HEPATOCELLULAR CYTOPROTECTION AGAINST THE PATHOBIOLOGIC EFFECTS OF THIACETARSAMIDE *IN VITRO* AND *IN VIVO* USING N-ACETYL-L-CYSTEINE

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ABSTRACT

The recommended treatment of adult heartworm disease in canines is 2.2 mg/kg of the arsenical drug, thiacetarsamide (T). This results in approximately a 73% kill of adult heartworms. Use of 3.3 mg/kg has been reported to be 100% efficacious. Thiacetarsamide is a known hepatotoxin at the lower dosage with a reported increase in severity of hepatotoxicity occurring at the higher dosage. The suspected predominant mechanism of T hepatotoxicity involves covalent binding of As to enzymatic sulfhydryl groups. Sulfhydryl (thiol) containing substances, such as glutathione, have been beneficial in reversing the toxic effects of As. N-acetyl-L-cysteine (NAC) is a monothiol, precursor substance to glutathione. It was hypothesized that concurrent use of NAC may allow the higher dose of T to be used without increasing the risk of hepatotoxicity.

This project investigated NAC for its effect on T-induced cytotoxicity *in vitro* using NCTC 1465 (murine transformed hepatoma) cell culture. Experiments were performed on confluent cell cultures still in log phase. Viabilities were evaluated by dye exclusion with 0.2% Trypan blue. Initial concentrations (1X) were determined using estimated extracellular fluid concentrations (EFC) as based upon canine IV dosages of 3.3 mg/kg of T and 140 mg/kg of NAC. Sigmoid lethality concentration curves (LC) were established for T using varying times of exposure to T,

incubation following exposure, and varying concentrations of T. An LC_{50} was consistently found between the [1 to 3X] of T with 4 hours exposure and 24 hours incubation. A series of 3 experiments were performed with simultaneous of NAC and T. NAC was in the media during the exposure to T (4 hr) in the first, during the incubation (24hr) in the second, and during both (28 hr) in the third. Cytoprotection in all three experiments was found as a shift of LC curves to the right using the [1 to 10X] of NAC.

Pretreatment effects of NAC and dextrose (D) upon hepatocellular toxicity of T *in vivo*, in normal dogs, were also investigated. Serial percutaneous transabdominal hepatic needle biopsies and blood samples for ALT levels were obtained from each dog prior to any treatments. Pretreatments were given 5 minutes before each injection of T. Group I was pretreated with .7 ml/kg of .9% NaCl IV (n=9). Group II was pretreated with 10 ml/kg of 10% D IV (n=6). Group III was pretreated with .7 ml/kg of 20% NAC IV (n=9). T was then given at 3.3 mg/kg IV BID for 2 days to all groups. Blood tests were repeated each day during and for 2 days after the treatments. Another hepatic biopsy sample was obtained from each dog 24 hours after the last treatment. Thiacetarsamide caused a predominance of periportal (acinar zone 1) hepatocellular necrosis with accompanying inflammation. Mean differences of ALT at 72 hours and biopsy results between groups I and II were

consistent with a worsened effect of dextrose ($p = 0.06$). A significant cytoprotective effect of NAC (I vs III and II vs III) was seen with biopsy scores ($p = 0.036$) and ALT values ($p < 0.05$).

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ABBREVIATIONS USED IN THE TEXT

ADP	-	adenosine diphosphate
ALT	-	alanine aminotransferase
ANOVA	-	analysis of variance
As	-	arsenic
ATP	-	adenosine triphosphate
B-OH	-	benzyl alcohol
C	-	celsius
CF	-	conversion factor (μg/ml x CF = μmol/l)
CHD	-	canine heartworm disease
ECF	-	extracellular fluid
EDTA	-	disodium ethylenediamine tetraacetate
EFC	-	extracellular fluid concentration
ELISA	-	enzyme linked immunosorbent assay
GSH	-	glutathione
H&E	-	hematoxylin and eosin
ICG	-	indocyanine green
IV	-	intravenous
kg	-	kilogram
mg	-	milligram
ml	-	millilitre
NAC	-	N-acetyl-L-cysteine
NCTC	-	National cancer tissue culture
P	-	phosphorous
PAS	-	periodic acid Schiff
SH	-	sulfhydryl groups
T	-	thiacetarsamide (or arsenamide)
TCA	-	tricarboxcyclic acid cycle

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1. GENERAL INTRODUCTION

1.1 Canine heartworm disease and use of thiacetarsamide

Thiacetarsamide sodium, an arsenic compound, is the most commonly used (since 1947) and only licensed drug for the treatment of mature heartworms (Dirofilaria immitis) in dogs (1,2,3,4,5). Canine heartworm disease (CHD) is an endemic problem in much of North America, Europe, Japan, Australia, and South America, as well as other regions of the world (6,7,8,9). It was first described in 1679 and first treated with thiacetarsamide in 1945 (10). Dirofilaria immitis is a nematode parasite whose primary hosts are both domestic and wild canid species including the domesticated dog (11,12). Female mosquitoes are the vector for heartworms, serving as the intermediate host. Over 60 species of mosquitoes worldwide are reported as capable of transmitting Dirofilaria immitis (13). First stage larvae (L_1 or microfilaria) are ingested by the mosquito during a blood meal from the infected dog and develop to an L_3 stage in the mosquito. After 2 to 2.5 weeks Dirofilaria immitis is transmitted as the L_3 when the mosquito bites another dog. These infecting larvae migrate through the host's tissues to the lumen of the pulmonary artery and its branches while developing into immature adults (L_5). This takes approximately 3 to 3.5 months. By six months post

infection the heartworms are adults and produce a microfilaremia (L_1) (7,12,14). Diagnosis is currently made using tests for blood born microfilaria or circulating adult heartworm antigens (ELISA) (15). In an endemic area of Louisiana, 13.6% of a veterinary teaching hospital case load is reported to be positive for heartworms (16). The infection rate in a hyperendemic area (Grand Bahama Island) is reported as 58% (17).

Clinical signs at the time of detection include none, cough, haemoptysis, exercise intolerance, right heart failure, hepatic congestion, loss of condition, protein losing nephropathy, ascites, and anorexia (7,15,16). The majority of pathology from CHD is related to endothelial damage and inflammation with subsequent induction of a myointimal proliferative response in the pulmonary arteries (7,18,19). This response within the pulmonary arteries is the result of immunologic deposition of antigen as well as the resultant platelet aggregation with release of platelet derived growth factors (4,20). The clinical presentation of the infected dogs varies with the number and acquisition rate of infective larvae. A few heartworms garnered over several months to years may result in little evidence of clinical disease; whereas many acquired in a few days or weeks, and maturing simultaneously, can cause acute, adverse alterations in right side cardiac hemodynamics (7,9,21).

