffusion or transcytosis[170]. Permability can be affected by injury to the endothelial cells as a result of lethal or sublethal injury. Inflammatory mediators and toxins are known to cause a mild injury sufficient to

increase permeablilty[171]. Thus, BOPP may act to injure the endothelium and lead to an enhancement of vessel wall permeability. As BOPP crosses this barrier, it may be unbound or bound to plasma proteins. Upon entering the wall of the artery, proteins can continue to migrate through into the periphery, or be accumulated in the wall[172]. The accumulation of lipoproteins, especially LDLs, in the vascular intima (Figure 1.15) is part of the pathogenesis of atherosclerosis, although the details of this accumulation are still under intense study[171]. It has been shown that adrenaline can increase the accumulation of LDL in rat aorta[173]. Thus, stress on the dogs in this study may have had the effect of increasing the accumulation of BOPP-LDL complexes.

Once in the intima, a number of factors determine the fate of lipoproteins: (i.) the capability of the smooth muscle cells and macrophages to degrade these complexes; (ii.) binding of the lipoproteins to the stroma of the intima and; (iii.) the ability of the lipoproteins to migrate back into the circulation. LDLs either return to the circulation or are degraded by smooth muscle cells or macrophages. In the pathogenesis of atherosclerosis, LDLs bind to the stromal elements of the intima and are subsequently engulfed by macrophages[171]. The reasons for this engulfment may be related to oxidation of the LDLs. Oxidized LDLs have been shown to have increased accumulation in arterial walls[174], but whether these LDLs become oxidized within the vessels or the intima is not known. The formation of oxidized LDLs can take place in *vivo* (by cells) or in *vitro* (using transition metals, etc.)[175]. BOPP binding to LDLs may influence its susceptibility to oxidation, or cause BOPP to be "handled" like an oxidized LDL. In addition to their increased uptake by phagocytic cells, oxidized LDLs have been shown to exhibit toxicity to endothelial cells resulting in increases in permeability[171, 176]. In summary, BOPP bound to LDLs may be affecting LDLs either in the vascular system or in the intima resulting in increased toxicity to endothelial cells, increased permeability across the endothelial layer or increased uptake by macrophages in the intima.

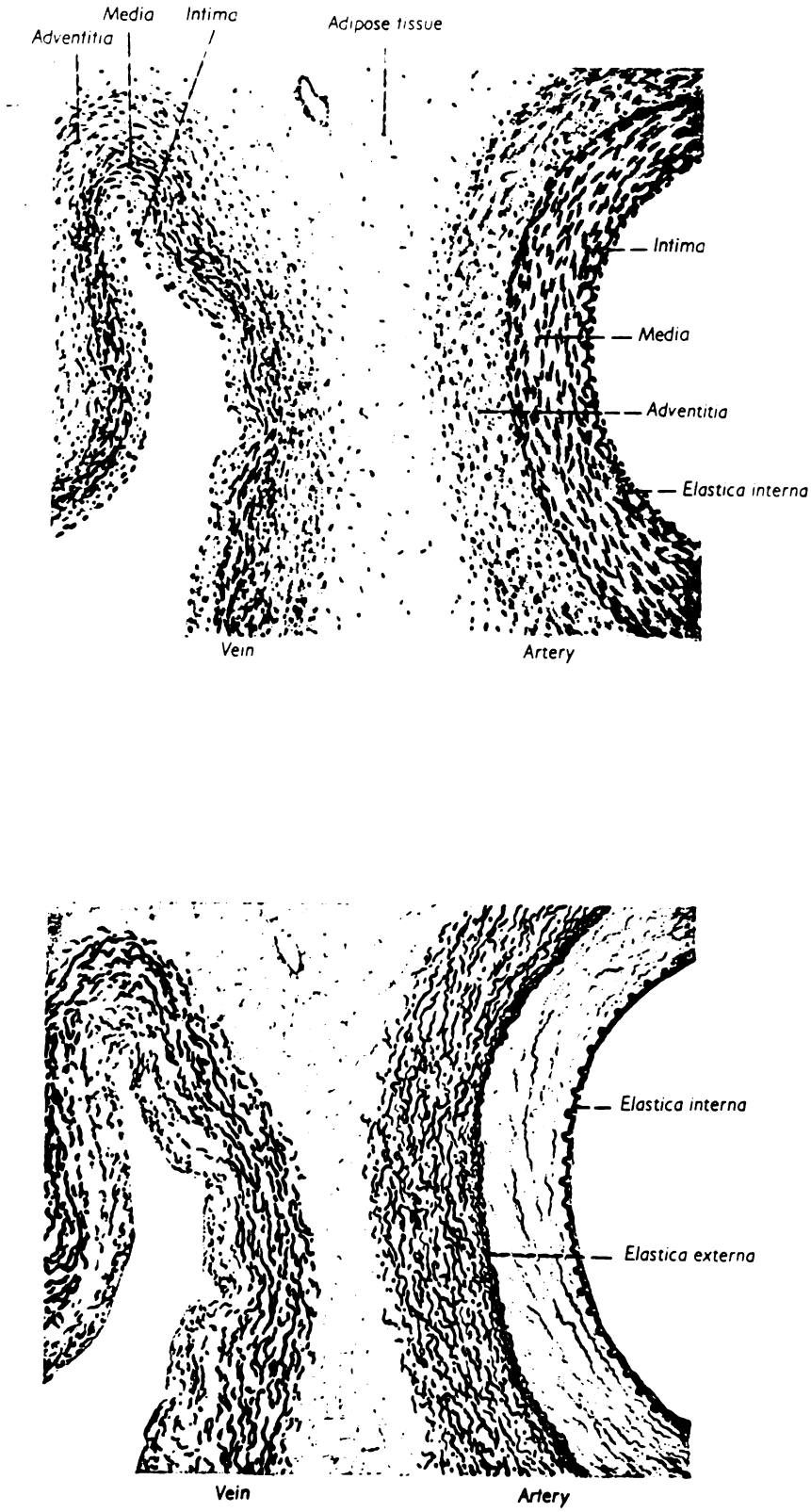


Figure 1.15. Cross-section of normal artery and vein. Upper panel shows hematoxylin-eosin staining which stains cell nuclei, lower panel shows resorcin-fushcin which highlights collagenous fibers. From Hammersen, F. (au.) *Histology.* Urban and Schwarzenberg, Inc. Baltimore, 1980, p. 108.

The accumulation of BOPP in the carotid artery may be related to the ability of BOPP to bind to components within the blood vessel. Porphyrins have been shown to bind to collagen and elastin[168]. The extracellular matrix of large vessels are rich in collagen and elastin[177]. Thus, BOPP that enters this millieu may bind to these proteins and be accumulated. Plates 1.1-1.2 show sections of carotid artery from dogs 4480 and 4481. These samples were obtained 28 days following BOPP administration. The fluorescence caused by porphyrin (red color in plates 1-upper, 2-upper) appears to be localized in the adventitial layer of the vessel (See Plates 1.1-lower, 1.2-lower, and Figures 1.15 for reference). This would support the theory of porphyrin binding to collagen and elastin[168], which are abundant in this layer. The presence of BOPP-associated porphyrin in this area of the blood vessel would pose little threat of serious damage upon irradiation with light or neutrons due to the sparse population of cells. While this data is encouraging, it is not clear from this data whether the porphyrin accumulates only in the adventitia or whether other layers of the blood vessel contain porphyrin at times less than 28 days.

The accumulation of boron in blood vessels may be due to a combination of the previously discussed mechanisms, and certainly merits further study in light of the potential damage that may result from neutron activation of blood vessels containing high levels of boron. An important part of this study will be examination of blood vessels at various times following BOPP administration to more completely characterize the location and mechanism of porphyrin accumulation.

The study of the pharmacokinetics and tissue distribution of boron in the dogs following BOPP administration has led to some promising results in the utility of BOPP as a sensitizing agent for BNCT and PDT. The pharmacokinetics have shown a rapid initial decline in plasma concentrations, with a long terminal half-life. The initial decline will be beneficial in allowing the plasma boron levels to decrease to a level allowing neutron activation of the boron without damage to tissues rich in blood. The long terminal half-life

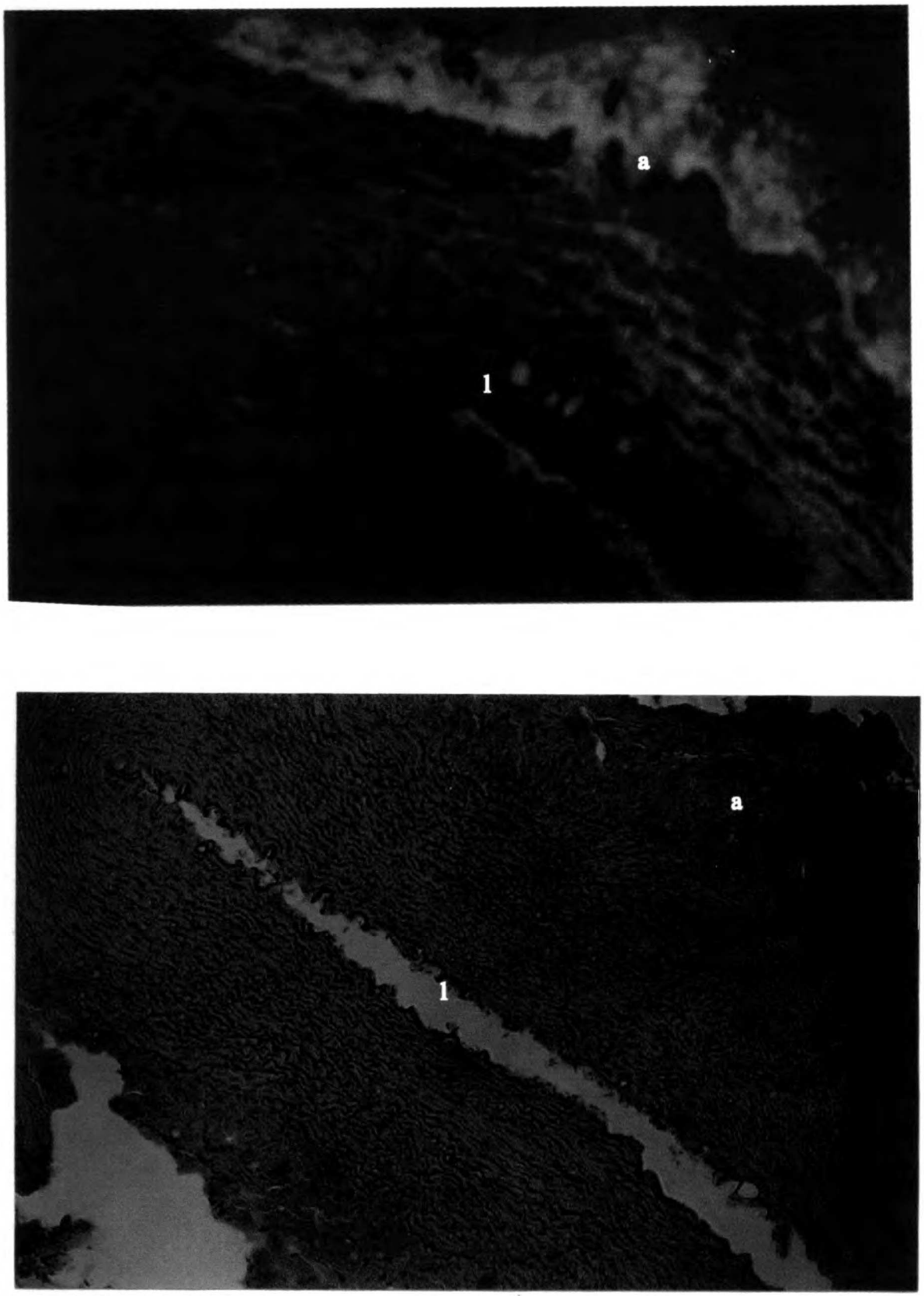
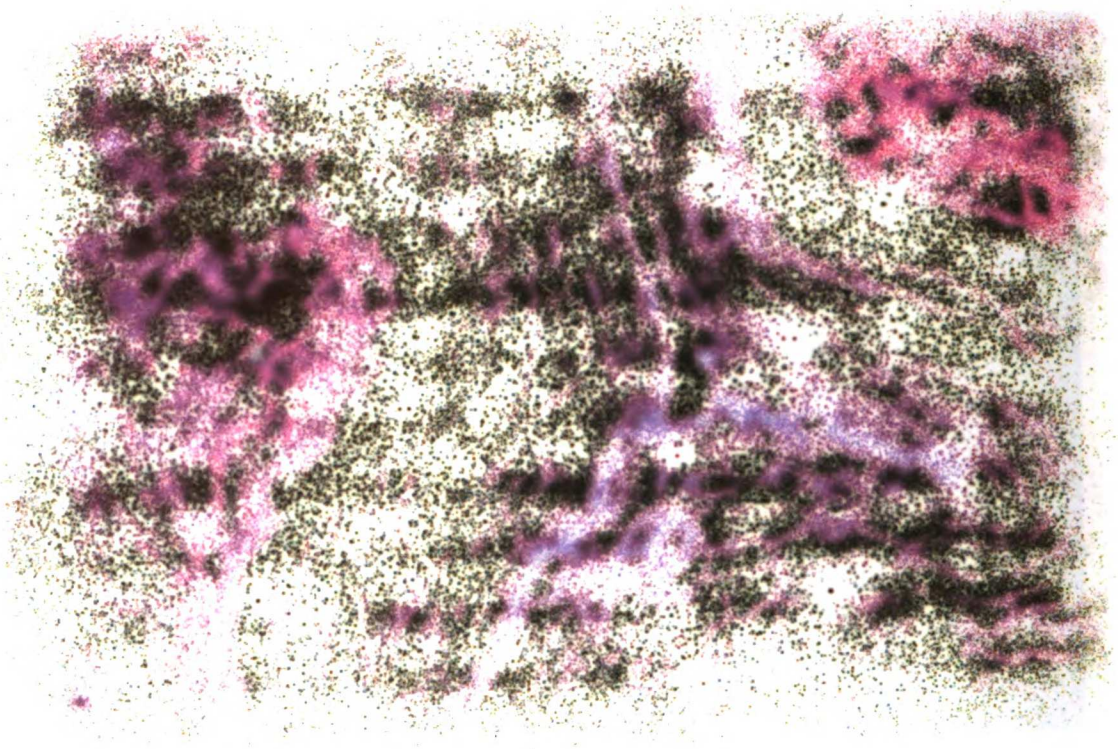
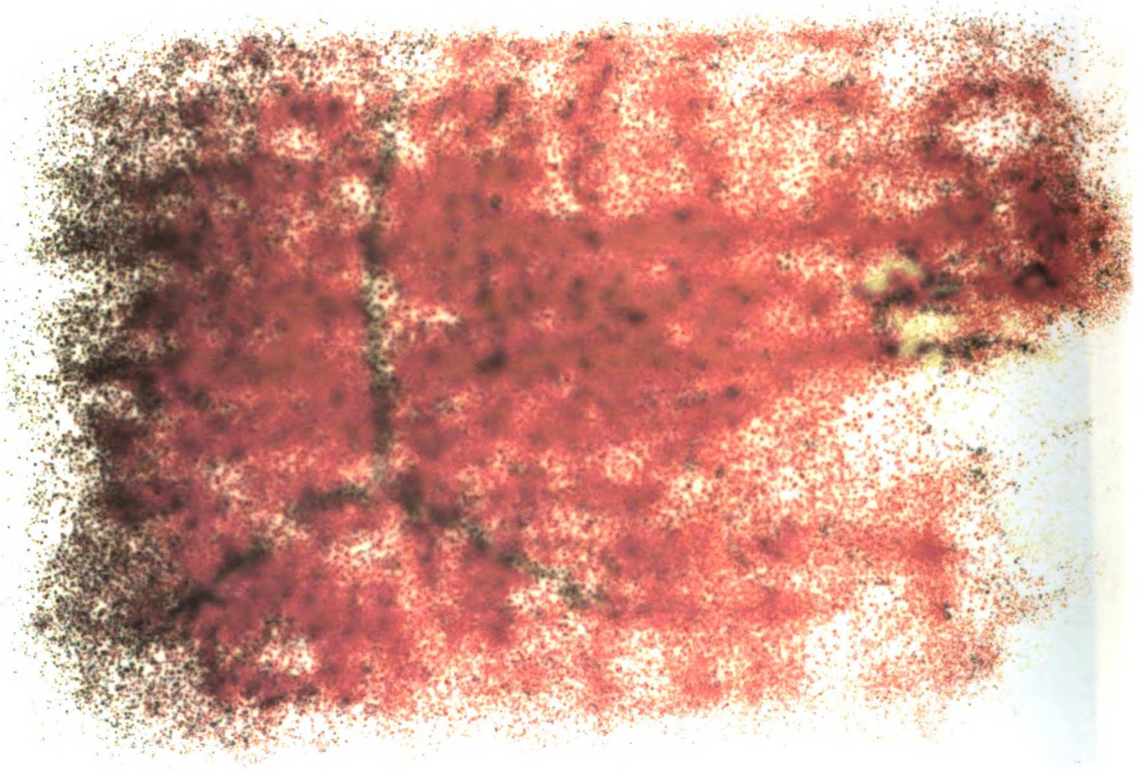


Plate 1.1 Cross section of dog carotid artery 28 days following BOPP administration.
Upper plate UVfluorescence microscopy, lower plate hematoxylin/eosin stained light microscopy
"l" indicates vessel lumen, "a" indicates adventitial tissue