Thiacetarsamide is currently recommended, for the

treatment of adult heartworms (adulticide therapy), to be administered intravenously at a dosage of 2.2 mg/kg of body weight given twice daily for 2 days (9,22). This dosage is thought to result in the best balance between effectiveness and adverse drug reactions. Immature (< 12 months) female heartworms are the most resistant to treatment. Mature (>24 months) male heartworms are most sensitive (23). Overall efficacy with the above regimen is reported as 20-40% for complete worm kill and a 74% mean (48-86%) worm kill (24,25). Because of this residual worm burden in treated dogs, a superior regimen for use of thiacetarsamide is sought.

The most common adverse reactions seen in dogs resulting from use of thiacetarsamide include perivascular cellulitis at sites of intravenous injection, hepatotoxicity manifested by anorexia and icterus, pulmonary thrombosis by dying adult heartworms, and renal tubular nephrosis. Thiacetarsamide is directly toxic to endothelium. Repeated administration at one site results in endothelial proliferation, thrombus formation, and occlusion of the venous lumen (7,26). Marked perivascular cellulitis occurs only when the drug is injected improperly or leaks back from the lumen of the vein. The severity of the inflammation is proportional to the amount injected incorrectly. The routine use of intravenous catheters at different sites, with confirmation of proper placement by saline injection,

precludes these problems. Pulmonary arterial thrombosis due to emboli of dyeing worms is unavoidable if there are a large number of heartworms present (27,28). Pretreatment of dogs with CHD using aspirin and/or heparin is recommended to minimize the problem of pulmonary thromboembolic disease (28,29,30). Renal tubular nephrosis occurs infrequently and may be related to preexisting renal disease, prerenal causes, or to the direct exposure to low levels of free arsenic (2,7,31,32). The occurrence of hepatotoxicity, and its attendant clinical signs of anorexia, vomiting, and icterus, is of great concern to veterinarians administering thiacetarsamide (2,7,12,26,33). Hoskins et al, reported 237 cases of naturally infected dogs with CHD which were treated with the standard regimen of thiacetarsamide as stated above. Thirty-nine of these dogs (14.1%) had clinical signs consistent with hepatotoxicity. The treatment regimen with thiacetarsamide was not completed in seven of the 39 cases due to severity of clinical signs. Preclinical evaluations using signalment (age, breed, sex), clinical status, thoracic radiographs, serum chemistries, hemograms and urinalysis cannot identify dogs at risk for this problem (16,33). The risk of hepatotoxicity (< 15%) is considered acceptable by all authors cited when the factors of moderate efficacy and lack of alternative, superior adulticidal drugs are considered. Of note is the anecdotal ability to administer thiacetarsamide to the same reactor dogs at a

later time (approximately 30 days) without recurrence of the hepatotoxicity (2,5,7,26). No explanation for this is offered in the literature. The mechanism of thiacetarsamide related hepatotoxicity in dogs is not known but its pattern is similar to reports of trivalent arsenical toxicity in humans (34,35). A hypothesis for the mechanism of thiacetarsamide induced hepatotoxicity is presented later in this chapter. The ability to predict hepatotoxicity in individual animals is desired and efforts have been directed to this end (25). But wide variation in response of individual dogs and relatively low numbers of animals have made interpretation of the results difficult.

1.2 Pharmacology of thiacetarsamide in the dog

Thiacetarsamide is a disubstituted, organic, trivalent arsenic compound. It has a molecular weight of 377.26 and its chemical formula is $C_{11}H_{12}AsNO_5S_2$ (Figure 1). Available as a white crystalline powder (arsenamide), it is dissolved in water at > 34° Celsius (C). The Pka = 4 and it remains in solution at a pH of 7 to 8 despite lower temperatures. It is stable in aqueous solution for at least 18 months if stored appropriately at 2-8° Celsius (C) and removed from light (36,37). Thiacetarsamide (arsenamide) is a 1% aqueous solution of the neutral dibasic salt. Solutions of thiacetarsamide exist as a mixture of p-arsenosobenzamide

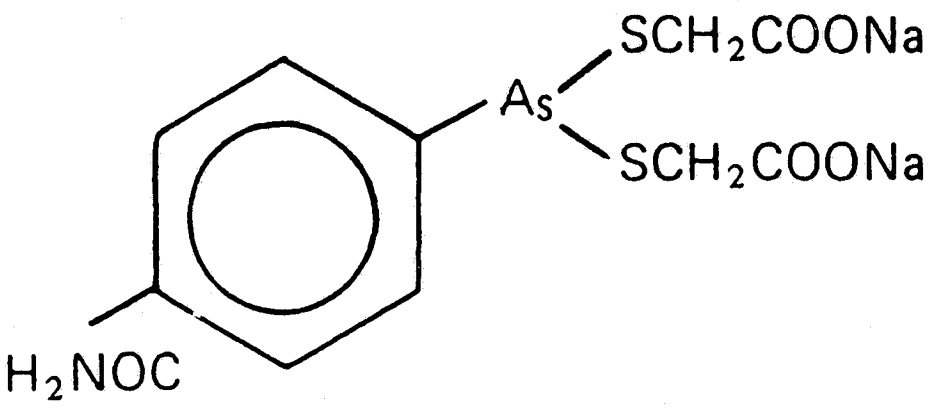

Figure 1. **Chemical structure of thiacetarsamide molecule.**

and thiacetarsamide. Both of these compounds are organic trivalent arsenicals. Thiacetarsamide is formed by condensation in a reversible (hydrolysis) reaction with p-arsenosobenzamide and mercaptoacetic acid (38). Both arsenosobenzamide and thiacetarsamide are toxic to adult heartworms. Retention by As of the trivalent state in the organic para-amide group is essential to maintain its efficacy against Dirofilaria immitis (12,37,39,40,41). Upon exposure to oxygen and light, deterioration results in production of unidentified degradation products (37). The oxidation to a pentavalent arsenic moiety partially explains the reduction of its therapeutic efficacy (37,41,42). Thiacetarsamide is considered to have a steep dose-response curve and a relatively narrow margin of safety (12).

The commercially available form of thiacetarsamide used in the *in vivo* project is Caparsolate™ (Caparsolate™ sodium, CEVA Laboratories, Overland Park, Kansas). It is a solution containing 10 mg of thiacetarsamide per millilitre (ml) with .9% benzyl alcohol as a preservative. The reported pharmacokinetic data for this formulation, when administered IV at 2.2 mg/kg to six healthy dogs, is based upon whole blood determination of arsenic (As) since degradation of thiacetarsamide to unidentified As metabolites is extremely rapid, probably occurring with the first passage through the liver. This may not accurately reflect true pharmacokinetics of thiacetarsamide since

differences between thiacetarsamide and whole blood arsenic probably exist. It is reported that the parasiticidal action of thiacetarsamide is dependent upon its being in the organic molecular form and a trivalent state (1,39,41,43). The interconversion of arsenic begins immediately so that trivalent, pentavalent, and inorganic forms are quickly present, along with various methylated compounds (44,45). These are not necessarily relevant forms with regards to the toxic mechanisms or parasiticidal actions of thiacetarsamide (34,36). A biphasic pattern of elimination is present for varying doses of thiacetarsamide (2.2 mg/kg, 2.4 mg/kg, 2.66 mg/kg, and 3.3 mg/kg). Blood concentrations of As decline rapidly in the first hour followed by a post distribution phase of slowly declining values to non-detectable levels. With repeated dosages, the post distribution phase reaches an equilibrium between the tissues and elimination. The result is increasing values for As with each subsequent dose and detectable levels for up to 2 weeks. However, the post distribution levels cannot be correlated with the IV dose used (46). Over 85% of administered As is recovered in feces by 48 hours suggesting that hepatic elimination is very important for thiacetarsamide (40). This implies a very different route of elimination than is found when elemental As is injected into dogs and 90% is reported cleared in the urine (44). Tissue concentrations of As are highest in the liver and to a lesser extent in the kidney