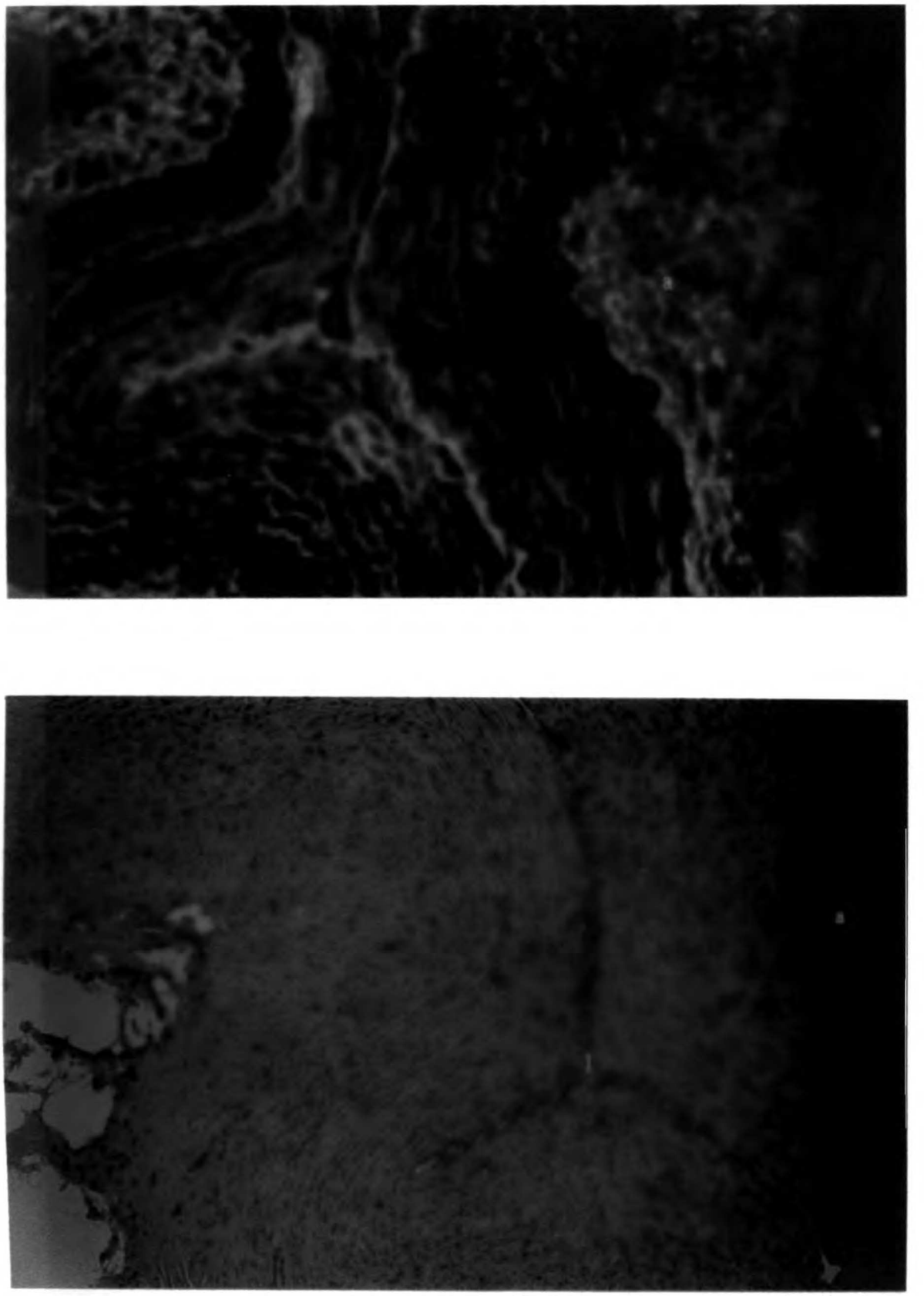


Plate 1.2 Cross section of dog carotid artery 28 days following BOPP administration.
Upper plate UVfluorescence microscopy, lower plate hematoxylin/eosin stained light microscopy
"l" indicates vessel lumen, "a" indicates adventitial layer

should not be a problem when one considers that the important therapeutic issue at the time of neutron treatment is the ratio between tumor and blood. Thus, if tumor levels are sufficiently high, blood can contain low levels of boron without causing significant adverse effects. For PDT, high levels of BOPP in the blood may be a distinct advantage due to the damage that would result to the blood supply of the tumor upon light activation. Tissue distribution results are generally promising. The presence of high levels of boron in tissues of the body cavity should not preclude the use of BOPP as a BNCT sensitizing agent in the treatment of peripheral tumors. It should also have no effect on the use of BOPP as a PDT sensitizing agent against tumors in most tissues. The low levels of boron in most tissues of the head is also encouraging. Extremely low levels in normal brain, skin and bone are of particular importance in the use of BOPP as a BNCT sensitizer for brain tumors. On the other hand, the high levels noted in the carotid artery may be detrimental to the use of BOPP for BNCT. High levels of boron in vascular tissue at the time of neutron irradiation may be very damaging to the normal tissues surrounding the tumor. Conversely, because of the more limited area of activation this accumulation, and the potential for damage to the vascular supply to the tumor, may be beneficial in the use of BOPP as a PDT sensitizer. Certainly, the key to determining the utility of BOPP for either application will be the determination of the levels of uptake of this compound into the target tissue, the tumor. If high tumor levels can be achieved and maintained, the uptake into vasculature and the retention of small amounts of boron in the plasma may not be problematic at the time of activation by light or neutrons.

CHAPTER 2.
IN VIVO HEMOSTASIS FOLLOWING BOPP ADMINISTRATION

2.1 INTRODUCTION:

Upon discovery that the administration of BOPP resulted in thrombosis of the cephalic vein in some dogs, we became interested in the cause of this problem and the potential for systemic effects. The cause for localized thrombosis can be quite complex, but a brief review of the hemostatic mechanisms will provide the necessary background for this discussion. The mechanisms by which a clot forms are defined by four major components.

The first component is the coagulation cascade, which is a stepwise series of proteolytic reactions whereby inactive proenzymes are converted to active enzymes which then act on the next proenzyme in the cascade[178]. The proenzymes in this system are produced primarily in the liver and are present in the plasma; they are designated with Roman numerals (Figure 2.1). Classically, this system is divided into an intrinsic and extrinsic pathway. These two pathways converge in a common pathway that results in the cleavage of fibrinogen by thrombin to form fibrin. Current knowledge suggests that there is significant interaction between the intrinsic and extrinsic pathways, but this classification remains useful due to its clarity[179]. The various components of the intrinsic pathway are found in the circulating blood. The activation of the pathway begins with the adsorption of factor XII and kininogen to sites of vascular damage[179]. This activates factor XII, setting the remaining cascade in motion. The extrinsic pathway is initiated by tissue factor, which is expressed on the surface of fibroblasts, white blood cells and endothelial cells[179]. Tissue factor on endothelial cells is expressed in response to cell injury or other noxious stimuli[180] and its presence is responsible for the rapid formation of thrombi. Both pathways lead to the common pathway, which is usually defined as beginning with the generation of activated factor X, which is pivotal in the generation of thrombin from prothrombin. Thrombin has several actions in the hemostatic system. Its

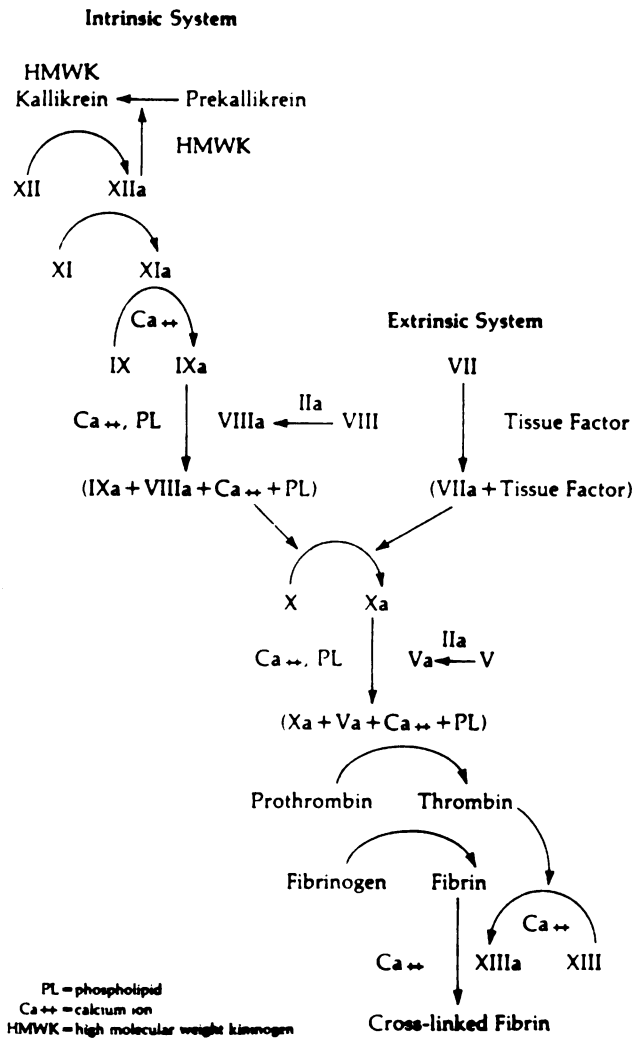


Figure 2.1. Simplified schematic of blood coagulation cascade. From Hougie, C. The biochemistry of blood coagulation. In Triplett, D.A. (ed.)*The laboratory evaluation of coagulation*, ASCP Press, Philadelphia, PA, 1982, p. 3.

principal function is to cleave fibrinogen to fibrin, but it is also able to induce platelet aggregation, cleave and activate factor XIII, can affect the fibrinolytic mechanisms[181] and has various feedback roles in the coagulation cascade[182]. The coagulation cascade is a highly complex system, linked with the platelets, endothelial cells and the fibrinolytic system.

The second component of clot formation is the endothelial cell (EC). These cells line the blood vessels and under normal circumstances act to prevent thrombosis[183]. Unperturbed ECs have potent activity preventing the activation and adhesion of platelets to the vessel wall[182]. ECs produce several receptors and factors that can inhibit the soluble factors (such as factor IX, X and thrombin) that allow blood coagulation[181, 182]. ECs also release substances that prevent fibrin formation on their surface[182]. When perturbed or injured endothelial cells may retract or die which exposes the underlying subendothelium to the circulation. The subendothelium is a highly thrombogenic surface, consisting of collagen and von Willebrand factor (vWF), a protein synthesized by endothelial cells that plays a role in platelet adherence to vessel walls and also serves as a carrier for factor VIII[184]. A less severe injury to the endothelial cells results in a number of changes in EC function and activity. Injured ECs express tissue factor on their surface[181], resulting in the initiation of the extrinsic coagulation pathway. Enhanced activity of endothelial factor V also increases thrombin formation[181]. Disturbed ECs can express an activator of factor XII, resulting in the activation of the intrinsic pathway[183]. Other effects on the coagulation pathway and the fibrinolytic pathway have been reported as well[183]. A number of factors have been identified that are capable of causing insult to ECs. These factors are both endogenous (interleukin 1, thrombin) or exogenous (infection, endotoxin, mechanical injury).

The third component of the clotting mechanism is the platelet. Platelets are circulating cells that are produced in the bone marrow[127]. Platelets are non-nucleated and have numerous granules that contain various hemostatic factors[179]. The role of platelets

in the formation of thrombi involves both their adherence and mechanical action, and their chemical interaction with the cells and proteins in the forming clot. Platelets adhere to exposed subendothelial components in the injured blood vessel[182]. These components consist of collagen and vWF which bind to receptors on the platelet surface. The binding to the subendothelium results in a shape change in the platelets and the release of their granular contents[179]. Platelets also undergo aggregation with each other upon binding to collagen[182]. The aggregation and release of granule contents has a strong positive feedback effect on further platelet aggregation, the coagulation cascade, inflammation (via the release of prostaglandins and thromboxanes) and vascular constriction[179, 182].

The fourth component of the clotting mechanism is the fibrinolytic system. Fibrinolysis is the dissolution of fibrin. This system counteracts the very efficient coagulation cascade and prevents uncontrolled deposition of fibrin in the vascular system. More importantly, the fibrinolytic system is involved in the repair of damaged vessels. The active fibrinolytic enzyme in this system is plasmin. Plasmin, when activated, can lyse fibrin to fibrin degradation products (FDPs), cleave fibrinogen to inactive fragments, and degrade factors V and VIII[182]. Thus plasmin has several roles in counterbalancing the coagulation cascade. Plasmin circulates in the vascular system as an inactive proenzyme, plasminogen. Plasminogen can only be activated at sites containing fibrin and fibrin-bound plasminogen activator[179]. In addition, there are circulating inhibitors of plasmin that prevent the enzyme from having systemic effects. Plasminogen activators are released by vascular cells into the circulation, and also are released into the forming fibrin clot during thrombosis[182]. The binding of plasminogen to fibrin in the presence of plasminogen activator allows the proteolysis of plasminogen to active plasmin. This local activation, in addition to the presence of circulating plasmin inhibitors, ensures that plasmin activity is limited to areas affected by the formation of a fibrin clot.

The hemostatic system is therefore an intricate and exquisitely balanced mechanism. The formation of abnormal thrombosis can occur as a result of perturbations in any of the

parts of the hemostatic system. Studies of porphyrin based compounds and their relationship with the hemostatic system has focused largely on hematin. Hematin (hemin hydroxide) is an iron protoporphyrin IX compound and is the product of the treatment of hemin with Na_2CO_3[85]. Hematin in solution has been used for decades in the treatment of porphyric illness and has been seen to cause coagulation abnormalities in humans to whom it is administered intravenously. These effects include: thrombocytopenia, endothelial cell effects and clotting factor alterations[85]. Despite the large volume of literature on the use of porphyrins in animals and humans, no studies have reported clinical or other effects on the hemostatic system. BOPP, while not a iron-containing porphyrin, has shown some effects similar to those seen in humans administered hematin. Both dogs adminstered BOPP and humans administered hematin exhibit decreases in platelets[85] and thrombosis[115]. A single report by Anderson et al.[119] describes the toxic effects of the administration of hematin in dogs. That study found significant renal, reticuloendothelial and vascular toxicity. Vascular changes included hemorrhages and thromboses. Thus, based on the similarity of the vascular effects seen in vivo the effects of BOPP may resemble those of hematin .

As a first step in evaluating the effects of BOPP on the coagulation system, studies were performed to examine the hemostatic cascade following BOPP administration. The tests performed were chosen to evaluate the extrinsic, intrinsic and common pathways of the coagulation cascade. In addition, assays of fibrinogen levels and other coagulation proteins were performed to provide further information regarding the effects of BOPP on the hemostasis.

2.2 MATERIALS AND METHODS:

Samples for hemostatic analysis were obtained from 8 dogs, all of which were 240 and 672 hour subjects. Dogs were sampled prior to and at several time points following BOPP administration (Table 1.1). Samples were obtained by jugular venipuncture using a

polypropylene syringe containing the appropriate volume of 0.102 M sodium citrate solution drawn out of blue top Vacutainer® tubes. The blood sample was then placed into the blue top tubes from which the sodium citrate was drawn. Samples were centrifuged at 2000 rpm (approximately 20*g*) for 15 minutes and decanted via plastic pipette into polyethylene tubes. These samples were frozen at -70° C until use. Tests performed on these samples included those listed in Table 2.1. Samples were submitted to Consolidated Veterinary Diagnostics (CVD) or the Comparative Hematology Section, Diagnostic Laboratory/Cornell University (Cornell) for analysis.

Table 2.1 Hemostatic tests performed

Consolidated Veterinary Diagnostics, West Sacramento, CA	Comparative Hematology Laboratory, Cornell University
Activated Partial Thromboplastin Time	Prothrombin Time
Thrombin Clot Time	Thrombin Clot Time
Fibrinogen (functional)	Fibrinogen (immunologic assay)
	Factor VII:C (Factor VII coagulant activity)
	von Willebrand Factor antigen (vWF:Ag)
	Fibrin degradation products (FDP)
	Soluble fibrin monomer complexes

Eight dogs were tested for the presence of fibrin degradation products. Samples for fibrin degradation product (FDP) were drawn via vacutainer needle directly into the appropriate (thrombin/soybean trypsin inhibitor containing) tubes. Samples were refrigerated (4° C) until analysis. FDP samples were submitted to Cornell for analysis by the latex agglutination assay that is semiquantitative for the detection of fragments X,Y, X oligomers, D, and E. Lower limit of detection is 2.5 μg/ml.

Two dogs were tested for the presence of antithrombin III (ATIII) in the plasma. These samples were obtained in the same manner as those obtained for routine hemostatic tests. These samples were submitted to Cornell for analysis. ATIII measurement is via a colorimetric, chromogenic substrate assay and is reported as percent (%) activity.

Samples from four dogs were obtained to test for the presence of soluble fibrin in plasma. Again, samples were prepared in same manner as those for routine hemostatic tests and submitted to Cornell for analysis. Soluble fibrin assay was performed using a hemagglutination assay which is semi-quantitative Reaction strength was graded as 0, trace, 1, 2, 3.

As a control, blood samples from two untreated dogs were obtained as described and submitted to Cornell for hemostatic analysis.

Statistics

Due to the presence of a test-defined maximum value, data for TCT, APTT, PT, and vWF are reported as median values with error bars indicating the reported range of values. Sample numbers for the soluble fibrin monomer test were small, thus the data are presented as individual subjects data over the time of the study. This allows for better visualization of the changes in soluble fibrin in individual animals.

2.3 RESULTS:

The administration of BOPP to dogs resulted in significant derangements in the clinical hemostatic parameters. Figure 2.2 shows the effect of BOPP treatment on the prothrombin time (PT) and activated partial thromboplastin time (APTT) and the thrombin clot time (TCT). The PT is performed by adding calcium and whole tissue cell phospholipoprotein membranes (thromboplastin) to the citrated sample. The time required for clot formation is then measured. The purpose of this test is to evaluate the extrinsic and common coagulaton pathways. Prolongation of this test indicates a disorder in one or both of these pathways[185]. The APTT is performed by the addition of calcium, phospholipids (partial thromboplastin), and a negatively charged contact factor (celite or kaolin) to citrated plasma. The time required for clot formation is then measured. This test is designed to detect abnormalities in the intrinsic or common pathways of coagulation[185]. TCT is simply the addition of thrombin to plasma with the measurement of time until clot formation[185]. Prolongation of this test suggests abnormalities in the level of fibrinogen or the activity of thrombin.

Figure 2.3 shows the median fibrinogen levels in dogs following BOPP administration. Fibrinogen was assayed in two ways. Cornell performed a ELISA-based assay that is designed to determine the total amount of fibrinogen in the plasma. The assay performed by CVD is a modification of the TCT that relies on the ability of thrombin to cleave fibrinogen. This assay provides a measure of the functional fibrinogen in the sample. As seen in Figure 2.3 the fibrinogen levels, as measured by both assays, were decreased following BOPP administration. By 48 hours following BOPP, the levels had returned to near their pre-treatment levels.

von Willebrand factor antigen levels following BOPP administration are reported in Figure 2.4. vWF:Ag levels are decreased immediately following BOPP administration and exhibit a strong rebound by 24-48 hours after administration. It was noted that this strain

of beagles had a low level of vWF:Ag prior to BOPP treatment. This was not thought to influence the effect of BOPP on this factor[186].