(49). The reported mean elimination phase half life for thiacetarsamide (as determined by whole blood As levels) and the clearance rate are 43 minutes and 200 ml/kg/min (47). The majority of the drug is thought to be metabolized in the liver. This is an extrapolation from information in the literature as follows. First, the half-life of total blood As is correlated with the half-life of indocyanine green (ICG) ($r^2 = 0.90$). Second, in partially hepatectomized dogs, the half-lives are significantly longer and the clearance rates significantly lower for both T and ICG indicating the dependence on the liver for metabolism (47,48). Third, thiacetarsamide given orally results in the same rapid blood As levels as intravenous administration but there is no death of heartworms suggesting that portal circulation prior to systemic circulation reduces efficacy (40). Efficacy of heartworm kill in response to administration of thiacetarsamide is reported to be related to post distribution phase total blood As coccentration (25,47). No correlation is reported for the concept of adulticidal activity being related to clearance rate for arsenic e.g., the lower the clearance rate of As, the longer the exposure of As to the worm, the greater the killing efficacy (47). This forms an apparent contradiction with the concept that it is the form of arsenic (thiacetarsamide rapidly metabolized) and not the arsenic concentration that has the toxic effect upon the heartworm. No correlation

exists between the resulting values for hepatic leakage enzymes (e.g., alanine aminotransferase (ALT)), ICG clearance, and the dose of thiacetarsamide used; however a wide variation for these values is noted within thiacetarsamide-treated groups of normal and heartworm infected dogs (2,4,25). The probable hepatotoxic mechanisms of thiacetarsamide are hypothesized from basic knowledge of arsenic, As metabolism and arsenic's mechanisms of toxicity.

1.3 Arsenic toxicology

1.3.1 Nature of arsenic

Arsenic is considered to be the twentieth most common element and is a metalloid. It is element 33 in the periodic table. It is a group V (column) element similar in its chemical properties to nitrogen, phosphorus, and bismuth. Arsenic is in period 4 (row) along with the elements calcium, manganese, copper, zinc, and selenium. Arsenic exists in various valence states: 0, 3+ (trivalent), and 5+ (pentavalent). It tends to form relatively stable covalent bonds with organic molecules. However, these are dissociable depending on the reactivity and concentration of new substrates since As has a moderate redox potential (34,35). It is found in three distinct forms: arsine (AsH_3), inorganic, and organic. Another

classification system for As is whether it exists in a disubstituted or monosubstituted form (Figure 2). Disubstituted As compounds are generally more stable than monosubstituted ones; however, interconversion between the two forms occurs frequently. The form, and hence nomenclature, of an arsenic compound *in vivo* is only valid for a short period of time before reactions occur which result in variably stable intermediaries or metabolites e.g., valence changes, organic versus inorganic compounds, monosubstituted versus disubstituted (Figure 3) (34,35,42,45,49,50).

1.3.2 Comparative metabolism of arsenic

Arsenic is well absorbed via inhalation, ingestion, and dermal contact (45). Distribution, metabolism, and elimination vary considerably between species of animal, form of As compound, and dosing regimen (34,45). In monkeys, dogs, and mice the clearance of As from blood is fairly rapid usually following a 2 phase pattern (31,42,46,47,51,52,53). Greater than 90% of blood As is distributed (diluted) and metabolized at a high rate, the half-life being 1-2 hours (first phase).

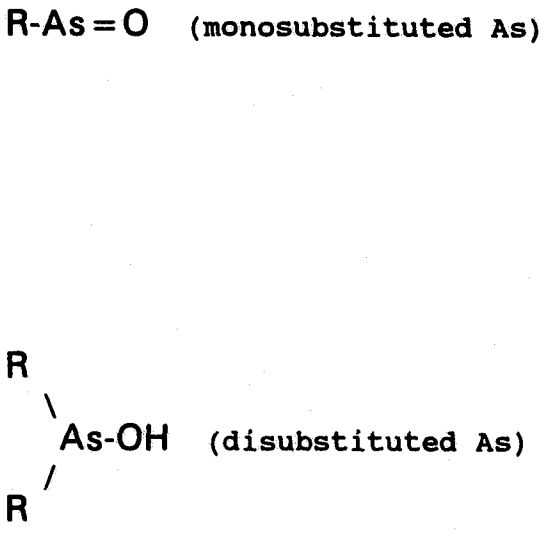

Figure 2. Chemical structure of mono and di-substituted arsenic.

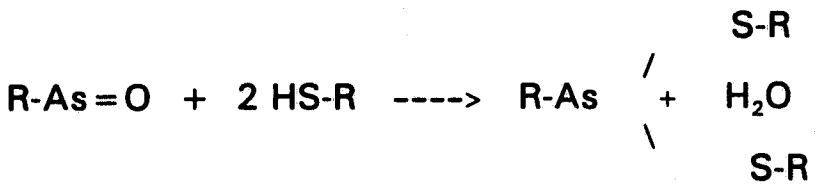

Figure 3. **Formation of dithiol bond upon interaction of arsenic with thiol groups.**

Then the As levels decline at a much slower rate with a second phase or post distribution phase of approximately 30-200 hours. Rats are not comparable to mice or dogs due to erythrocyte binding of As and resultant marked prolongation of blood arsenic levels (45). A third phase can be ascribed to As if the prolonged presence of As in keratinized tissues is considered (54). This tissue sequestration of As is reported in dogs as well (55). The valence state of As appears to be very important to the pharmacokinetics of As. The trivalent form appears to remain in blood, liver and kidney for a longer period then pentavalent forms of As (45,51). The principle metabolic path for pentavalent As is conversion to the trivalent form followed by methylation and renal elimination. Formation of water soluble dimethylarsinic acid and methylarsonic acid are most common with dimethylarsinic acid predominating in analysis of urine. Methylation of As appears to be dose related. With increasing or repetitive doses, a decrease in dimethylarsinic acid is noted. In mice, parenteral administration of trivalent As causes longer retention in blood than oral administration (42). In epithelial (HeLA S_3) and hepatoma (Hep G_2) cell cultures, biotransformation of arsenate was predominantly by methylation with the Hep G_2 cells excelling in reduction of arsenate. Most of the As was excreted into the media (56). In L-A mouse fibroblast cell culture suspension, arsenic metabolites (both the 3 and

5 + valence forms) were methylated to monomethylarsonic and dimethylarsinic acids which were then released into the media (57). This could be significant for thiacetarsamide if higher concentrations of non-methylated forms (active) of As persist after IV dosing as opposed to oral administration, helping to explain the loss of efficacy against CHD when thiacetarsamide is administered orally. There is marked variation in biliary excretion of As for various species. Biliary excretion of As is usually less than 5% of an administered dose in mice, rats and hamsters; however, with acute toxicity, the percentage is higher (45). In Marmoset monkeys, the hepatic retention of As is 20% after 4 days (52). In dogs, the elimination of As can be up to 85% in feces for arsenosobenzamides or 65 to 90% in urine (31,40,44). It becomes apparent that the pharmacokinetics for different As compounds, in different species, administered by different routes, cannot be predicted. Based on this information, it also seems likely that the total blood As values obtained by Holmes *et al* (47) and Sundloff *et al* (46), following administration of thiacetarsamide, are comprised of multiple forms of As, some of which are potentially toxic or efficacious, and some of which are not.