Fibrinogen degradation products (FDPs) were not found to be increased in any sample. ATIII levels in two dogs were found to be decreased slightly immediately following BOPP administration. Mean (± S.D.) ATIII levels prior to treatment were 113.5% (± 2.1) and 81% (± 2.8). Reference normal values for this assay are 70-126%. Soluble fibrin monomer levels are shown in Figure 2.5. Soluble fibrin monomer is the product of thrombin cleavage of fibrinogen prior to fibrin crosslinking to form a fibrin polymer. Normal values for this assay are 0 (negative). Soluble fibrin levels were increased in 3 of 4 dogs tested.

2.4 DISCUSSION:

The very complex equilibrium of the hemostatic system is disrupted by the presence of BOPP. The most significant finding, in spite of all the changes in the hemostatic test results, is that there was no evidence of a clinical bleeding abnormality in any of the dogs. Based strictly on the findings of these tests, one might expect that these dogs would experience clotting problems ranging from abdominal bleeding, joint swelling and severe bruising at venipuncture sites. On the contrary, none of these were observed. Thus, while the test results are alarming, the clinical picture is not significantly altered.

The mechanism of BOPP's effect on the coagulation pathway and the tests used to evaluate that system are likely due to an effect on the common pathway. Prolongations in the PTT, PT and TCT (Figure 2.2) are strongly suggestive of some abnormality in the portion of the system that is common to all of these tests. Since the TCT involves only the addition of thrombin to citrated plasma, it is the simplest test with the fewest complicating factors. The severe prolongation of this test indicates that BOPP is having an effect on one

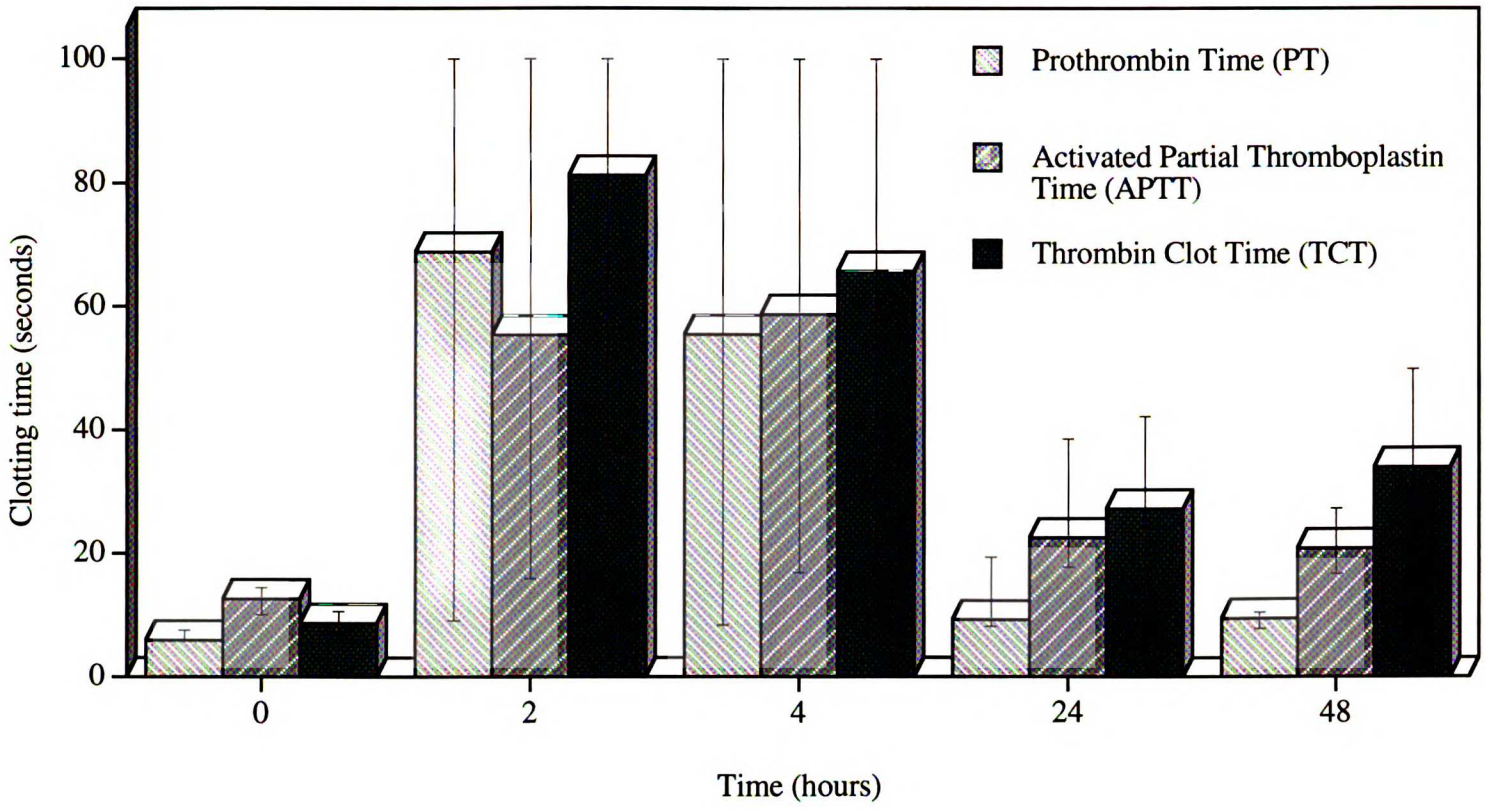


Figure 2.2. Median values for coagulation tests following BOPP administration in dogs. Error bars indicate range. Reference values are <17 seconds, <18 seconds and <9 seconds for APTT, PT and TCT, respectively. Maximum measurable value for all tests is 100 seconds.

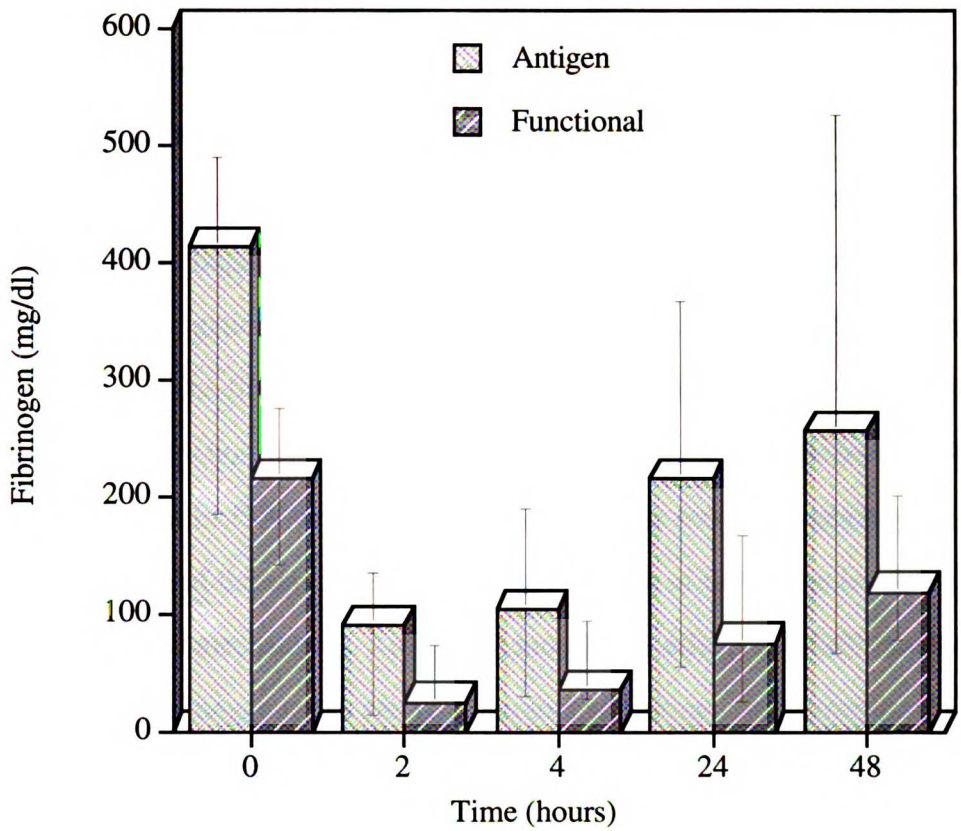


Figure 2.3. Median functional and total (antigen) fibrinogen levels following BOPP administration. Error bars indicate range. Reference values for functional fibrinogen are >200 mg/dl and for total fibrinogen are >180 mg/dl. Minimum measurable value for functional test is 25 mg/dl.

of only a few possible components. Thrombin acts on fibrinogen to cleave fibrinogen and produce soluble fibrin which subsequently undergoes polymerization in the presence of thrombin and factor XIII to become the fibrin clot that is measured in the TCT. Inhibition of thrombin or its role in the cleavage of fibrinogen or the polymerization of soluble fibrin could cause prolongation of this test. In addition, low levels of fibrinogen would also cause the prolongation of this test since there would be inadequate fibrinogen as substrate for thrombin. The fibrinogen levels in the plasma following BOPP administration were much lower than normal (Figure 2.3). This is the case for both the total fibrinogen (as measured by an ELISA-based antibody assay) and the functional fibrinogen (as measured by the addition of thrombin). This apparent lowering of fibrinogen levels could be the result of several mechanisms. Among the most likely effects of BOPP resulting in the lowering of fibrinogen levles are (i.) thrombin activation, (ii.)thrombin inhibition and (iii.) fibrinogen binding.

(i.) BOPP is activating thrombin in vivo and causing a widespread increase in the cleavage of fibrinogen and subsequent lowering of fibrinogen levels. Were this the case, one would expect to find high levels of soluble fibrin and fibrin degradation products (FDP) in the plasma. Tests of FDP were negative in all dogs, and soluble fibrin monomer levels (Figure 2.5) were increased in some dogs but not to the degree that might be expected as a result of excessive thrombin activity. ATIII levels were seen to decrease in those dogs tested (2 of 2). ATIII forms a tight complex with thrombin (and other serine protease factors such as factors X, IX, Xi, and XII[187]) and renders it inactive[185]. ATIII levels will decrease in response to increases in thrombin activation[187], thus there may be some evidence that thrombin activation is occurring in response to BOPP administration although activation of thrombin to the degree necessary to cause the observed decreases in fibrinogen would be expected to result in a more dramatic drop in ATIII levels[188]. In addition, these dogs did not show evidence of disseminated intravascular coagulation (DIC), a severe and often

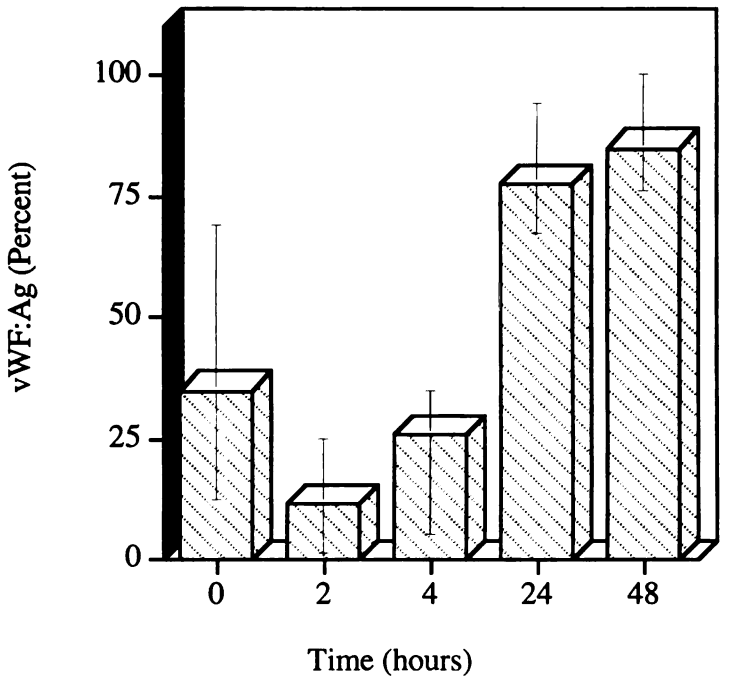


Figure 2.4. Median vWF:Ag following BOPP administration in dogs. Error bars represent range. Reference values are >70.

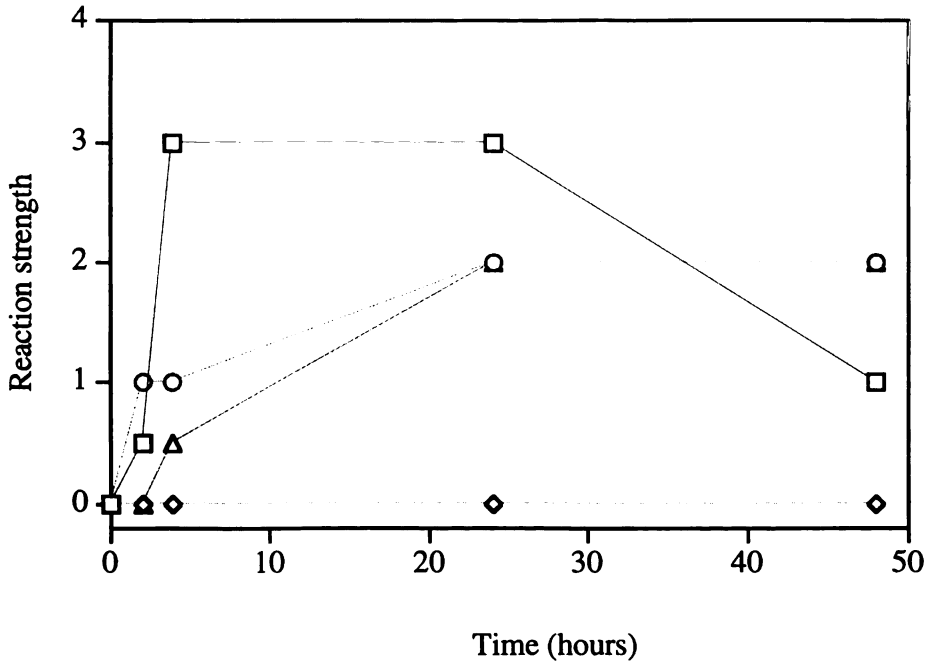


Figure 2.5. Soluble fibrin monomer levels in dogs following BOPP administration. Each line represents an individual subject. Maximum measurable value is 3, trace values reported as 0.5

fatal syndrome that is the result of excessive stimulation of the coagulation cascade[179]. While the hemostatic profile was similar to that seen in dogs with DIC, (decreased fibrinogen, prolonged clotting test times, decreased platelets, decreased ATIII) its clinical picture shows signs such as bleeding, circulatory collapse and generalized thromboembolism, none of which were found in the BOPP-treated dogs. A decrease in vWF:Ag has been reported in dogs that have been subjected to conditions that generate thrombin[189], similar to that seen in dogs in this study (Figure 2.4).

(ii.) In the presence of BOPP, thrombin is inhibited and cannot act on the fibrinogen in the plasma. This would cause an increase in TCT and an apparent decrease in the functional fibrinogen due to its reliance on thrombin. There would be no increase in fibrin degradation products or soluble fibrin due to the inability of thrombin to act on fibrinogen to form these products. The dogs did show some evidence of an increase in soluble fibrin, but no increase in FDPs. The decrease in the total fibrinogen, a test that is independent of thrombin, in the dogs is more difficult to explain using this theory, unless BOPP has some interfering effect on this test in addition to inhibiting thrombin.

(iii.) Another explanation is that BOPP is binding to fibrinogen and inhibiting thrombin cleavage of fibrinogen to soluble fibrin. If the binding site for the antibody in the fibrinogen ELISA test is also affected, this would be seen as a decrease in total fibrinogen. Thus, binding of BOPP to fibrinogen might inhibit thrombin activity resulting in a prolonged TCT and a decrease in functional fibrinogen. BOPP could also interfere with antibody binding resulting in a decrease in total fibrinogen. There would be no increase in FDPs since fibrinogen is not being cleaved by thrombin. The increase in soluble fibrin seen in some dogs does not fit into this mechanism or that described in (ii.). The available evidence from this study does not unambiguously support any of these explanations, but the data do suggest that BOPP affects the coagulation system at the level of the interaction of thrombin and fibrinogen and may have effects on other portions of the system as well.

The effect of BOPP on the levels of vWF:Ag are dramatic (Figure 2.4) The beagles used in this study were found to be vWF:Ag deficient with levels between 30 and 50% of normal. The dogs had no history of coagulation abnormalities, and routine coagulation profiles performed by the supplier (Marshall Farms USA, Inc.) were normal. The administration of BOPP resulted in a decrease in vWF:Ag followed by a dramatic rebound in vWF:Ag levels at 24-48 hours after BOPP administration. As discussed above, the generation of thrombin can cause a decrease in the level of vWF, presumably by a consumption of vWF[189]. vWF levels in the study by Toh et al.[189] rebounded to normal levels 1 hour after thrombin activation. The generation of thrombin (by administration of factor X and lipid vesicles) was very brief and not sustained for an extended period. The administration of BOPP could cause a more sustained activation of thrombin resulting in a longer decrease in vWF levels. Toh et al. also suggest that there may be some relationship between the decrease in vWF and the decrease in platelets noted in the same study. They postulate that thrombin activation may be the cause of both of these events. Thrombin can cause sequestration of platelets, possibly by causing the binding of vWF to the platelets and thus stimulating platelets to temporarily bind to the endothelial wall[189]. This theory might explain the concurrent findings of a decreased platelet count (Figure 1.8) and the decreased levels of vWF:Ag seen in BOPP treated dogs. Alternatively, a loss of vWF without replacement may explain the delay in the rebound of vWF in BOPP treated dogs. vWF is produced by endothelial cells[190] and its synthesis and release may be affected by the presence of BOPP. Finally, BOPP may be binding to vWF and affecting the binding of the antibody used in the ELISA to detect the protein. vWF binding has been described with hematin, and is thought to affect the binding of vWF to factor VIII and platelets[191]. vWF:Ag levels at 24-48 hours after BOPP administration were above those found at time 0. It is not known if this is due to release of newly synthesized and stored vWF, or the release of previously sequestered vWF. Toh et al.[189] showed a rebound in vWF levels soon after thrombin-induced depletion, but did not note an overshoot of vWF levels. They

also noted an increase in multimeric forms of vWF, indicative of newly synthesized vWF[192]. Studies of the multimeric forms of vWF after BOPP administration may provide clues to the cause of this phenomenon.