1.3.3 Mechanisms of arsenic toxicity

Arsenic has been used throughout the last several millennia as a herbicide, fungicide, metallurgical additive, medicament, and poison. It has toxic effects upon prokaryotes and eukaryotes alike (35). The mechanisms of arsenic toxicity at the cellular level are multiple. Each toxic mechanism generally applies to all forms of As; however, the form of As and the organism involved will influence the relative importance of each mechanism (34,35,49). Squibb states that:

> "Present knowledge concerning biochemical mechanisms of arsenic toxicity in mammals is far from complete, although aspects of this problem have been studied in considerable detail. Single cell studies have provided evidence that specific forms of arsenic can be cytotoxic; however, this potential is modified in more complex systems by a variety of factors, including the oxidation/reduction and/or methylation pathways for arsenic *in vivo*, differences in uptake and loss of arsenic by different cell types and organ systems and, perhaps, differences in the susceptibility of different cell types to toxic effects of arsenic due to cellular differences in such things as respiration rate and mitotic activity resulting from specialized functions of the cells."

The major emphasis for scientific study of the toxic mechanisms of As came about at the end of World War I, when the need for antidotes to arsenical gas warfare agents was perceived. Lewisite (diphenyl chlorarsine) was one of the more potent gas warfare agents stimulating this interest. Medicinal use of As also was increasing in the early 1900's. Use of organic arsenical compounds for treponemal infections

(e.g., syphilis) and trypanosomiasis (African sleeping sickness) was popular. Filarial disease of humans became a concern at the onset of World War II as soldiers from the western hemisphere were sent to the jungles of Indonesia and Indochina. The only agents found effective were a few organic trivalent arsenicals. It is thought that organic arsenicals penetrate cells better, and in general, additional side groups reduce toxic and therapeutic potentials (58). There is little recognized medicinal use of arsenicals today except for thiacetarsamide as an antifilarial agent for CHD.

The inorganic trivalent form of As is the most toxic (if it is in a soluble compound to enhance absorption) because of its highly reactive nature in biologic systems. The pentavalent form has some inherent toxic action, but its conversion to the trivalent form *in vivo* probably accounts for much of its toxicity (50,51,54,59). Arsine occurs primarily as a gas and is considered the most toxic form of As, causing direct haemolysis and mucosal or endothelial cell injury. In general, the inorganic forms are more toxic than organic forms and the trivalent compounds are more toxic than the pentavalent ones (45,60). Hence, the pattern of toxic potential for As compounds is: arsine, inorganic trivalent, organic trivalent, inorganic pentavalent, and organic pentavalent. As a point for reference, it should be recalled that thiacetarsamide is a disubstituted organic

trivalent arsenical. Exposure of animals and people to arsenic remains common through herbicides, wood preservatives (pressure treated lumber), metal industries, and the environment (34,49,60,61,62,63,64,65).

The most common clinical signs ascribed to acute As poisoning of mammals include abdominal pain, weakness, trembling, depression, anorexia, icterus, vomiting, diarrhoea, and death. Chronic As poisoning is more often associated with poor health, weight loss, pigmentation of skin, cirrhosis, neurologic deficits and psychological problems (34,60,66). Capillaries of all organs are affected due to endothelial injuries. Subsequent gross findings at post mortem examination frequently include edema and haemorrhage from many mucosal and serosal surfaces (35,49,58,60).

In 1920, Voegtlin and Smith established the ability of trivalent As to bind to sulfhydryl groups (-SH) within cells. This is now considered to be the most important mechanism of As toxicity (34,35,49,58). Voegtlin reported in Physiology Review of 1925, as cited by Squibb 1983, that a protective effect could be achieved both *in vitro* and *in vivo* with use of glutathione. He theorized that a reversible reaction with -SH groups occurred. Arsenic binds to -SH in a monosubstituted or disubstituted reaction (Figure 3) with the disubstituted being the more stable form, especially in 5 or 6 member ring formations (49,67).

This ability of trivalent As to bind -SH (thiol) groups accounts for the inhibition of many enzyme systems of cells, including alanine-amino transferase, pyruvate decarboxylase via binding to lipoic acid, multiple tricarboxcyclic acid cycle (TCA) enzymes, cytochrome and mixed function oxidases (35,58,68). The resultant cellular dysfunctions include uncoupling of the electron transport chain and the TCA (aerobic respiration) as well as loss of specific enzymatic functions necessary for cell viability (49,58,69,70). Binding of As to substrates of enzymes may slow the reactions. As well, free electrons, released from electron transport, are then potentially available to form lipid peroxides and other free radicals within the cytoplasm (71,72,73). The ability to bind to -SH groups may also be partially responsible for ultrastructural changes seen in various organelles of mice and rats exposed to As (69,74,75,76). Oxidation of sulfhydryl groups on membrane surfaces can affect the actions of adenylate cyclases, thereby impeding the actions of regulatory agents such as isoproterenol and glucagon (77).

The pentavalent form of As is closely related in the periodic table with phosphorus. Pentavalent As can substitute for, and interfere with, phosphate in enzymatic reactions (35). Of particular interest is the effect of arsenic in carbohydrate metabolism where it supplants phosphorus in some reactions. Arsenic is reported to be

interactive with many substances involved with carbohydrate and pyrimidine metabolism with initial thoughts that it disrupted the kinetics of these reactions by a process termed arsenolysis (78). More recently, arsenate (a form of pentavalent As) has been utilized by mitochondria from beef heart to synthesize ADP-arsenate. The ADP-arsenate replaces the formation of ATP by adding the arsenate to ADP in lieu of a third phosphate. This uncouples the process of oxidative phosphorylation. The arsenate ester, ADP-arsenate, is inherently unstable compared to phosphate esters. It does not therefore store high energy like ATP. The ADP-arsenate ester bonds are rapidly hydrolysed. The presence of As also seems to stimulate ATPase and decreases the P-ATP exchange reaction (79). In guinea pigs, a single dose of trivalent As (with conversion of some to pentavalent As) resulted in a decrease in fructose-6-diphosphate and glyceraldehyde-3-phosphate, both involved in glucose and glycogen metabolism. An increase in pyruvate concentration was used as evidence of a blockade of the TCA cycle enzymatic systems. In multiple dose groups, decreases in glycogen, glucose-6-phosphate, fructose-6-phosphate, glycerolaldehyde-3-phosphate, dihydroxyacetonephosphate, and pyruvate were recorded. There also was a marked depletion of glycogen and a loss of glucose tolerance (prolonged hyperglycaemia in response to a glucose challenge) (80). Similar results are reported for rats using parenteral

pentavalent As and mice with trivalent As (76,81). The chemical similarity of As to P is evident when it is seen that glucose-6-phosphate dehydrogenase shows similar K_m and V_{max} values for either glucose-6-phosphate or glucose-6-arsenate (35). In fungi, the use of additional P will reverse the effect on metabolic inhibition by As (82). The inhibition of cellular respiration has also been correlated in mice with ultrastructural damage to hepatocellular mitochondria (69,74).

Alteration of a cell's cytoskeleton has been demonstrated for several toxic heavy metals including As. Using 3T3 cell culture, it is reported that time and dose dependent alterations of the cytoskeleton occur (83). The microtubules and microfilaments become disorganized and may no longer allow proper organelle organization within the cells. This disarray would probably affect secretion, mitosis, cell wall integrity, endoplasmic reticular enzymes, and other cellular functions (58,83,84,85,86,87,88).

Evidence for further interference with organelles is found in the potential for mutagenicity and carcinogenicity. First, the creation of peroxy free radicals damages the DNA (89). Then inhibition of DNA repair processes and resultant cross-linking of chromatids may result in cell death, failure of mitosis, or cell transformation (35,50,90,91,92,93,94).

Adaptive responses of animals to chronic As exposure is probably mediated by several mechanisms as discussed by Squibb *et al* (35); however, none are clearly proven. The major hypotheses include decreased gastrointestinal absorption, enhanced methylation, increased production of thiol proteins e.g., glutathione, and increased conversion to pentavalent As (35,95,96,97,98).

In summary, Arsenic is hepatotoxic. The liver is one of the organs to which As is distributed following exposure. There is variable biliary excretion (as previously described) dependent upon the form of As, dose, and species. As well, there is considerable variation between individuals as to whether the exposure results in cell injury (leakage of ALT), cell death, or both. Cirrhosis and hepatic necrosis with biliary proliferation are common following acute toxic exposures and occur with As related hepatotoxicity (2,12,61,99,100,101). Ultrastructural (mitochondrial and ribosomal) and functional injuries are reported *in vivo* for As exposed hepatocytes (69,74,76,102,103). In cell cultures, cytoplasmic blebbing through cell membranes and cytoskeletal changes are seen to be As dose-dependent (83,91). And in other laboratory species, aberrations in cellular metabolism such as carbohydrate, high energy phosphate, and aerobic respiration are reported (79,80,93). With these last comments in mind, it is then difficult to point at a "most" likely hepatotoxic

mechanism for thiacetarsamide in dogs, when all may be present and possible.

1.3.4.1 Cytoprotection against arsenic toxicity

Because binding of As to thiol groups is the most pronounced of the toxic mechanisms, the dissociability of this bond is of great interest (49,67). Disubstituted As with two -SH groups is more stable than a monosubstituted As, however, this does not preclude reactions or dissociation of the disubstituted As. As pH rises above 7, the dissociability of dithiol As bonds increases (35,104). Kinetic factors, such as substrate concentrations, play roles in the degree of protection against the detrimental bond between As and -SH groups of various enzymes in cells. Evidence provided initially by Voegtlin *et al*, in 1924 and 1925, demonstrates this principle using the monothiol substance glutathione (GSH) to prevent (protect) against the effects of the trivalent arsenical, mapharside. The efficacy of dissociating (reversing) this relatively strong dithiol-As binding of mapharside to -SH containing molecules is much enhanced by use of a dithiol compound (49,67). The dithiol compound most commonly reported is British anti-lewisite (BAL) which is 2,3-dimercaptopropanol, which acts as an antidote for As poisoning (60,67,105). The administration of BAL in clinical veterinary medicine is

impractical because of its cost, necessity of repeated intramuscular injections, and predictable adverse reactions. BAL does not restore GSH levels nor provide a precursor for GSH regeneration. It actually inhibits a number of metal containing metallothionein enzymes (106,107). Use of thiol compounds, either monothiol or dithiol, for therapeutic purposes probably has benefits beyond reversal of the As-SH binding (84). These may include enhanced elimination of As from the cells, scavenging of As prior to its binding to -SH groups, and induction of other cytoprotective substances such as metallothioneins (50,68,108,109).

1.3.4.2 Glutathione

Glutathione is a tripeptide (gamma-glutamylcysteinylglycine). It is a compound containing glutamic acid, cysteine, and glycine. N-acetyl-L-cysteine (NAC) is rapidly deacetylated by cells to L-cysteine and then incorporated into GSH. L-cysteine is a -SH containing amino acid which is a GSH precursor substance. The actions of glutathione transferase enzymes are important in many detoxification and metabolic processes of the liver, providing for sulfonation reactions in phase II (synthesis) metabolism:

$$R + GSH \xleftrightarrow[\text{transferase}]{\text{glutathione}} R\text{-}S\text{-}G$$

Xenobiotic substances (R) conjugated to GSH are then further metabolized to mercapturic compounds via acetylation with acetyl-CoA and can then be excreted in the urine. Glutathione also participates in the decomposition of peroxides by the reduction of oxidized glutathione (GSH+GSH or GSSG) (110,111). Glutathione is the most abundant nonprotein sulfhydril compound in animal tissues (112).

Inhibition of GSH, using buthionine sulfoximinein, in hamster livers resulted in marked hepatotoxicity following the administration of arsenite (113). This suggests that GSH provides a protection against *in vivo* arsenic induced hepatotoxicity. Increased GSH levels and enhancement of cysteine transport into cells have been reported in mouse peritoneal macrophages following administration *in vitro* of sodium arsenite (114). Transport of other amino acids is decreased. Glucose also increases transport of cysteine into cells. The cysteine transport proteins may be stress-induced proteins (114). Inhibition of GSH results in delayed elimination of As and increased toxicity (at lower concentrations of As) in hamster kidneys (115). Glutathione can partially reduce the toxicity of carbon tetrachloride induced liver necrosis in rats. This may be the result of enhanced cellular response to the presence of a xenobiotic instead of simply reducing lipid peroxidations and covalent binding to reactive metabolites (116). Intracellular depletion of GSH from periportal hepatocytes, but not

pericentral hepatocytes, is seen following exposure of isolated rat hepatocytes to allyl alcohol (117). This supports the idea that hepatocytes from different regions of the lobule (acinus) may have different sensitivities to hepatotoxins based upon site of initial presentation of toxicants, concentration of toxicants in bile, and metabolism of the offending agent with GSH (toxic metabolites) (117,118).