The presence of soluble fibrin monomer in plasma (Figure 2.5) following BOPP administration suggests that thrombin is present and capable of acting on fibrinogen. As discussed earlier, soluble fibrin monomer is the product of thrombin on fibrinogen (Figure 2.1). Soluble fibrin is then polymerized by thrombin and factor XIII to form crosslinked fibrin polymers[187]. An increase in soluble fibrin could result from an increase in thrombin cleavage of fibrinogen with or without a concurrent inhibition of the crosslinking step. BOPP may be acting on thrombin to increase fibriongen cleavage, as discussed above, or inhibiting the ability of fibrin monomers to crosslink. This inhibition may be due to decreased factor XIII or thrombin activity, enzymes important in crosslinking, or binding of BOPP to the fibrin monomers at sites that would interfere with crosslinking. The amount of soluble fibrin monomer found in these subjects is elevated in 3 of 4 subjects tested, but the degree of elevation is variable, making it difficult to speculate on the exact mechanism responsible for this result.

The effects of porphyrins on the coagulation system are not well known, so extrapolations from other porphyrin compounds are not easily made. The compound that has seen the most intense study has been hematin. Hematin has been shown to have effects on several components of the hemostatic system, including the coagulation cascade. Increases in PT, PTT, and TCT have been reported in human patients following hematin administration[85, 115, 117]. Hematin treatment has resulted in decreases in functional, but not total fibrinogen[117], no effect on ATIII levels[117], evidence of increases in FDPs[85, 117], without an increase in soluble fibrin monomer[117]. The conclusion of the authors in Glueck et al.[117] is that hematin acts by making fibrinogen non-clottable by thrombin inactivation and studies of hematin ex vivo have found that hematin binds to both thrombin and fibrinogen[193]. Hematin's effects on vWF are noted above. Thus the effects of

hematin and BOPP demonstrate some similarities (decreased functional fibrinogen, prolonged clotting times), but there are notable differences (BOPP causes decreased total fibrinogen, no change in FDP, increases in soluble fibrin monomer, decreases in ATIII). BOPP and hematin may act by similar mechanisms, including the possibility that both act by binding to thrombin and/or fibrinogen and affecting the interaction between these two proteins. Hematin may be used as a model compound for further studies regarding the effects of BOPP on the coagulation system.

The intricately balanced system of coagulation makes determining a simple cause for the effect of BOPP difficult. BOPP may act on numerous enzymes and cells resulting in additive or conflicting effects on the coagulation cascade. Of course, this would make interpretation of clinical tests difficult. Clearly, BOPP acts on the common pathway of coagulation, but the exact location of this effect remains to be determined and is the subject of ex vivo studies described in other sections of this dissertation.

This study has focused on one aspect of the hemostatic system, the coagulation cascade, and the effect that BOPP has on its function. As discussed in the introduction to this chapter, there are other components to the hemostatic system that may be important in the generation of thrombi at the site of injection. Further studies into the role of these components are necessary to fully understand the effect of BOPP, and possibly other porphyrins, on the hemostatic system.

CHAPTER 3.
EX VIVO HEMOSTASIS

3.1 INTRODUCTION:

The cephalic thrombosis associated with the administration of BOPP to dogs could be a significant problem. Experiments examining the *in vivo* effects of BOPP on the hemostatic parameters of dogs is reported earlier. The hemostatic system is very complex and involves not only the proteins that make up the hemostatic cascade, but also the endothelial and other cells lining the vessels, the platelets, and other blood-borne cells[182]. To examine the hemostatic tests in the presence of BOPP without the presence of blood vessels or other cellular structures, we chose to look at whether we could reproduce the *in vivo* effects of BOPP on the hemostatic cascade in an *ex vivo* system. The premise of these experiments is simple. By adding varying amounts of Na_2BOPP dissolved in 0.45% saline (the vehicle used in the *in vivo* experiments) to dog blood or plasma and then performing hemostatic tests on these samples, we could determine if blood vessels (or other stationary elements) or circulating cells are necessary for the effect of BOPP on the hemostatic system. In addition, varying concentrations of BOPP could be added to the plasma or blood to determine if a dose-dependency exists. The BOPP concentrations used were up to and greater than the concentration of BOPP in the IV solution administered to the dogs. The concentration of BOPP in the administered solution was approximately 1 mg/ml. The concentration of BOPP in the plasma following BOPP administration was estimated to be as high as 0.45 mg/ml. The tests that were performed included the thrombin clot time (TCT), fibrinogen level as measured by ELISA, vWF:Ag as measured by ELISA and soluble fibrin monomer. The thrombin clot time assesses the ability of exogenously added thrombin to act upon fibrinogen in the sample. A depletion of fibrinogen or the inability of thrombin to cleave fibrinogen would result in a prolongation of the TCT[179]. Prolongation of the TCT was found following BOPP administration to dogs in the *in vivo* experiments. In contrast to the TCT, the measurement of fibrinogen using an ELISA test determines the levels of fibrinogen present without relying on the ability of thrombin to cleave fibrinogen. Decreases in this test of fibrinogen were found in

dogs administered BOPP solution. vWF:Ag levels were also found to decrease in dogs administered BOPP, thus prompting us to determine if this phenomenon could be repeated *ex vivo*. Soluble fibrin monomer is the product of thrombin's activity on fibrinogen prior to the crosslinking of fibrinogen[182]. The presence of high levels of fibrin monomer, coupled with a significant decrease in fibrinogen would suggest that BOPP is capable of causing an increase in the cleavage of fibrinogen, probably via an activation of thrombin. Mild increases in fibrin monomer were noted in some dogs following BOPP administration, but these increases were not thought to be of significant magnitude to explain the dramatic decrease in fibrinogen levels seen in those dogs. We hypothesize that BOPP is binding to fibrinogen and interfering with the ability of thrombin to cleave fibrinogen and the ability of the fibrinogen antibody to bind to the protein. In this case, the addition of BOPP to dog blood or plasma should result in an increase in TCT, a decrease in fibrinogen, no change in fibrin monomer and an unknown effect on vWF:Ag.

3.2 MATERIALS AND METHODS:

BOPP stock solution

Na_2BOPP prepared by M-S. Koo in the Kahl laboratory was dissolved in 0.45% sterile saline to a form a stock solution. Stock solution was protected from light until use.

Blood and plasma samples

Subjects were normal male beagle dogs, approximately 1 year of age. Blood samples were obtained by removing the citrate fluid from 3-4.5 ml Vacutainer® citrate tubes (0.105M sodium citrate) by drawing this fluid into a 12 cc syringe. This syringe was used to obtain blood from the subjects via jugular venipuncture. Blood/citrate mixture was returned to the citrate tubes. Blood/citrate mixture was poured into plastic centrifuge tubes.

Using a plastic pipet, blood was aliquoted into polyethylene tubes. The remaining blood/citrate mixture was centrifuged at 2000 rpm (approx. 20*g*) for 15 minutes and the plasma removed to a polypropylene cryo tube.

BOPP addition to plasma or blood

BOPP solution was added to plasma or blood samples in varying concentrations. BOPP solutions did not exceed 5% of the total volume of the sample. After BOPP addition, samples were vortexed. Blood/BOPP mixtures were centrifuged at 2000 rpm for 13 minutes and plasma was pipetted into cryo tubes. All samples were protected from light and frozen at -70° C until analysis.

Heparinized samples

In addition to citrated blood samples, blood was drawn via jugular venipuncture into a plastic syringe and placed into a Vacutainer® tube containing sodium heparin (100 IU / 7 ml). Samples were centrifuged at 2000 rpm for 13 minutes and plasma pipetted into cryo tube. BOPP solution was added to the plasma and samples were vortexed. BOPP/plasma mixtures were frozen at -70° C until analysis.

Control samples

Aliquots of 0.45% saline, without BOPP, were added to citrated blood and plasma and also to heparinized plasma. These samples were treated as above and considered negative controls.

Analysis

Samples were sent to the Comparative Coagulation Section at the Diagnostic Laboratory of Cornell University. Tests performed were: thrombin clot time (TCT), quantitative fibrinogen levels using an ELISA assay based on the method of Lominadze et

al.[194], vWF:Ag levels via ELISA using the method of Benson et al.[195], and soluble fibrin monomer complex (via a latex agglutination, semi-quantitative assay, FS Test Unit, American Bioproducts, Parsipany, NJ).

Statistics

The paired T-test was used to test the significance of changes in vWF:Ag and fibrinogen in heparinized samples. Comparison was between the samples obtained from the same dog but treated with saline or BOPP as described above.

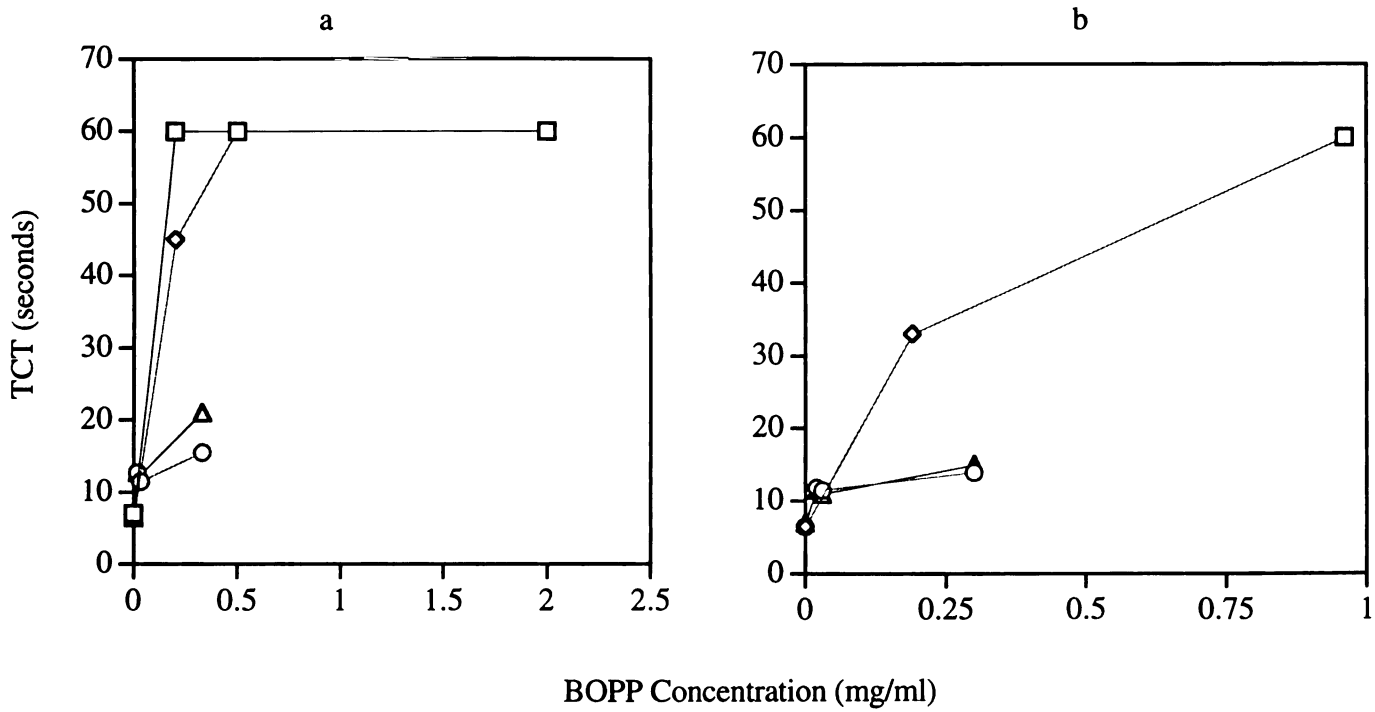
3.3 RESULTS:

The *ex vivo* addition of BOPP to blood and plasma samples resulted in changes in measured hemostatic tests. Figures 3.1-3.4 illustrate the changes in these parameters. Reference values for each parameter are listed in the caption. Symbols represents an individual animal from whom the plasma was obtained. The reference value is the value at which the reference lab considers that parameter to be within the normal range. Thrombin clot times in blood or plasma were found to increase with the addition of BOPP (Figure 3.1). Figure 3.2 illustrates the decrease in plasma and blood fibrinogen levels upon the addition of BOPP. vWF:Ag levels were also found to decrease (Figure 3.4). Changes in soluble fibrin monomer levels were variable in the presence of BOPP (Figure 3.3). The addition of BOPP to heparinized plasma resulted in changes in the fibrinogen levels and vWF:Ag levels. The addition of 0.03 mg/ml of BOPP to heparinized plasma resulted in a non-significant decrease ($p = 0.054$) in fibrinogen levels compared to heparinized plasma with added 0.45% saline (data not shown). vWF:Ag levels showed a trend to decrease in the heparinized plasma to which BOPP had been added, but the decrease was not statistically significant ($p = 0.3$, data not shown). No consistent change in soluble fibrin monomer levels was seen in heparinized plasma to which BOPP had been added.

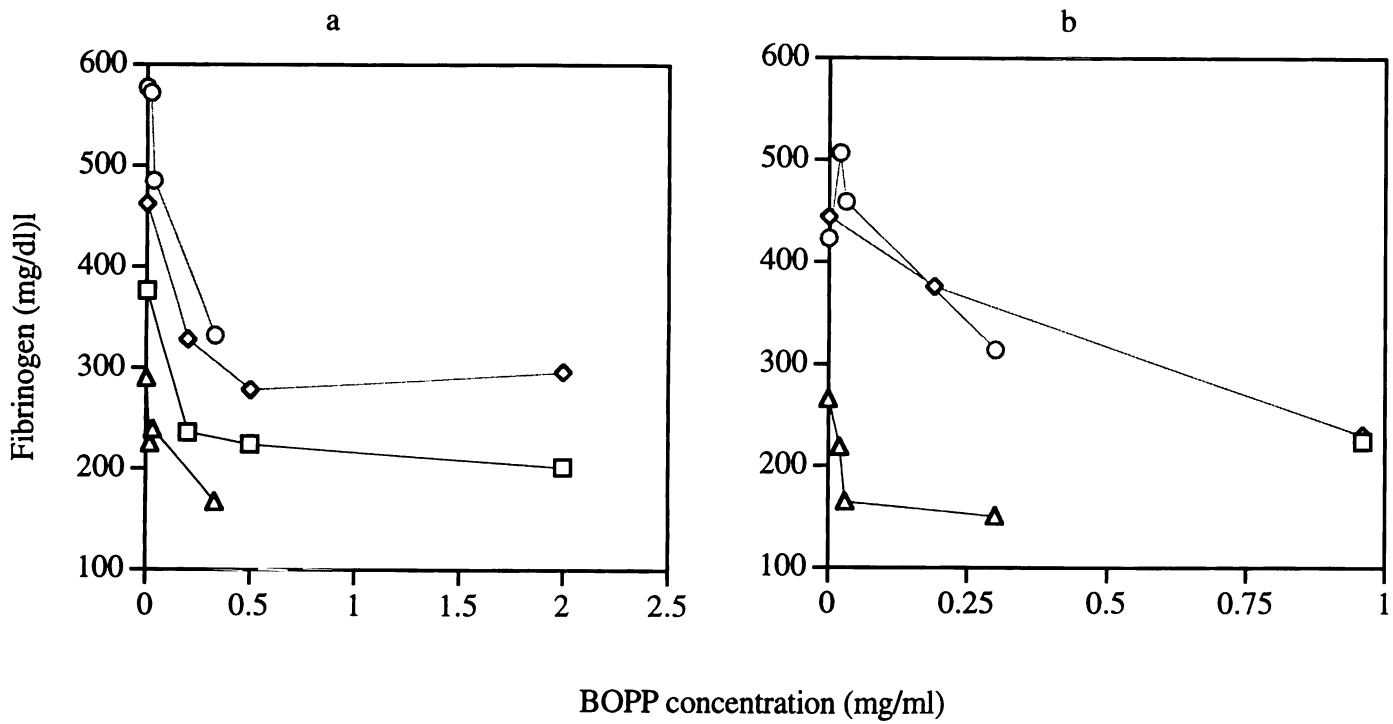
3.4 DISCUSSION:

BOPP appears to have a significant effect on the measured hemostatic parameters, regardless of whether the BOPP is added to blood or plasma. BOPP addition to blood or plasma results in a concentration dependent increase in the TCT (Figure 3.1). Since the TCT is a measure of the ability of a small amount of exogenously added thrombin to cleave fibrinogen to fibrin monomers and the subsequent polymerization of these monomers[179], BOPP can act by inactivating thrombin or by binding to fibrinogen and preventing thrombin cleavage. Also, BOPP may be causing an increase in thrombin activity which cleaves much of the fibrinogen prior to analysis. Thus when exogenous thrombin is added during the TCT test, there is insufficient fibrinogen available to demonstrate clotting. Finally, it is also possible that BOPP may be preventing the polymerization of fibrin monomers. To further investigate this mechanism, the measurement of fibrin monomers in the presence of BOPP is shown in Figure 3.3. Fibrin monomer levels were increased in the presence of high levels of BOPP suggesting that thrombin activity is still present. The presence of fibrin monomers indicates that fibrinogen cleavage is occurring or that fibrin polymerization is inhibited. If the presence of BOPP caused an increase in thrombin activation and a resultant increase in the cleavage of fibrinogen, the levels of fibrin monomer would be expected to be much higher[186].

Fibrinogen levels (Figure 3.2) appear to decrease upon the addition of BOPP to the plasma or blood samples. The causes for this phenomenon are similar to those that might result in an increased TCT. Increased activation of thrombin would cause cleavage of



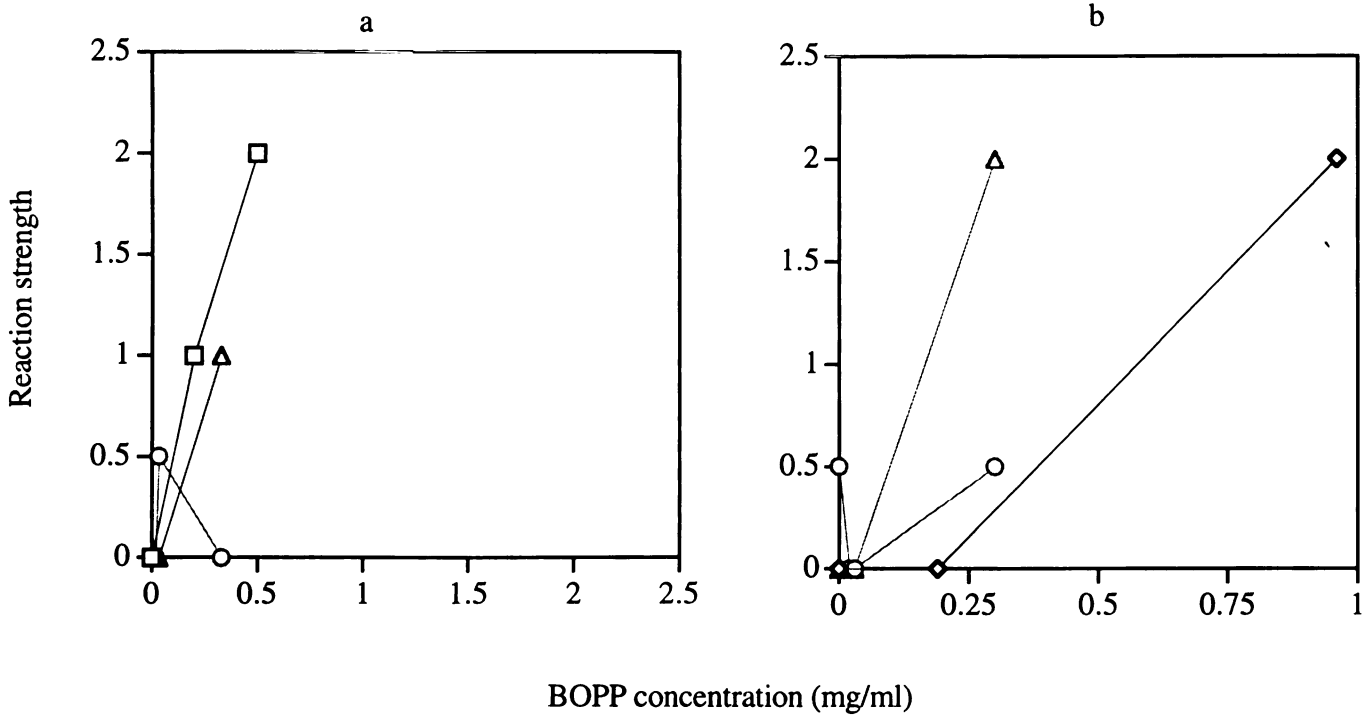
Figures 3.1a,b. Thrombin clot time in blood (a) or plasma (b) following addition of BOPP. Maximum measurable value = 60 seconds. Reference value for TCT is < 9 seconds.



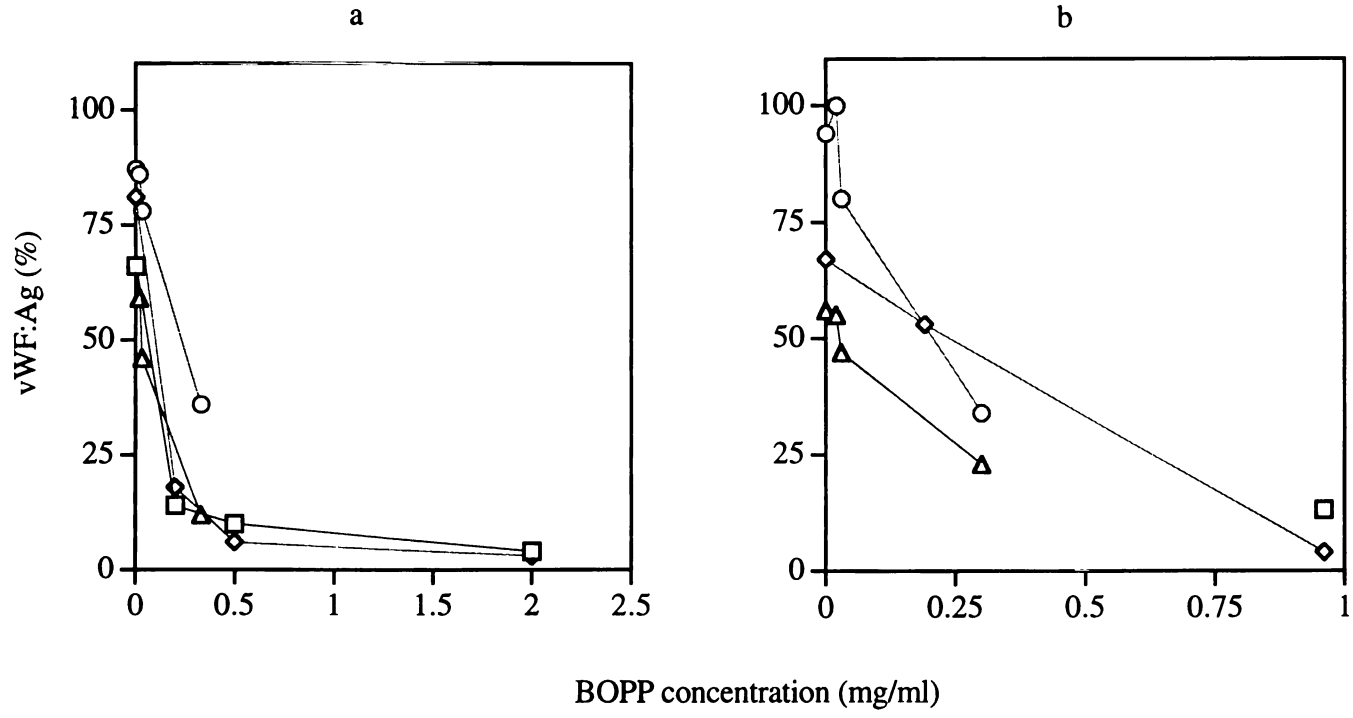
Figures 3.2a, b. Blood (a) and Plasma (b) fibrinogen levels after ex vivo addition of BOPP. Reference value for fibrinogen is > 175 mg/dl.

fibrinogen leading to a decrease in measured amounts. The binding of BOPP to fibrinogen might interfere with the fibrinogen-antibody interaction resulting in a decrease in measured, but not actual, fibrinogen levels. Again, an increase in fibrin monomer levels, as seen in this study, would suggest some activation of thrombin but the magnitude of the decrease in fibrinogen is unlikely to be completely explained by this activation. Also arguing against the increase in thrombin activity is the absence in the dogs of a strong increase of fibrin degradation products which would be expected to be elevated in response to increased fibrinogen cleavage. Also, heparinized plasma to which 0.03 mg/ml of BOPP had been added were found to have a trend toward decreased levels of fibrinogen compared to heparinized plasma with added saline as control, although more extensive studies of heparinized samples are needed to further characterize this effect. Heparin, by binding to antithrombin III, is a strong potentiator of the anti-thrombin activity of ATIII[179]. Upon the binding of ATIII to thrombin, heparin is released and may act on other ATIII molecules[187]. Since the concentration of ATIII in plasma is 10 times that of prothrombin[178], there is adequate ATIII to inhibit thrombin in the presence of heparin. Thus, the presence of heparin in the sample would result in the inhibition of any active thrombin and prevent the cleavage of fibrinogen. Thus, the finding of decreased fibrinogen in heparinized plasma samples containing BOPP suggests that BOPP is not causing thrombin activation.

vWF:Ag levels (Figure 3.4) were decreased in the presence of BOPP. A BOPP concentration-dependent decrease in vWF:Ag is suggested by the *ex vivo* data. The mechanism for this phenomenon is not known. It is possible that BOPP is interfering with the test used to determine vWF:Ag levels. Alternatively, thrombin activation *in vivo* is associated with a decrease in vWF:Ag[189], and is thought to be due to increased consumption of the vWF. Conversely, vWF:Ag levels were seen to have a trend toward decrease in heparinized plasma to which BOPP had been added. As noted above, heparin potentiates the inactivation of thrombin thus making consumption of vWF a less



Figures 3.3a, b. Soluble fibrin monomer levels (measured as reaction strength) in blood (a) and plasma (b) following ex vivo addition of BOPP. Maximum value is 3. Reference range for soluble fibrin is 0.



Figures 3.4a,b. vWF:Ag levels in blood (a) or plasma (b) following ex vivo addition of BOPP. Reference values for vWF:Ag are >70%

likely scenario. Finally, BOPP may also be stimulating the binding of vWF to factor VIII resulting in the binding of the entire complex to platelets. This might explain a decrease in the vWF:Ag levels in BOPP added to whole blood, but will not explain the same finding in plasma which is deficient of platelets.

These *ex vivo* data can be compared to that seen in the dogs following BOPP administration. TCT times were increased following BOPP administration in dogs and in the presence of BOPP in the *ex vivo* system. There appeared to be a concentration dependence *ex vivo* which resembles the return to more normal TCT values seen in the dogs at 24 and 48 hours after BOPP administration, when plasma BOPP concentrations had decreased. Fibrinogen levels in the *ex vivo* system were found to decrease in the presence of BOPP, similar to the decrease in both functional and total (as measured by ELISA) fibrinogen levels seen *in vivo*. The decrease in fibrinogen in the dogs after BOPP administration appears to be more pronounced than that seen in the *ex vivo* system. Fibrin monomer levels in the ex vivo system were found to exhibit a moderate and inconsistent increase in the presence of BOPP. This was also seen in the *in vivo* experiments.

Hematin and other porphyrins have been reported to have effects on *ex vivo* tests of hemostasis. Glueck et al.[117] demonstrated that low concentrations of hematin are capable of prolonging TCT in an *ex vivo* system. Hematin was not found to interfere with fibrin polymerization. The authors suggested that hematin acted by interference with coagulation factor mechanisms. Jones[118] confirmed the effect of hematin on TCT using solutions of hematin of various ages. He found that aged solutions, which are thought to contain polymers or aggregates, were more active in prolonging the TCT. Green et al.[193] also reported similar findings with hematin and demonstrated the ability of hematin to inhibit thrombin activity on a synthetic thrombin substrate. Conversely, Becker et al.[121] found that protoporphyrin and hematin could activate factor XII, the first step in the intrinsic clotting pathway. In that study, they found that the addition of protoporphyrin or hematin to normal human citrated plasma caused a decrease in the prothrombin time (PTT). This is

in conflict with the other reports that indicate that hematin causes an increase in PTT *in vivo*[85, 115, 117, 118]. Since the PTT relies on the generation of active thrombin, and most studies have indicated that hematin causes an inhibition of thrombin action on fibrinogen, the results of Becker et al. may be due to differences in methodology or experimental endpoint. Green et al.[191] found that hematin was capable of binding to the vWF-Factor VIII complex *in vitro*. This binding affected the function of the complex, and by binding to platelets may be capable of inducing platelet aggregation. This might explain the decrease in platelet concentrations seen following BOPP administration and may also be involved in the decrease in vWF:Ag. Thus, it appears that porphyrins and porphyrin-like compounds can have significant effects on the hemostatic system. BOPP may behave much like hematin in the *ex vivo* systems. Both these substances are capable of prolonging the TCT, and both may be binding to thrombin or fibrinogen to cause this effect.

CHAPTER 4.
CELL CULTURE

4.1 INTRODUCTION:

The thrombosis of the cephalic vein found in several dogs following BOPP administration may be the result of several mechanisms. One of those mechanisms is injury to the endothelial cells lining the cephalic vein. Veins are lined by endothelial cells which act as an anatomic and functional barrier between the intravascular compartment and the rest of the body (Figure 1.15). These cells have a diverse and complex function that includes modulating hemostasis. The endothelial cell plays a vital role in maintaining the delicate equilibrium that exists between coagulation and bleeding.

Endothelial cells are involved in a number of antithrombotic mechanisms. They have antiplatelet activities that prevent adhesion of unactivated platelets to the vessel wall. This is accomplished by the secretion of prostaglandins that inhibit platelet function[183]. Endothelial cells also are capable of inhibiting the hemostatic cascade by regulating the formation of thrombin, the pivotal protease enzyme in the formation of fibrin. This regulation is achieved by a number of mechanisms such as the production of inhibitory proteins, the expression of membrane receptors capable of binding thrombin and decreasing its activity, and by the production of Protein C. Protein C has many functions, one of which is to decrease the production of activated thrombin[Gertler, 1992 #242]. In addition, endothelial cells can counteract the formation of thrombi by secreting plasminogen activators which activate plasminogen. This activation occurs via the cleavage of plasminogen to plasmin which is responsible for localized digestion of fibrin[183].

Conversely, endothelial cells are intimately involved in the generation of thrombi. Intact, unperturbed endothelial cells do not initiate coagulation, but these cells do express prothrombotic properties[183]. Injury to endothelial cells results in a rapid response with the result being the formation of local thrombosis. The mechanisms by which this occurs are numerous and complex. Injury to the endothelial cell can elicit the expression of receptors for circulating coagulation factors such as Factors IX or X[183] and also elicit the expression of tissue factor on the surface of cells. Injured endothelial cells also are known

to produce several soluble factors that modulate coagulation. Included among these factors are Factor V and vWF. vWF acts as an adhesive protein that allows for the attachment of platelets. The damage to endothelial cells often exposes the underlying subendothelium which contains high levels of vWF, thus providing an adhesive surface for the attachment of platelets.

Under normal circumstances in the venous circulation, antithombotic properties tend to predominate[183] but the stimuli that can cause conversion to a thrombotic state are varied. Mechanical injury, endotoxin, hypoxia, and numerous endogenously produced factors (thrombin, tumor necrosis factor) can cause injury to endothelial cells resulting in thrombosis[181]. These stimuli have been found to act by various mechanisms to result in a procoagulant state.

As part of our study of the causes of cephalic vein thrombosis following BOPP administration, we performed some studies to determine the effects of BOPP on cultured endothelial cells. A simple, easily propagated cell line for these studies are Human Umbilical Vein Endothelial Cells (HUVECs). These cells are hardy, well-characterized and strongly representative of the *in vivo* system[196]. They divide to reach a confluent layer and maintain that single layer. HUVECs have been used extensively in the study of hemostasis and the role of the endothelial cell. HUVECs can be grown on a variety of strata, including simple tissue culture plasticware. Cultured HUVECs maintain many of the hemostatic functions found *in vivo* including the synthesis of hemostatic factors including vWF[197], cell surface expression and secretion of tissue factor[197-199], and the adhesion of platelets[200]. BOPP may be acting on endothelial cells in many ways, but the most likely mechanisms for the induction of local thrombosis are that BOPP is causing death or retraction of the endothelial cells resulting in exposure of the subendothelium. Exposure of the subendothelium to the circulation results in the adhesion of platelets, the activation of the coagulation cascade and subsequent thrombosis. BOPP may also be acting as a milder toxin to the endothelial cells, not causing death or morphologic changes

but instead inducing the cells to express procoagulant properties. These properties may include increased production of tissue factor or factor V, or a decrease in fibrinolytic activities. Previous studies have shown the ability of cultured endothelial cells to demonstrate all of the properties in response to various perturbations[183]. Our initial studies determine the concentrations of BOPP that cause significant toxicity in endothelial cells; these studies examined the effects of BOPP on HUVEC viability.

4.2 MATERIALS AND METHODS:

Materials

Human umbilical vein endothelial cells were kindly provided by Dr. Glenn Gobbel (Brain Tumor Research Center, UCSF). Isolation media was M199 with Earle's Salts (GIBCO) with 5% heat-inactivated horse serum (UCSF Cell Culture Facility, CCF), 2.5μg/ml fungizone (CCF) and 0.1 mg/ml Gentamicin (CCF). Isolation media was used within 14 days of preparation. Cultivation media was isolation medium with added 2.0 mM L-glutamine (Sigma Chemical Co. St Louis, MO), 5% fetal calf serum (CCF), 10% heat inactivated horse serum (CCF), 20 mM HEPES buffer (CCF), 0.1mg/ml heparin sulfate (Sigma), 0.1 mg/ml endothelial cell growth supplement (from bovine pituitary, Sigma). Cultivation media was used within 7 days of preparation. Trypsin (0.05%) versene (0.02%) in saline was obtained from CCF.

Cell culture methods

Cell culture methods were based on those of Gobbel et al.[201]. Cells were grown in polystyrene T-75 flasks (Corning) flasks, without coating, with 15 ml of culture media. Media was changed every 2-3 days. Cells were incubated at 37°C with 5% CO_2. When cells reached confluence, they were passaged by rinsing quickly with 3 ml of cold trypsin-

versene which was removed and replaced with 3 ml of clean trypsin-versene. Cells were allowed to detach from the flask surface and 10 ml fetal bovine serum added. Cells were suspended by gentle mixing, collected into a 15 ml centrifuge tube, and pelleted by centrifugation at 200g for 5 minutes at 10°C. Supernatant was removed and cells were resuspended in 10 ml of isolation media. Centrifugation was repeated to pellet cells. Cells were resuspended in cultivation media and re-seeded into T-75 flasks.

Determination of BOPP toxicity in HUVECs

Stock solutions

BOPP stock solution was prepared by adding BOPP disodium salt to culture media to a concentration of 1 mg/ml. This mixture was mixed until dissolved and filtered through a methylcellulose 0.22 μ filter into a sterile centrifuge tube. The BOPP solution was protected from light and used within 2 hours of preparation.

Methods

T-75 flasks with confluent cultures of HUVECs (passage 25-30) were passaged as described above and the cells from each flask split equally into 3 24-well flat bottom plates without coating (Corning No. 430262). Total volume of media in wells was 0.5 ml. Cells were re-fed with culture media every 48 hours. Cells became confluent in the wells by 3-4 days following culture.

All procedures from this point were performed under low light conditions. Upon reaching confluence, cells were treated with concentrations of BOPP ranging from 0.075-0.75 mg/ml. The BOPP treatment was performed by first adding an appropriate amount of culture media to the well, followed by an appropriate amount of BOPP stock solution to reach the desired BOPP concentration. Total volume in each well was 0.5 ml. Plates were swirled to mix, then foil wrapped to shield from light and still allow gas penetration. Plates

were incubated at 37°C with 5% CO_2 for incubation times ranging from 1 hour to 48 hours.

Following incubation, BOPP-containing media was removed via suction and cells were rinsed with 0.5 ml isolation media at 37°C. Rinse solution was removed by suction, and 0.5 ml of fresh culture media (at 37°C) was added. Wells were photographed using phase contrast microscopy (Zeiss MC80X camera mounted on an Axiovert 100 phase contrast microscope, 400 ASA Kodak Gold film) at 200X magnification. Immediately following photography, cells were fixed and stained with crystal violet solution (5 %) for 10-15 seconds. Wells were rinsed thoroughly with warm tap water and allowed to air dry.

Cell toxicity was assessed by two methods. Photographs of wells were examined for changes in cell morphology and decreases in cell density. For a more quantitative measure, plates stained with crystal violet used to measure spectrophotometric density using the following assay.