1.3.4.3 N-acetyl-L-cysteine

N-acetyl-L-cysteine, a monothiol substance, has a molecular weight of 163 and exists as a white powder with an acidic pH of 2 when dissolved in water (Figure 4). It has an approximate LD_{50} in dogs of 700 mg/kg given IV as reported by Sheffner, 1970, and cited by Hatch, 1982. The oral LD_{50} in rats is 5050 mg/kg (36,107,119). The commercial product, Airbron^{tm}, comes as a 20% aqueous solution with .05% w/v of disodium ethylenediamine (EDTA). The EDTA serves as an antioxidant (preservative) (107). The most common use for Airbron in human and veterinary medicine is as an inhaled mucolytic. However, it is also used for treatment of acetaminophen toxicosis in humans, dogs, cats, and mice. For this purpose, it can be administered

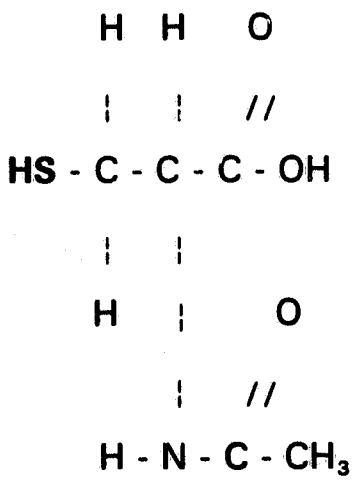

Figure 4. **Chemical structure of N-acetyl-L-cysteine.**

intravenously or orally at an initial dosage in dogs and cats of 140 mg/kg of body weight (120,121). One disadvantage of glutathione use for treatment *in vivo* is its limited solubility, poor transport into the cells, and failure to penetrate to the mitochondria which are rapidly depleted of endogenous GSH (84,98,122). A monothiol substance that does have good absorption and transport into cells *in vivo* is N-acetyl-L-cysteine (NAC) (95,120,121,123). NAC may impede As reactions with cellular -SH groups by binding and formation of stable ring structures (Figure 5 and 6). As a precursor of GSH, NAC provides a substrate for regeneration of cellular GSH (57,111,120,121,122,124). Other monothiol compounds are able to serve as GSH precursors and protect against various cytotoxic agents, but none have proven superior to NAC, especially in hepatocytes *in vivo* and *in vitro* (125,126,127,128).

1.4 Cell culture cytotoxicity testing

The ability to detect toxicity of chemicals in simple cell culture models is of considerable interest from several perspectives. First, the ability to screen large numbers of chemicals inexpensively is desirable because of the current concern for exposure to drugs, additives, and chemicals within our environment.

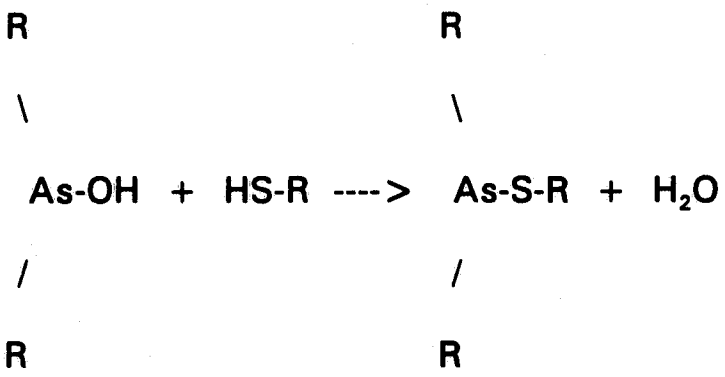

Figure 5. **Interaction of disubstituted As and dithiol forms a monothiol arsenical bond.**

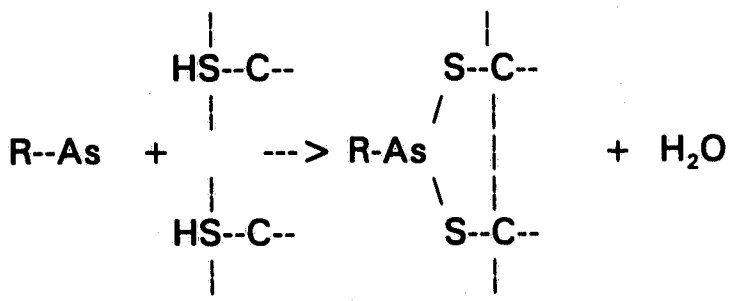

Figure 6. Five member Arsenic-S-C ring structures are very stable.

Second, the ability to detect specific organ sensitivities *in vitro* to various substances may concentrate research resources on a particular tissue during *in vivo* testing for toxic effects. And third, the reduction in numbers of laboratory animals used, in determination of toxicity and standard lethality dose responses, is a prime interest of scientists, animal welfarists, and budget minded administrators. However, *in vitro* testing cannot fully account for the complexity of interactions which occurs in the living animal (129,130,131,132,133). Not all of the *in vivo* toxicities are detected at similar concentrations in cell culture. However, correlation of *in vitro* and *in vivo* toxicologic data has been shown to occur (134,135). Those toxicities that are detected *in vitro* have importance in selection of trial dosages or tolerable exposure limits *in vivo* (134,136,137). The correlation of *in vitro* and *in vivo* toxicologic injury is based upon the assumption that mechanisms of cell death are of two basic types in both systems: interference with basic cell function and structure or interference with organ specific cell functions (organ specific cytotoxicity) (136). As well, cell death may be mediated by direct irreversible cytotoxicity (necrosis) or terminal metabolic dysfunction (apoptosis).

Another key discussion concerns the advantages of using cells obtained from fresh isolated organs over those from transformed cell lines. The advantage of freshly isolated

cells lies in the completeness of metabolic function which is carried to the model from the live animal. However, a supply of freshly killed animals must still be maintained since these cell cultures generally remain viable for only a few hours to several days. The advantages of using transformed cell lines include ease of maintenance of the model for replication and comparison of experiments, growth characteristics which reduce the difficulties of experimental cell isolation protocols, and purity of desired cells for detection of tissue specific cytotoxicities. The disadvantage is primarily the potential difference in metabolic capabilities of transformed cells compared to cells which have been freshly isolated from normal organs. This is somewhat dependent upon the degree of differentiation (anaplasia) of the malignant cell line used (131,138).

1.5 Hepatoma cell cultures in toxicity testing

The use of transformed hepatoma (malignant hepatocytic origin) cells in cell culture and cytotoxicity testing is common. Many hepatoma cell lines maintain their normal functions and ultrastructural characteristics as cells of hepatic origin (139,140,141,142).

Methods for assessing cytotoxic effect of substances in cell cultures include cell counting (mitotic activity and

viability), cell weight, DNA content, media protein concentration, uptake (synthetic activity) of radiolabeled compounds, plating efficiency, cell cycle time, enzyme levels, and cytometry (131,138,143). Cytoplasmic blebbing, without the uptake of vital dyes, is one of the earliest signs of cellular toxicity (130,144). The use of cell culture to study toxicity of arsenicals and the use of hepatoma cell lines (transformed hepatocyte cell lines) is reported widely in the literature (57,90,91,92,93,143,145,146,147,148). As well, investigations of glutathione levels in a human melanoma cell line and as cytoprotectants in alveolar macrophage cell cultures have been reported (149,150).