HUVEC dilution assay

Following crystal violet staining, 24 well plates were allowed to air dry. Using an automated plate reader (Spectrafluor, TECAN Austria, Ges.m.b.H., running X-fluor software) in the absorbance mode, the absorbance of each well of the plate at 620 nm was measured . Triplicate absorbance measurement of each grid and a 5x5 grid of measurements per well were averaged to obtain the reported absorbance value. 620 nm was chosen as the measurement wavelength based on the low absorbance of BOPP at this wavelength and the linearity between absorbance and cell dilution seen in test plates. These test plates were prepared as follows. HUVEC cells were grown to confluence in T-75 flasks. Cells were passaged as described above, and resuspended in 10 ml of culture media. This suspension was diluted with culture media in 24 well plates resulting in dilutions from 100% suspension down to 10% suspension. Each dilution was represented by 4 wells/plate. These plates were incubated for 24 hours to allow cell attachment and

were stained with crystal violet. Plates were read by the plate reader at 620 nm. Three plates were prepared and measured.

Stastistical analysis

HUVEC dilution assay: Data obtained in the HUVEC dilution assay was plotted as absorbance vs. dilution (Figure 4.2). It was apparent that the data were not linear at absorbances above 4.5. Thus, the 1.0 dilution value was outside the linear range. The remainder of the data appeared linear and a linear regression line fitting these data was calculated.

Differences in cell survival as a function of incubation time: Data obtained from the addition of BOPP to HUVECs was used. Comparisons between incubation times, with the BOPP concentration held constant, were made using Analysis of Variance (ANOVA). Those groups demonstrating a difference between means of $p<0.05$ were compared in a pairwise fashion using Bonferroni's t-test.

Differences in cell survival as a function of BOPP concentration: Data obtained from the addition of BOPP to HUVECS was used. Comparisons between BOPP concentrations, with the incubation time held constant, were made using ANOVA. Those groups demonstrating a difference between means of $p<0.05$ were compared to a control value (0.0 mg/ml BOPP) using Bonferroni's t-test.

4.3 RESULTS:

To establish the linearity of the plate reader method for determining cell numbers in 24 well plates, we measured the absorbance of crystal violet stained HUVECs at various dilutions. Figure 4.1 shows this data. The absorbance at 620 nm appears to plateau at an absorbance above approximately 4.5. We calculated the linear regression for the data on the linear portion of the curve (dilutions of 0.1-0.8) and obtained the equation seen on

Figure 4.1. The slope and intercept (± S.D.) of the line are 0.449 (0.010) and 0.094 (0.005), respectively. HUVECs were incubated with BOPP at concentrations ranging from 0.075 - 0.75 mg/ml and for times from 1-48 hours. The results of these incubations are reported in Figure 4.2. Absorbance of wells were obtained at 620 nm. Data is reported as mean absorbance (±S.D.) vs. BOPP concentration. Each line represents the data from one incubation time following BOPP addition.

Statistical evaluation of the data obtained from 620 nm absorbance measurements of crystal violet stained HUVECs following BOPP administration revealed the following differences. When comparing incubation times at constant BOPP concentration, the BOPP concentrations that showed significant differences ($p < 0.05$) as a function of incubation time were 0.75, 0.3, 0.2 and 0.1 mg/ml. The bonferroni t-test matrices for these comparisons are shown in Tables 4.1a-d. The left column and top row indicate the incubation times of BOPP with HUVECs. If there was a significant difference ($p<0.05$) between HUVEC absorbances at two incubation times, that difference is listed in the Table as a "Y". Non-significant differences are listed as "N". At BOPP concentrations of 0.75 mg/ml (Table 4.1a), there was a significant decrease in HUVEC absorbance at 24 and 48 hours of incubation compared to shorter incubation times (1, 2, 4 hours). Significantly higher HUVEC absorbance was noted in the 48 hour incubation group at BOPP concentrations of 0.3, 0.2 and 0.1 mg/ml (Tables 4.1b-d)

Table 4.2 shows the matrix of bonferonni t-tests between HUVEC absorbance at different concentrations of BOPP. Comparisons were made between absorbance at

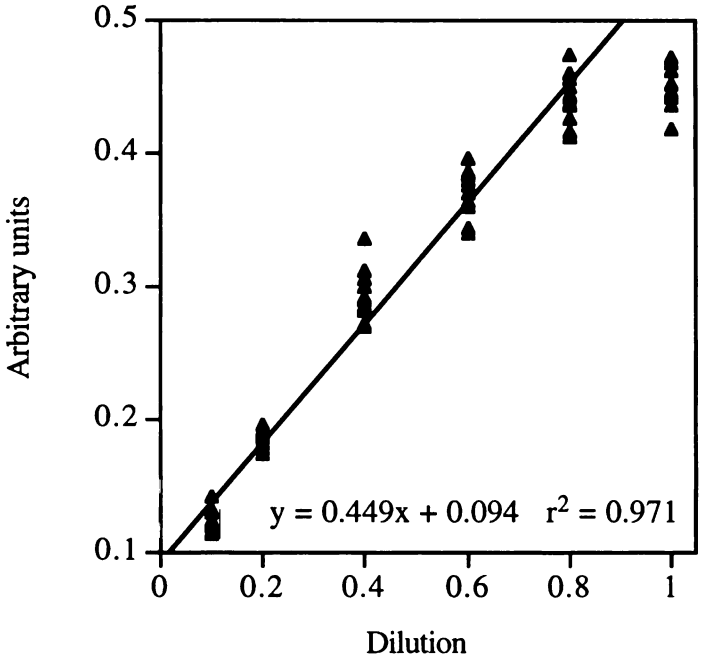


Figure 4.1. Absorbance of crystal violet stained HUVEC at 620 nm vs. dilution. Linear regression shown describes data from dilutions of 0.1-0.8.

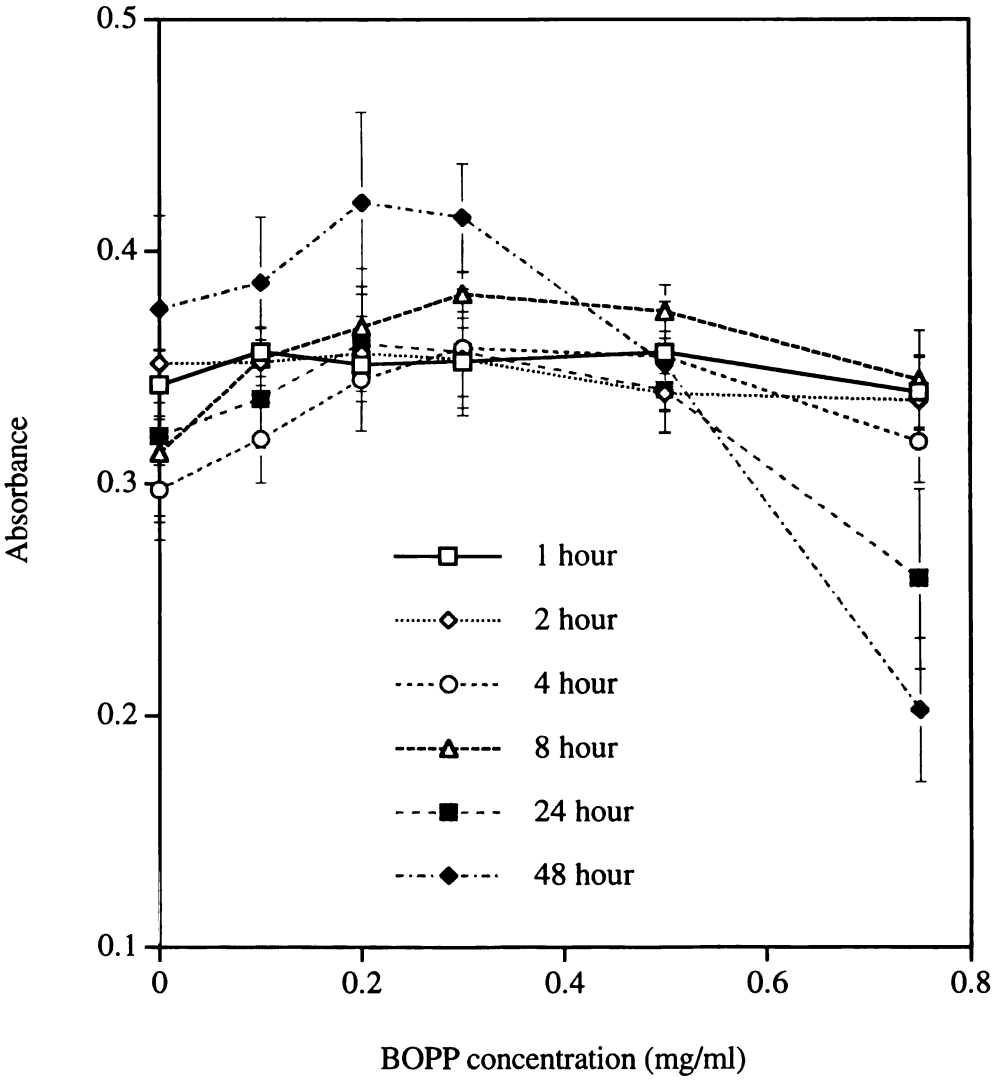


Figure 4.2. Absorbance of crystal violet stained HUVECs following BOPP treatment. Symbols indicate mean values. Error bars indicate S.D.

different BOPP concentrations and the absorbance of HUVECs without BOPP (0 mg/ml). Differences ($p<0.05$) in absorbance between groups are listed with a "Y". Non-significant differences are listed with a "N". HUVEC absorbance was significantly increased compared to controls in 4 and 8 hour incubations when BOPP was present at 0.1, 0.2. and 0.3 mg/ml. HUVEC absorbance was decreased when BOPP at 0.75 mg/ml was present for 24 or 48 hours.

Plate 4.1 illustrates the decreased number and the morphologic changes noted in cells exposed to BOPP. HUVECs treated for 48 hours with BOPP (0.75 mg/ml) showed significant changes in morphology with cell retraction and filamentous matrix material in bare areas.

Tables 4.1a-d Bonferroni t-test matrix of comparisons between absorbance of HUVECs at different incubation times in the presence of BOPP. Top row and left column indicate incubation times. Y= significant difference between absorbances of HUVECs between incubation times in row and column. N= no significant difference

Table 4.1a. BOPP concentration 0.75 mg/ml

Incubation time (hrs)	1	2	4	8	24	48
1	X					
2	N	X				
4	N	N	X			
8	N	N	N	X		
24	Y	Y	N	Y	X	
48	Y	Y	Y	Y	N	X

Table 4.1b. BOPP concentration 0.3 mg/ml

Incubation time (hrs)	1	2	4	8	24	48
1	X					
2	N	X				
4	N	N	X			
8	N	N	N	X		
24	N	N	N	N	X	
48	Y	Y	Y	N	Y	X

Table 4.1c. BOPP concentration 0.2 mg/ml

Incubation time (hrs)	1	2	4	8	24	48
1	X					
2	N	X				
4	N	N	X			
8	N	N	N	X		
24	N	N	N	N	X	
48	Y	Y	Y	N	N	X

Table 4.1d. BOPP concentration 0.1 mg/ml

Incubation time (hrs)	1	2	4	8	24	48
1	X					
2	N	X				
4	N	N	X			
8	N	N	N	X		
24	N	N	N	N	X	
48	N	N	Y	N	Y	X

Table 4.2. Bonferroni t-test matrix of comparisons between BOPP concentrations at constant incubation times. Incubation times are listed in top row. Y= significant difference ($p<0.05$) between absorbance of HUVECs at the BOPP concentration at left and absorbance of control (HUVECs with no BOPP). N= no significant difference.

BOPP concentration (mg/ml)	1 hour	2 hours	4 hours	8 hours	24 hours	48 hours
0	X	X	X	X	X	X
0.1	N	N	Y	Y	N	N
0.2	N	N	Y	Y	N	N
0.3	N	N	Y	Y	N	N
0.5	N	N	N	N	N	N
0.75	N	N	N	N	Y	Y

4.4 DISCUSSION:

The thrombosis noted in dogs following BOPP administration may be due to several mechanisms, one of which is a toxic effect of BOPP on endothelial cells lining the cephalic vein. These experiments were designed to provide some information regarding the toxicity of BOPP on these cells. In the most extreme case, BOPP could cause death of

endothelial cells resulting in the exposure of the subendothelium. In less extreme cases, the toxicity of BOPP may elicit more subtle prothrombotic properties such as tissue factor expresssion and vWF release. This study was designed to look at the more serious toxicity. The use of BOPP was associated with numerous difficulties in the quantitation of the effect on endothelial cells. Most available tests for cell toxicity (lactate dehydrogenase, bicinchoninic acid, TO-PRO 1, MTT) rely on colorimetric and/or enzymatic reactions to provide a quantitative measure of the number of cells that survived a particular insult. BOPP interferes with these tests due to its strong color, and its ability to interfere with enzymatic activity. Tests utilizing other measures of viability were attempted (cell counting, Coulter counter, hemacytometer) without success. Unacceptable error levels and non-linearity in the desired cell population range were problematic with these methods. The methods chosen to determine cell toxicity in the presence of BOPP were phase contrast microscopy and 620 nm absorbance following crystal violet staining. Phase contrast microscopy is a qualitative method for determining the morphology and density of cells following BOPP treatment. A more quantitative method is the application of a plate reader to determine the absorbance of crystal violet stained cells in 24 well plates. A major assumption of this method is that the cells that stick to the plates are viable. Dead cells are either floating in the media or are rinsed from the plate surface during washing of the plate. Another assumption is that the cells that are floating in the media are not viable. This assumption was tested by removing the supernatant from confluent cultures of HUVECs in 24 well plates and replating the supernatant on fresh plates. Upon evaluation with a phase contrast microscope 48 hours following re-plating, no HUVECs were found to have attached to the plate. Thus, this data suggests that detached cells are not viable.

The BOPP concentrations used in this study were similar to those that might be seen in blood or plasma in the dogs used in earlier experiments. The concentration of BOPP in the administration solution was approximately 0.9 mg/ml. Peak plasma concentrations of BOPP were approximately 0.4 mg/ml. The effect of BOPP on HUVECs

appears to be most pronounced at higher concentrations and longer incubations. Figure 4.3 shows the absorbance of crystal violet stained HUVECs at different BOPP concentrations and incubation times. The absorbance of cells in the 48 hour group was significantly higher than those of the other groups at lower BOPP concentrations (<0.4 mg/ml) suggesting a higher number of cells survived at these concentrations. The cause for this finding is not known, but may suggest that 48 hours is sufficient time for significant cell division to occur in the presence of low concentrations of BOPP. Incubation times of 24 and 48 hours at concentrations of 0.5 and 0.75 mg/ml BOPP resulted in significantly lower absorbance and thus lower cell survival. This suggests that BOPP is toxic to endothelial cells at these concentrations. Morphologic examination of HUVECs at these concentrations confirms damage to the cells, with significant retraction of cell bodies (Plate 4.1). The reversibility of this phenomenon, and its importance in the initiation of coagulation, was not studied but would be valuable information in understanding the mechanism of BOPP thrombosis. BOPP concentrations in the dogs were not maintained at these levels for this length of time. At the concentrations of BOPP to which the dog vessels were exposed, there was no significant evidence of toxicity leading to cell death. This does not mean that BOPP has no effect on endothelial cells and the thrombosis seen in the dogs. As discussed earlier, the thrombotic effect of endothelial cells can be elicited by numerous stimuli that do not cause cell retraction or cell death[183]. Thus, studies of the sublethal effects of BOPP on endothelial cells are necessary to fully understand the role endothelial cells play in the thrombosis associated with BOPP administration. Surprisingly, there was an increase in cell numbers at concentrations of 0.3, 0.2 and 0.1 mg/ml in some cultures. No data have been reported showing a stimulatory effect of porphyrins on endothelial cells. One possibility for this finding may be the uptake of BOPP by endothelial cells during incubation resulting in increased absorbance at 620 nm. BOPP absorbance at 620 nm is very low, and would not be expected to add significantly to the strong color of crystal violet, especially after rinsing of the cells prior to staining. If this phenomenon occurred,

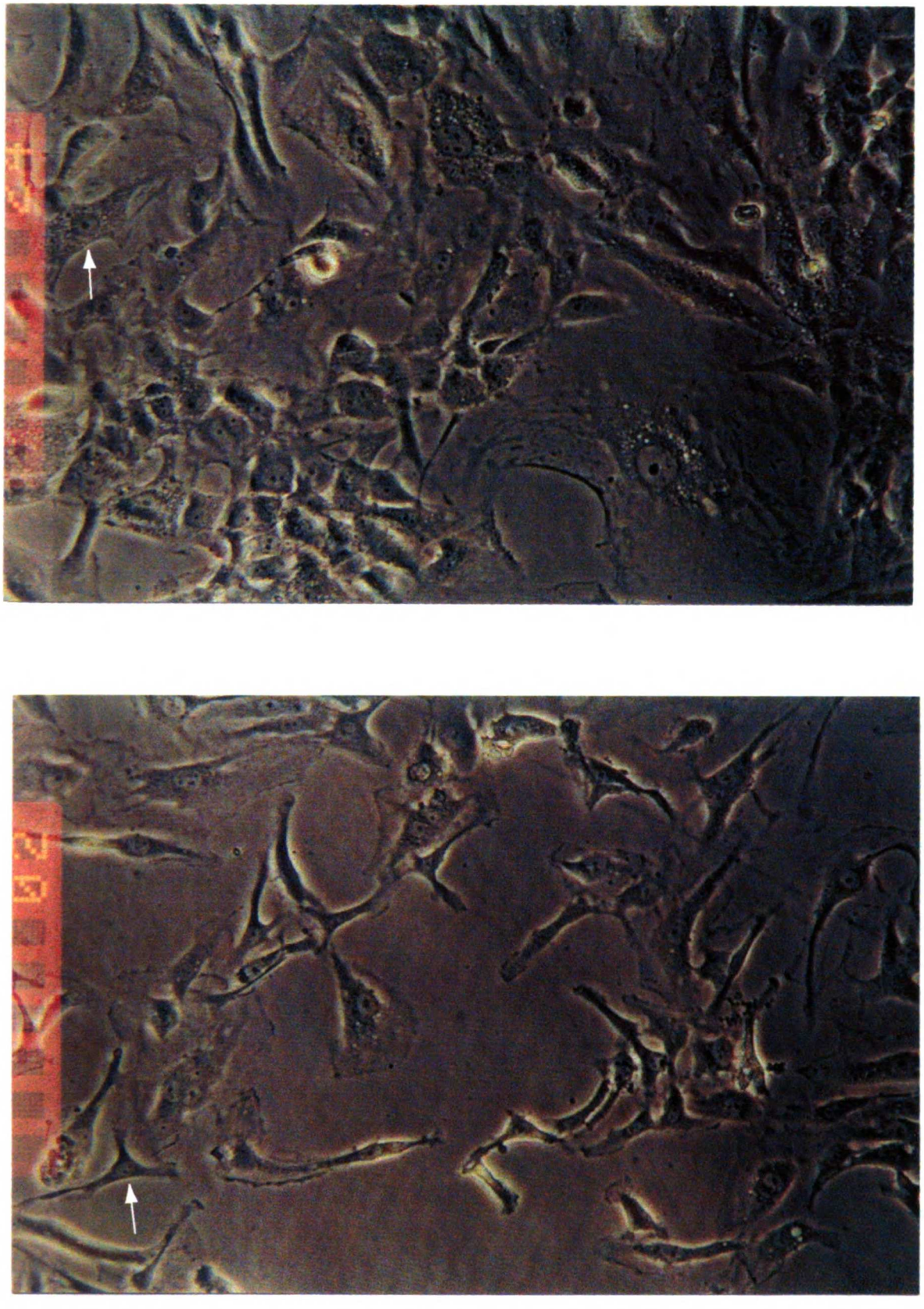


Plate 4.1 Phase contrast micrographs of HUVECs 48 hours following incubation with 0 mg/ml BOPP (upper panel) and 0.75 mg/ml BOPP (lower panel). Arrow in upper panel indicates normal HUVEC, arrow in lower panel indicates retracted HUVEC.

one might expect an increase in the absorbance with time for all concentrations of BOPP, until one reached a concentration or incubation time that resulted in cell death. It is difficult, from the available data, to determine if this phenomenon is occurring.

While this data is preliminary, it is indicative of a potentially toxic effect of BOPP on endothelial cells *in vitro*. The concentrations and incubation times of BOPP needed to cause significant HUVEC death is higher than that seen in the dogs, but lower concentrations may cause sublethal injury that results in the expression and release of procoagulant factors. Ben-Hur et al.[202] reported that the release of procoagulant factors was a more sensitive indicator of injury to endothelial cells due to photosensitization than the release of ^{51}Cr, a commonly used cytotoxicity assay. Concentrations of hematin as low as 0.04 mg/ml for 1 hour were capable of causing reversible morphologic changes in cultured endothelial cells[120]. Concentrations of 0.1 mg/ml were required to cause cell detachment. Platelet adhesion to endothelial cells was enhanced by the presence of hematin, thus suggesting that low levels of hematin can elicit procoagulant properties in endothelial cells[120].

If BOPP and other porphyrin based compounds are to be administered intravenously, a better understanding of the effects of these compounds on endothelial cells and the hemostatic system is vital. This knowledge may help provide information useful in designing formulations and administration methods that can prevent the formation of thrombi.

CHAPTER 5.
BOPP-FIBRINOGEN INTERACTION

5.1 INTRODUCTION:

The data presented in Chapter 3 suggest that BOPP has an effect on the interaction between thrombin and fibrinogen, and may also affect the interaction between fibrinogen and the antibody used in an immunoassay. One possible mechanism for this effect is the binding of BOPP to fibrinogen resulting in the inability of thrombin to act on fibrinogen and the antibody to recognize fibrinogen. The effect of this interaction would be prolongation of the hemostatic tests dependent on thrombin cleavage of fibrinogen (TCT, APTT, PT) and a decrease in the functional and total fibrinogen concentrations. As shown in Chapters 2 and 3, these effects were seen both *in vivo* and *ex vivo* in the presence of BOPP.

The binding of porphyrins to plasma proteins is well known and has been discussed in detail in other sections of this thesis. Data regarding the binding of porphyrins to fibrinogen is limited. El-Far and Pimstone[72] presented data showing binding of porphyrins to fibrin and fibrinogen. Hematin has also been shown to bind to fibrinogen resulting in inhibition of thrombin cleavage[193]. Hematin binding is responsible for significant abnormalities in hemostatic tests following hematin administration, and has also been associated with deleterious clinical effects[85]. The hemostatic test abnormalities seen following hematin administration are similar to those seen following BOPP administration.

The purpose of this study was to determine if BOPP binds to fibrinogen and to explore the nature of that interaction. The use of spectroscopic methods in the study of protein binding, in particular porphyrins, has become commonplace[143, 193, 203]. In this study we used absorbance and fluorescence spectroscopy as tools in examining the BOPP-fibrinogen interaction.

5.2 MATERIALS AND METHODS:

Materials

Na_2BOPP was obtained from M-S. Koo. Canine fibrinogen (Fraction I) was obtained from Sigma (St. Louis, MO, Lot 110H9307). Fibrinogen consisted of 60% protein, of which 85% was clottable. Bovine serum albumin was obtained from Sigma. Phosphate buffered saline (PBS, pH 7.4) was magnesium- and calcium-free and was purchased from the UCSF Cell Culture Facility.

BOPP fibrinogen absorbance experiment

BOPP stock solutions of 740 μM were prepared and dilutions to 7.4 -185 μM solutions were prepared in the presence of 6 μM fibrinogen in PBS. The solutions were prepared in polypropylene tubes and vortexed for 5 seconds. Following mixing, tubes were centrifuged for 5 minutes at 1500 rpm. Supernatant was removed and absorbance measured at 405 nm and 280 nm.

BOPP with albumin and fibrinogen

BOPP, bovine serum albumin and fibrinogen were mixed at various concentrations and ratios in PBS. Samples were prepared in polypropylene tubes, vortexed for 5 seconds and centrifuged at 1500 rpm X 5 minutes. Supernatant was decanted and absorbance measured at 280 and 405 nm.

BOPP fluorescence in the presence of fibrinogen

A stock solution of BOPP (45 μM) was prepared in PBS and mixed with stock fibrinogen solution (12 μM) to prepare samples containing 6 μM fibrinogen and 0.12 μM - 15 μM BOPP. Samples were vortexed for 5 seconds and centrifuge for 5 minutes at 1500 rpm. The supernatant was removed and the fluorescence spectrum obtained using an excitation wavelength of 405 nm and scanning the emission at 580-700 nm. The emission intensity at the highest peak was measured, as was the peak wavelength. Samples of fibrinogen in PBS and BOPP in PBS were also measured.

5.3 RESULTS:

The addition of BOPP to solutions of fibrinogen results in significant changes in the character of the solution. BOPP, at concentrations greater than 37 μM resulted in the formation of a precipitate in the sample (Table 5.1). This precipitate was red, flocculent and pelleted upon centrifugation. The presence of albumin in the BOPP-fibrinogen solution can prevent this precipitation. The concentrations of BOPP, albumin and fibrinogen and their molar ratios are listed in Table 5.1. The presence of fibrinogen had an attenuating effect on the absorbance (405 nm) of BOPP at high BOPP concentrations (Figures 5.1.a, b). Increasing concentrations of BOPP with fibrinogen result in decreased absorbance at 280 nm (Figure 5.1.b) even though the absorbance of BOPP at 280 nm increased linearly in the absence of fibrinogen (Figure 5.1.a). The absorbance at 405 nm of a solution of BOPP in the presence of albumin alone (450 μM) or albumin (450 μM) with fibrinogen (3 μM) is shown in Figure 5.2. The absorbance increases with increasing BOPP concentration, but the absence of fibrinogen results in a decrease and plateau of absorbance at BOPP concentrations above 74 μM.

The peak wavelength of fluorescence of BOPP solutions was affected by the presence of fibrinogen. In the presence of fibrinogen, the peak fluorescence was at 624 nm while in the absence of fibrinogen the peak was 618 nm (data not shown). The fluorescence intensity at the peak increased with BOPP concentration, and showed a plateau at concentrations above 10 μM (Figure 5.3).

Table 5.1 Presence of precipitation in samples of BOPP with fibrinogen and/or albumin

BOPP	Fibrinogen	Albumin	Ratio (BOPP/Fibrinogen/ Albumin)	Precipitation
	μM			
185	6	0	31/1/0	*y*
74	6	0	12.3/1/0	*y*
37	6	0	6.2/1/0	*y*
18.5	6	0	3.1/1/0	n
7.4	6	0	1.2/1/0	n
0	6	0	#na	n
370	0	450	0.82/0/1	n
74	0	450	0.16/0/1	n
37	0	450	0.08/0/1	n
7.4	0	450	0.016/0/1	n
3.7	0	450	0.008/0/1	n
0	0	450	na	n
185	3	450	62/1/150	n
37	3	450	12.3/1/150	n
3.7	3	450	1.2/1/150	n
0	3	450	0/1/150	n
185	3	225	62/1/75	n
185	3	150	62/1/50	n
185	3	75	62/1/25	n
185	3	30	62/1/10	n
185	3	15	62/1/5	*y*
185	6	15	31/1/3	*y*
185	6	30	31/1/6	*y*

#-not applicable

5.4 DISCUSSION

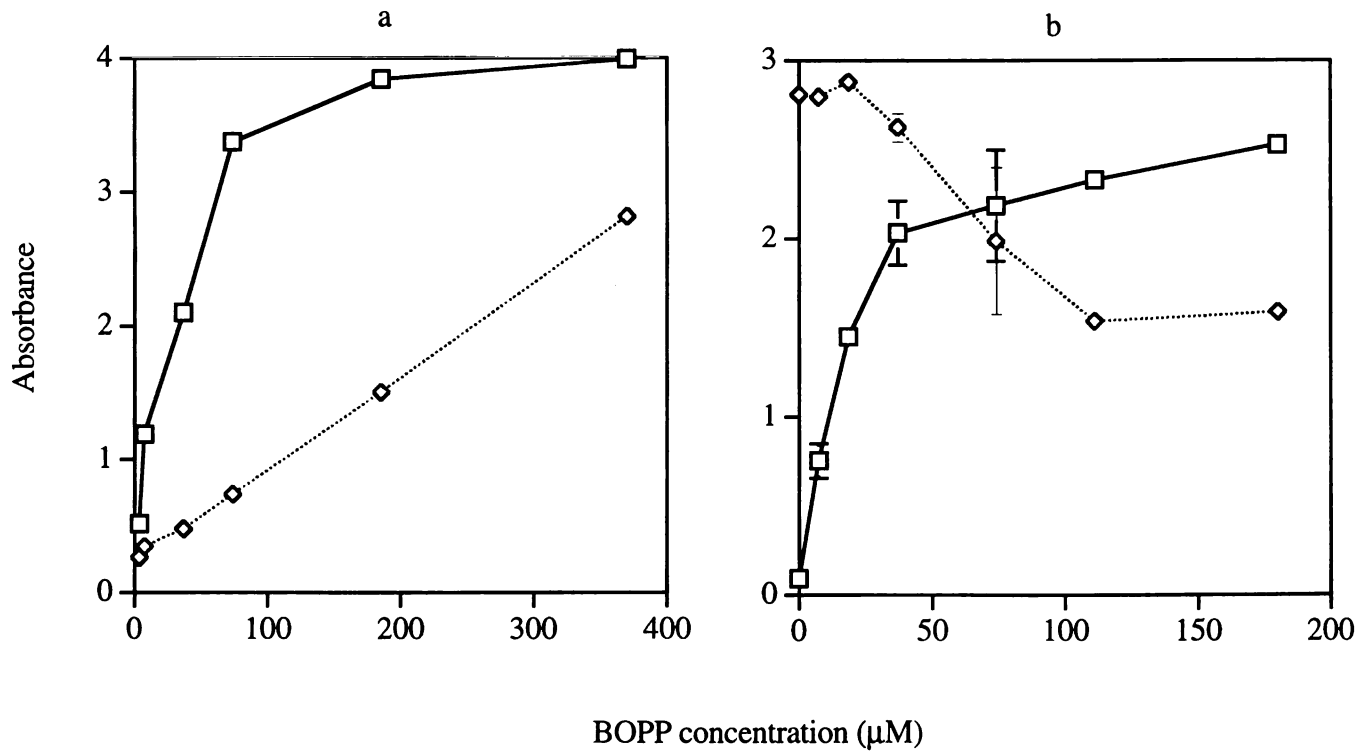
The effect of BOPP on the hemostatic parameters *in vivo* and *ex vivo* has been documented in previous chapters with the data suggesting that the interaction is occurring at the level of thrombin's action on fibrinogen. This study provides evidence that there is an association between BOPP and fibrinogen, and that this association is affected by the presence of albumin. This study was designed to look at concentrations of BOPP, fibrinogen and albumin that were similar to that seen *in vivo*. The BOPP concentration in plasma following BOPP administration is approximately 250 μM, the fibrinogen concentration 8 μM and albumin concentration 600 μM. The use of these concentrations in this work allowed us to evalutate the effect of BOPP on fibrinogen in a relevant, but somewhat simplified system.

The most striking finding in this study is the precipitate that resulted upon addition of BOPP to solutions of fibrinogen. This suggests that BOPP is interacting with fibrinogen to cause precipitation. The flocculent appearance of the precipitate indicates that it is proteinaceous and its red color that BOPP is associated with the precipitate, suggesting that this not simply a salting-out of the fibrinogen. Thus, it is possible that BOPP is binding to fibrinogen and causing its precipitation by affecting its solubility. Similar effects have not been seen with other porphryins[72] at concentrations of porphyrin of 30 μM or hematin[193] using hematin concentrations of 75 μM and fibrinogen concentrations of 5 μM. This precipitation occurs at BOPP concentrations as low as 37 μM and a molar ratio of BOPP to fibrinogen of 6:1 (Table 5.1). By examining Figure 5.1, it can be seen that the increase in BOPP absorbance at 280 nm is linear in the absence of fibrinogen. In the presence of fibrinogen, the increasing concentration of BOPP results in a decrease in the 280 nm absorbance indicating that this decrease in absorbance may be due to precipitation of a BOPP-fibrinogen complex.

At the concentrations studied, BOPP is likely to be > 50% dimerized[155]. This could result in a complex mixture of monomeric BOPP, dimerized BOPP and fibrinogen

molecules that may have one or both of these forms bound. The presence of fibrinogen led to a shift in the Soret band of +3-4 nm (BOPP 0.75 μM, data not shown). Similar shifts in the Soret have been seen in the binding of protoporphyrin to proteins[203]. The dimerization of BOPP results in a shift in the Soret band to a shorter wavelength[155]. The combination of these two phenomena make interpretation of spectra at physiologic concentrations difficult, but the increase in the Soret band in the presence of fibrinogen is suggestive of BOPP association with fibrinogen. The absorbance at 405 nm is also affected by BOPP concentration and the presence of fibrinogen (Figure 5.1). As confirmed by Spizzirri[155], the absorbance of BOPP at 405 nm plateaus at concentrations above 100 μM. The absorbance of BOPP at 280 nm is linear up to and above this range. The absorbance at 405 nm of BOPP in the presence of fibrinogen follows a similar pattern as that of BOPP without fibrinogen (Figure 5.1.b). There is a plateau in the absorbance at concentrations above 50 μM, but the absorbance at concentrations below 50 μM are similar to that of BOPP alone. Certainly, the precipitation of fibrinogen with BOPP is at least partially responsible for the decrease in absorbance seen at the higher BOPP concentrations. These changes in the absorbtion of BOPP, coupled with the presence of precipitate in mixtures of BOPP with fibrinogen strongly suggest an interaction between BOPP and fibrinogen at physiologic concentrations.

The *in vivo* situation is significantly more complex. Porphyrins are known to bind to plasma proteins[144, 151, 203] and are considered to be nearly completely bound in the plasma. Thus, the ability of free BOPP to interact with fibrinogen in the plasma may be limited. The ability of albumin to prevent BOPP precipitation of fibrinogen was demonstrated in this study and is shown in Table 5.1. The presence of albumin at concentrations exceeding 5 times that of fibrinogen prevented the formation of precipitate by high concentrations of BOPP (185 μM). This concentration of albumin is significantly lower than that found in the plasma. These data may suggest that albumin acts to increase the solubility of a BOPP-fibrinogen complex, or that albumin binds significant quantities of



Figures 5.1a, b. Absorbance of BOPP solutions containing no fibrinogen (5.1.a) and 6 μM fibrinogen (5.1.b). Squares represent 405 nm absorbtion and diamonds represent 280 nm absorbtion. Error bars indicate S.D.

BOPP resulting in less free BOPP to interact with fibrinogen. In either case, it is unlikely that fibrinogen precipitation *in vivo* as the result of BOPP treatment is the cause for the observed decreases in fibrinogen concentrations (Chapters 2,3). The absorbance at 405 nm of BOPP in the presence of albumin (Figure 5.2) results in a different picture than

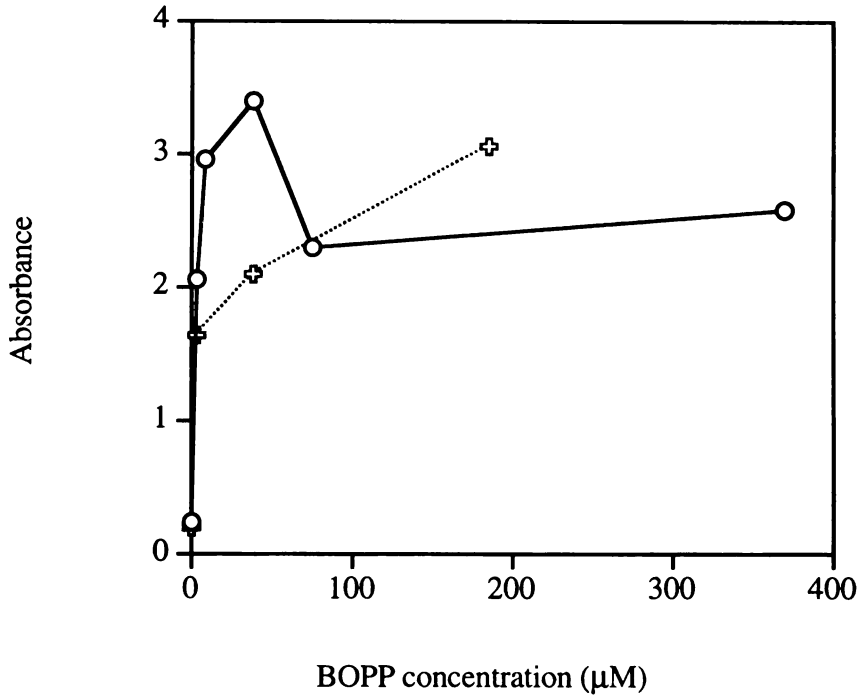


Figure 5.2 Absorbance at 405 nm of BOPP solutions containing albumin (450μM) or fibrinogen (3 μM) and albumin. Open circles represent BOPP with albumin, open crosses represent BOPP with albumin and fibrinogen.

that seen with BOPP and fibrinogen. The absorbance increased until BOPP concentrations reach 50 μM, then decreased and became level. No evidence of precipitation was seen, thus loss of BOPP from solution via this mechanism is unlikely. Any number of interactions between BOPP monomer, BOPP dimer and albumin are possible which may result in a decrease in the absorbance. The data presented here are insufficient to characterize these interactions and this data indicates a need for further study. The addition of fibrinogen to the BOPP-albumin mixture (Figure 5.2) results in an absorbance similar to

that seen with fibrinogen and BOPP alone (Figure 5.1.b). Thus the interaction between BOPP and albumin is affected by the presence of fibrinogen such that the absorbance of BOPP does not decrease at high BOPP concentrations. The presence of albumin in plasma may function to prevent the precipitation of a BOPP-fibrinogen complex which, if the BOPP-fibrinogen complex maintains some activity, may decrease the effects of BOPP on the cleavage of fibrinogen. Alternatively, albumin may bind a large percentage of BOPP, resulting in less free BOPP available to exert its effects on fibrinogen. In similar findings, Green et al.[193] found that the presence of albumin significantly decreased the hematin-induced prolongation of the thrombin time.