1.6 General Hypotheses

Based upon the use of arsenical compounds in cell cultures, particularly hepatomas, and the investigations of glutathione in cell culture and cytoprotection, it appears appropriate to evaluate the cytotoxicity of thiacetarsamide and the cytoprotective value of NAC in hepatoma cells *in vitro*. The lack of a canine hepatoma cell line currently precludes investigation in a transformed canine hepatocytic cell line. However, as previously discussed, there are similarities between canine and murine metabolism of

arsenic. Therefore, the evaluation of thiacetarsamide in mouse hepatoma cells can be considered reasonable. The demonstration of hepatocellular cytoprotection against the effects of thiacetarsamide by use of NAC in mouse hepatoma cells would allow for a more accurate determination of initial concentrations for both compounds to be evaluated *in vivo* in canines.

The hypothesis of this research is that the monothiol substance, NAC, is a cytoprotectant against the hepatotoxic effects of the thiol binding arsenical compound, thiacetarsamide; and that this can be demonstrated in an *in vitro* model with the results applied to the canine *in vivo*.

2.MURINE HEPATOMA (NCTC 1469, CELL CULTURE AS AN *IN VITRO* MODEL FOR THE EFFECTS OF THIACETARSAMIDE AND N-ACETYL-L-CYSTEINE

2.1 Introduction

The objective of this study was to develop an *in vitro* cell culture model which could be used to evaluate the potential cytoprotective effects of NAC against the pathobiologic effects of T. In order to accomplish this, several features of the model had to be investigated.

First, an appropriate cell line needed to be chosen. The murine hepatoma cell line NCTC (National Cancer Tissue Culture) 1469 was selected because of its ease of maintenance and the ability to readily obtain cells from tissue culture vessels for evaluation. Upon culture of this transformed cell line, viable cells are shed into the culture medium and maintain their viability. Those cells that remain adherent to flasks are easily dislodged without the requirement for trypsin pretreatment. Descriptions of this cell line indicate that it maintains many normal metabolic capabilities including those of the cytochrome mono-oxidase (mixed function oxidase) system and mitochondrial respiration. These are of relevance to the proposed toxic principles of arsenic on cells as stated previously in chapter one (151). It was necessary to

determine a concentration of NAC and T which could reflect clinically relevant levels of exposure for future *in vivo* experiments in dogs. The identification of a lethal concentration 50% (LC_{50}) on a dose-effect curve, with comparison of slopes and confidence limits was the logical starting point for in vitro experiments and examination of NAC as a cytoprotectant (129,152,153). The growth characteristics of NCTC 1469 cell culture were identified to develop a reproducible model. The preference for the model was to identify a point of log phase growth, when cell cultures had a high viability and active metabolism, and where cell activity was not inhibited by cell crowding (contact inhibition) (130,131,143,145,154).

Second, the effects (lethal concentration curve) for T and any potential effects of NAC on tissue culture cells had to be determined prior to exploring possible cytoprotective effects of NAC against the toxicity of T. The parameters for cytotoxic concentrations, times of exposure, and presence of on-going cellular injuries (identified by post exposure incubation periods) were priorities for later experiments.

Because of the ease in obtaining the commercial medical grade products of NAC (Airbron™) and T (Caparsolate™), and their relevance to proposed *in vivo* use, it was preferable to use them in these experiments. However, these products both contain additives. Caparsolate™ contains .9% benzyl

alcohol (B-OH) as a preservative. Airbron™ contains .05% w:v (.5 mg/ml) of disodium ethylenediamine tetraacetate (EDTA) as a preservative. Therefore the effects of these substances needed to be explored to identify their potential influences upon proposed studies of the effect of NAC on the cytotoxicity of T.

2.2 Materials and Methods

2.2.1 NCTC 1469 Murine Hepatoma Cell Culture System

The methods used for cell culture technique were as specified by the supplier (American Type Culture Collection (ATCC)) and as found in, Culture of Animal Cells, by Freshney (131). Cell culture medium was prepared in advance. It consisted of NCTC 135 medium supplemented with L-glutamine (SIGMA Chemical Co., Toronto, Ontario) and the addition of 5 ml of L-glutamine and 5 ml Penicillin (10000U/ml) / Streptomycin (10 mg/ml) (SIGMA) per 500 ml. Horse serum was added to give a concentration of 10%. The pH of the medium was adjusted by titration with the addition of 0.1N HCl (SIGMA) for a final pH of 7.35 to 7.40 (Beckman, Scientific Instruments Div., Fullerton, California, USA). This medium was filter sterilized (.22 micron Disposable Sterile Filter System/Stand 500 ml, Corning Inc., Corning,

New York, USA) then stored at 8°C. Prior to any use of medium with the cell cultures, it was warmed to 37°C (Precision Water Bath 183, Scientific Inc., Chicago, Illinois, USA).

The transformed murine hepatoma cell line NCTC 1469 was obtained (ATCC) frozen in an ampule containing 2.8 x 10^6 cells per ml. These were maintained in liquid nitrogen until used. The cells were thawed by rapid agitation of the ampule in a 37°C water bath. The ampule was then immersed in 70% ethanol at room temperature and placed in a laminar flow hood under aseptic conditions (131). The contents were transferred by sterile pipette to a sterile 15 ml test tube. The cell suspension was diluted with 10 ml of culture media and placed in a 25 ml culture flask (T25) (Disposable Sterile Tissue Culture Flask - canted neck, Corning Glass Works, Corning, New York, USA). This was then placed in a humidified 37°C incubator with 5% CO_2 (#6300 NapCo CO_2 Incubator, Tualatin, Oregon, USA). After 24 hours, the surface of the flask was scraped with a sterile rubber spatula and the contents were transferred to a 50 ml centrifuge tube and centrifuged at 100g for 10 minutes at 10°C. The supernatant was pipetted and replaced by 10 mls of fresh media which had been prewarmed to 37°C. This suspension was placed back into a new T25 and allowed to grow for 72 hours. Cell cultures were examined daily using an inverted microscope.

Because this cell line is not strongly adherent, complete confluence of cells on the surface of the flask is not achieved. The method described above for harvesting of cells from canted neck tissue culture flasks was standard throughout all experiments.

Viability of cells was assessed by dye exclusion. One milliliter of suspended cells was placed in a 10 ml plastic test tube (Falcon 2057, Becton Dickinson, Lincoln Park, New Jersey, USA) and then 0.1 ml of the vital stain, 0.2% Trypan Blue (#34078, Gurr, BDH Chemicals Ltd., Poole, England) was added. This mixture was agitated (Vortex Genie 2 #12-812, Allied Fischer Scientific, Bohemia, New York, USA). A drop of the stained cell suspension was placed on a microscope slide (M6147, Baxter Corp, CanLab/division, Toronto, Ontario) and coverslipped (M6041-2, CanLab, Toronto, Ontario). Viability of cells was quantitated by determining the ratio of stained cells to unstained cells. Trypan blue dye is normally denied penetration into viable cells so that only metabolically injured or dead cells will stain blue (118,131). This technique for assessment of viability was used in all experiments.