The fluorescence of BOPP in the presence of fibrinogen is also affected. The peak fluorescence observed following 405 nm excitation was found to shift by + 6 nm in the presence of fibrinogen. This is a strong indication of an interaction of BOPP with the protein. A similar shift has been reported with the binding of protoporphyrin with albumin[203]. Fluorescence spectra of BOPP in the presence of fibrinogen exhibited only the peak at 624 nm, with no peak at 618 nm. This suggests that much of the BOPP is associated with fibrinogen. Unlike Lamola, we found that there was no significant increase in the quantum yield of BOPP with increasing fibrinogen to BOPP concentration ratios. It is possible that the environments with which the porphyrins associate on their respective proteins is different resulting in divergent results. Figure 5.3 also shows that BOPP fluorescence intensity decreases with increasing BOPP concentration as a result of the formation of low quantum yield dimers[155].

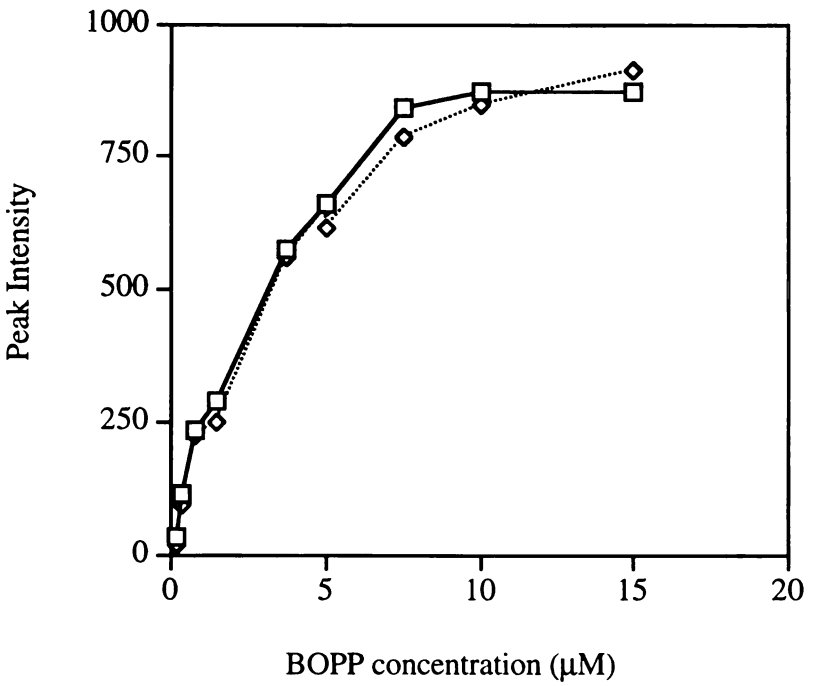


Figure 5.3. Peak fluorescence intensity with excitation at 405 nm of BOPP solution with and without fibrinogen. Error bars indicate S.D. Open squares represent BOPP with fibrinogen, open diamonds represent BOPP without fibrinogen.

The data in this experiment provide further evidence of a BOPP-fibrinogen association. Attempts to determine the dissociation constant (K_d) and maximum binding capacity of fibrinogen were complicated by the precipitation that results from large excesses of BOPP. The precipitation phenomenon also complicates other methods (equilibrium dialysis, gel filtration) of determining these constants. But based on the data obtained, one can predict that the K_d is sub-micromolar based on the lack of a 618 nm fluorescence peak even at 0.12 μM concentrations of BOPP[204].

The data presented in this chapter are by no means comprehensive, but provide strong evidence of an interaction between fibrinogen and BOPP. This interaction may be responsible for the abnormalities in the hemostatic tests reported in other parts of this

thesis. The relationship between BOPP and fibrinogen is complex, and is likely to be strongly influenced by the presence of other plasma proteins and the aggregation state of BOPP. Further study of BOPP, and other porphyrins, may provide valuable information regarding the potential hemostatic effects of these compounds and may help to prevent adverse effects caused by their administration.

CHAPTER 6.
BINDING OF BOPP TO RED BLOOD CELLS

6.1 INTRODUCTION:

An important characteristic of therapeutic compounds is their ability to bind to blood cells, in particular red blood cells (RBCs). Drug bound to blood cells is not available for passage into tissues and, in a drug that has a low extraction ratio in the liver, the binding to blood is an important factor in its hepatic clearance[205]. In addition, the concentration of boron in blood is an important therapeutic consideration in BNCT. The blood boron concentration has an effect on the damage caused to the vascular system in the vicinity of the tumor as a result of neutron irradiation. High concentrations of boron in the blood at the time of treatment are not desirable since the neutron capture reaction occurring within the blood will have an effect on the vasculature surrounding that blood[206]. The degree of this effect is the subject of significant debate[111, 206, 207]. With this in mind, we proposed a study of the blood and plasma boron concentration following BOPP administration.

6.2 MATERIALS AND METHODS:

To study the amount of boron bound to blood cells, we obtained blood and plasma from a dog at various time points following BOPP administration (as described in Chapter 1). These samples were submitted for boron analysis (Chapter 1). In addition, we analyzed the hematocrit of the dog at 0, 48 and 79 hours following BOPP administration. Data was reported as μg boron / mg of sample. We have made the assumption that the density of blood and plasma is close to one (± 5%). To determine the amount of boron contained in blood cells we used the following formula relating plasma, blood and blood cell concentrations:

Equation 1. $$C_b * V_b = C_p * V_p + C_{bc} * V_{bc}$$

where C_b = blood boron concentration, V_b = blood volume, C_p = plasma boron concentration, V_p = plasma volume, C_{bc} = blood cell boron concentration, and V_{bc} = blood cell volume. Since $V_{bc} = H*V_b$ and $V_p = (1-H)*V_b$, where H = hematocrit, Equation 1 becomes:

Equation 2. $$C_b = C_p*(1-H) + C_{bc}*H$$

One can then solve for the concentration of boron in the blood cells (C_{bc}).

To calculate the hematocrit at various times following BOPP administration, we plotted the hematocrit of the dog at the three available three time points (0, 48 and 79 hours). Least squares regression was used (Minim 3.0.8) to fit the data. This data fit was used to determine the predicted hematocrit for all time points at which boron measurements were made.

6.3 RESULTS AND DISCUSSION:

The blood and plasma concentrations of boron follow a similar decline as seen in Figure 6.1. The blood cell concentration is low, and appears to decline at a slightly slower rate than that of blood. This suggests that the blood cells are retaining the boron more avidly than the blood overall. Because of the uncertainty in the estimated hematocrit values and the error involved in measuring boron concentrations ($\leq 5\%$), it is difficult to predict the precise amount of boron in the blood cells or speculate on the avidity of binding of boron-containing species. It is possible that BOPP or a metabolite of BOPP is binding to high affinity sites on the red blood cell resulting in a slower decline in blood cell boron concentrations relative to plasma or blood. If so, this may have important implications for other porphyrin-based compounds, particularly if the binding of those compounds to blood cells results in toxicity or a significant pool of compound in the blood cell compartment.

The concentration of boron in the vascular lumen is an important factor in the determination of the utility of a BNCT compound. While it is generally agreed that the dose to the wall of the vessel is attenuated compared to that of the blood[111, 206, 207], it is also believed that the injury to the skin and other normal tissues as a result of treatment with BNCT is related to damage to the vascular system[111, 206, 207]. With this in mind, it is apparent that keeping the levels of boron in the blood at a minimum at the time of neutron irradiation would be advantageous in reducing the potential of local tissue injury. Thus, an important therapeutic implication from these data is that boron is not highly concentrated in the blood cells which would result in much higher blood levels than plasma levels. Nor does the boron concentrations in the blood cells increase over the time course of the study. Were boron to concentrate in the blood cells at high levels, this would make the blood levels of boron a more important measure of intravascular boron levels then plasma levels. In addition, avid binding of BOPP to blood cells might affect the pharmacokinetics of boron by decreasing the clearance of boron[205]. The low amount of boron found in blood cells suggests that blood cell binding of BOPP or its metabolites is not likely to be a cause of the low boron clearance seen in these dogs.

The binding of boron to blood cells appears to be small and should not affect the pharmacokinetics of boron or the utility of BOPP as a therapeutic agent. Further studies of this binding phenomenon would be valuable in determining the binding characteristics of BOPP to blood cells and the potential for toxic effects of BOPP or other porphyrin-based compounds on these cells.

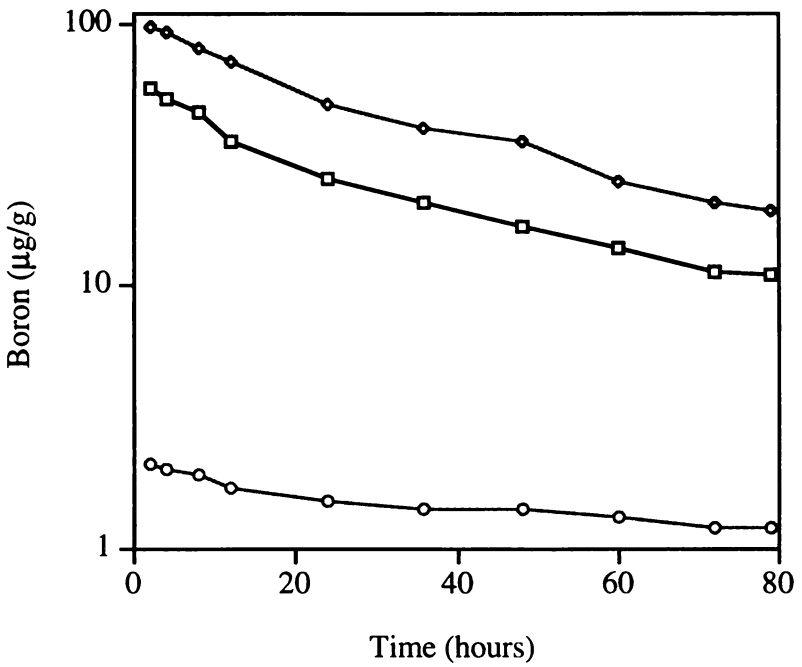


Figure 6.1. Blood, plasma, and blood cell boron levels following BOPP administration. Diamonds represent plasma, squares represent whole blood and circles represent blood cells.

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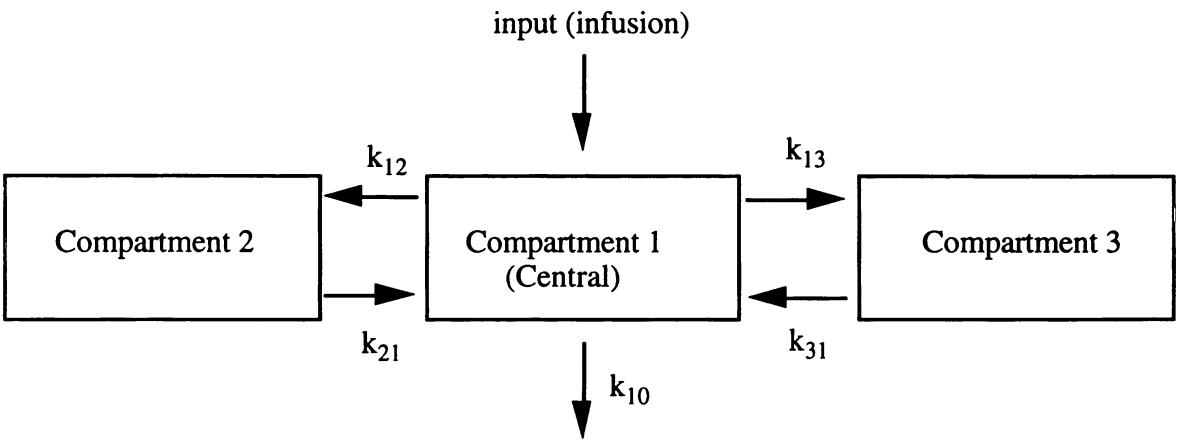
APPENDIX I.
Basic Pharmacokinetic Principles and Terms

This appendix serves as a brief glossary of pharmacokinetic terms and their relationships. The pharmacokinetic model used for this study is illustrated in Figure A.1



This model assumes all elimination of boron is from the central compartment.

AKAIKE

AKAIKE is a method for determining if the model used to fit the data is appropriate. This method considers the number of observations made, how well the model describes data, and the number of parameters used to fit the model to the data[208]. This technique allows you to determine the correct number of parameters to use when evaluating a data set.

Area under the curve (AUC)

This parameter is defined as that area under the plasma concentration vs. time curve[209]. This area can be extrapolated beyond the measured time points to time infinity. This extrapolated AUC is calculated using Equation A.1

$$AUC_{extrap} = C_{final}/k_{terminal} \qquad \text{Eq A.1}$$

where AUC is the extrapolated AUC, C_{final} is the plasma concentration at the last measured time point, and $k_{terminal}$ is the rate constant for the terminal half-life[209] (in this study, k_{gamma}).

Clearance (CL)

Clearance as used in this study is the total clearance of boron from the body. CL is a proportionality constant that relates plasma concentration with rate of drug elimination (Eq A.1)[205]. The units for CL are volume/time.

$$\text{Rate of elimination} = CL*\text{concentration} \qquad \text{Eq. A.2}$$

CL in this study was calculated by using the Equation A.3

$$Cl = k_{10}*V_c \qquad \text{Eq. A.3}$$

Compartments

This study used a compartmental modeling procedure to determine the appropriate number of exponential terms required to describe the plasma boron data in the dogs. Each exponential term represents a compartment. Without very extensive tissue concentration studies, it is difficult to determine which tissues are represented by each compartment. For this study, a three-exponential mathematical model best described the plasma boron data, thus plasma boron was found to fit to a three-compartment model.

Half-life ($t_{1/2}$)

As described above in the discussion of compartments, the plasma boron data was mathematically fit to a three-exponential function. Each exponential describes a portion of the plasma concentration vs. time curve and are called rate constants (k). The half-lives are calculated using these elimination rate constants using Eq. A.4[205].

$$t_{1/2} = 0.693/k \qquad \text{Eq. A.4}$$

The units for half-life is hours. Half-lives are defined as the time required for elimination of 1/2 of the dose. In a three compartment model there are three half-lives, the initial (alpha), middle (beta) and terminal (gamma). The initial half-life usually describes the distribution of the drug into tissues, although there is some elimination and metabolism occurring simultaneously. The terminal half-life describes the plasma concentration following distribution and during the release of drug from tissues and its subsequent elimination. The middle half-life is less well defined and reflects both the elimination of drug and its redistribution among tissues.

Maximum Likelihood

This technique uses statistical methods to determine the mathematical function that is most likely to describe a given data set[208].

Mixed effect modeling

This method of data analysis is used to estimate pharmacokinetic parameters from plasma concentration vs. time data[210].

Rate constants (k)

Figure A.1 shows the model used for the estimation of the pharmacokinetic parameters. The rate constants (k) are shown. These rate constants determine the rate at which boron distributes between the compartments. The rate constants area also used to describe the plasma concentration vs. time curve. The calculation of the multi-exponential model to describe the plasma concentration vs. time data results in the following equation[211] (Eq. A.5)

When $t \leq T_{inf}$ then $T_p = t$, if $t > T_p$ then $T_p = T_{inf}$;

$$C = \{A*(1-\exp[-k_1*T_p])*\exp[-k_1*t] + B*(1-\exp[-k_2*T_p])*\exp[-k_2*t] + D*(1-\exp[-k_3*T_p])*\exp[-k_3*t]\} \quad \text{Eq A.5}$$

where C is the plasma concentration, t is time of study, T_{inf} is the time during the infusion, and T_p is the time when the infusion ends. A, B, D are coefficients relating to the zero time intercept for this equation. k_n are the rate constants describing the plasma concentration. These rate constants are in units of t^{-1}. k_1, k_2, and k_3 are identical to k_{alpha}, k_{beta} and k_{gamma}, respectively.

Volume of distribution (V_c)

This parameter is a proportionality constant between the amount of drug in the body and the plasma concentration[205] and is defined by Eq. A.6.

$$V = \text{Concentration in plasma/Amount in body} \qquad \text{Eq. A.6}$$

In this study the V used is initial volume of distribution, that of the central compartment, which is the volume into which the drug distributes immediately upon entering the body

(ie. at time t = 0). V rarely corresponds with a real volume in the body since distribution to many tissues may occur[205].

Volume of distribution at steady state (V_{ss})

This term defines the volume of drug distribution when the drug is at steady state in the body. Steady state is defined as the condition where the input of the drug is equal to the elimination, as would occur with a constant infusion of drug. This volume often reflects nearly the total volume into which the drug can distribute in the body[205]. V_{ss} is calculated using the Eq A.7

$$V_{ss} = \frac{\text{Amount of drug in body at steady state}}{\text{Plasma drug concentration at steady state}} \qquad \text{Eq A.7}$$

For this study, steady state was not achieved during BOPP infusion. However, the V_{ss} can be estimated based on Gibaldi and Perrier[211] using Eq A.8

$$Vss = V\{(k_{13} + k_{12})/(k_{21}*k_{31}) + 1\} \qquad \text{Eq A.8}$$

using the rate constants illustrated in Figure A.1.