When cell cultures in the T25 flasks approached confluency, they were scraped gently with a sterile rubber policeman (spatula), pipetted, centrifuged, resuspended with 45 mls of medium, vortexed, and counted with a haemocytometer (Bright-Line, Superior Reichert, Buffalo, New

York, USA). The cell suspension was then divided and placed into three T75 flasks (Disposable Sterile Tissue Culture Flask - canted neck, #25110-75, Corning Glass Works, Corning, New York, USA). These flasks were monitored daily. Every 3 days, old media was pipetted and replaced by fresh media (each time-feeding equalled one feeding cycle). Cells suspended in the old media were either discarded, used to seed new flasks, or centrifuged and placed in 1 ml of cell freezing medium (DMSO and fetal calf sera) in 2 ml ampules (Cryogen Vials, Corning Glass Works, Corning, New York, USA) which were then frozen in liquid nitrogen for future use in initiating new cultures.

At the end of 3 feeding cycles, cells were removed from the T75s and counted as described above. Dilution was made with standard medium to provide 35 to 50 x 10^4 cells per ml. One ml of this cell suspension was then placed in each well of 24 well flat bottom tissue culture plates (#3047, Falcon/Becton Dickinson Labware, Lincoln Park, New Jersey, USA). A sufficient number of 24 well plates were inoculated to provide for each experiment, such that each individual experiment utilized plates inoculated and maintained in a similar manner and date.

Every 3 to 4 days each well or flask was examined for cell adherence. If adherence was present then the media was aspirated and discarded. An appropriate volume of fresh media was then added under sterile procedures (131). After

3 feeding cycles (9 to 12 days) adherent cells approached confluency and the media appeared turbid due to suspended cells in the media. This stage was considered to represent log phase of cell growth and was used for experiments (131).

2.2.2 Thiacetarsamide for *in vitro* use

Because the *in vitro* use of NCTC 1469 cells was designed to be a correlative model for *in vivo* studies, a level of T which would be appropriate in the live dog, as discussed in chapter one, was desired. Thiacetarsamide was provided as a solution of 10 mg/ml in water and .9% benzyl alcohol (Caparsolate™, Sanofi Animal Canada Inc., Victoriaville, Quebec). The pH was 7.37 and 7.41 for two separate lot numbers; this was consistent with manufacturer claims. A purified product (Certificate of Analysis 98.7% pure by HPLC), arsenamide was obtained from the originating manufacturer (Abbott Labs, North Chicago, Illinois, USA). Benzyl alcohol (SIGMA) was also obtained for independent cytotoxicity testing because of its use as a preservative in the commercial preparation.

Because arsenamide is water soluble and is rapidly distributed in body fluids, it was considered appropriate to assume that arsenamide is distributed into the extracellular fluid (ECF) compartment as would be expected to be

encountered by the in situ hepatocyte. The ECF is normally assumed to approximate 20% of mammalian body weight (155). The desired protocol for superior efficacy in the treatment of canine adult heartworm disease was the use of thiacetarsamide at a dosage of 3.3 mg/kg of body weight. At this dosage, the estimated concentration in the dog's ECF is 16.5 μg/ml. The calculation for this was: per 1000 g (body weight) x 20% (estimated ECF) = 200 ml; 3.3 mg T per 200 ml = 0.0165 mg/ml or 16.5 μg/ml. This concentration of 16.5 μg/ml was the basis for a theoretical 1X concentration of T. This theoretical dosage was intended to assist in correlating the *in vitro* and *in vivo* experiments. Dilutions of T were made to give .01, .03, .1, .3, 1, 3, 10, and 30X concentrations when added to cell culture plate wells. These concentrations were converted to more appropriate scientific units (Table I) for interpretation of individual experiments *in vitro*.

Formulation of the non-commercial arsenamide solution was accomplished by addition of 200 mg arsenamide in 20 ml of sterile water to create a 10 mg/ml solution. This was found to have a pH of 3.3 and the product was insoluble. A titrated addition of .1N NaOH (SIGMA) to the solution with stirring and heating (Nuova II Stir Plate, Thermolyne Corp., Dubuque, Iowa, USA) to 37°C was used to obtain a solution at a pH of 6.8.

TABLE I

RELATIONSHIP BETWEEN THEORETICAL DOSAGES OF THIACETARSAMIDE AND ITS MOLAR CONCENTRATIONS CALCULATED USING A CONVERSION FACTOR (CF)

MW = 377.26

CF = 1000/377.26 = 2.65 $\mu g/ml \times CF = \mu mol/l$

[1X] = 16.5 $\mu g/ml$ = 43.7 $\mu mol/L$

[X]	$\mu mol/L$	$\log_e \mu mol/L$
.01	.437	-0.83
.03	1.31	0.27
0.1	4.37	1.47
0.3	13.11	2.57
1.0	43.7	3.78
3.0	131.1	4.88
10	437.0	6.08
30	1311.0	7.18
100	4370	8.38

This pH was consistent with the measured pH of samples of commercial CaparsolateTM which was 6.7 to 7.2. Dilutions were made as for CaparsolateTM version of T, as noted (Table I).

Benzyl alcohol was serially diluted to be comparable to the dilutions used for T (Table II). The approximate concentration in the theoretic 1X exposure, both *in vitro* and *in vivo* is .0001% in ECF. Serial dilutions from .00001% to 1.0% of B-OH in culture medium were used.

2.2.3 N-Acetyl-L-Cysteine for *in vitro* use

Use of the commercial preparation of NAC (Airbron*TM* 20%, Allen & Hamburys, Glaxo Canada Ltd., Toronto, Ontario) was preferred for *in vivo* experiments. A 1X concentration was based upon .7 ml of a 20% solution per kg of body weight (121) and then similar calculations were utilized to calculate the concentration used *in vitro*. NAC is also water soluble and rapidly distributed to the ECF. The calculation for NAC was: 1000 grams (body weight) x 20% (estimated ECF) = 200 ml of ECF; .7 ml of a 20% solution = 1 mg NAC per 200 ml of ECF which equals .7 mg per ml of media to approximate the 1X anticipated ECF levels *in vivo* (Table III).

TABLE II

RELATIONSHIP BETWEEN THEORETICAL EXTRACELLULAR FLUID CONCENTRATION OF BENZYL ALCOHOL (B-OH) CALCULATED USING A CONVERSION FACTOR (CF) AND ITS MOLAR CONCENTRATION

MW = 108.1

CF = 1000/108.1 = 9.25 μg/ml x CF = μmol/l

[1X] = .0001% of media/ECF = 1 μg/ml = .00925 μmol/L

[X of T]	μg/ml	μmol/L	% in media
0.1	0.1	0.000925	0.00001
1.0	1.0	0.00925	0.0001
10	10	0.0925	0.001
100	100	0.925	0.01
1000	1000	9.25	0.1
10,000	10000	92.5	1.0

TABLE III

RELATIONSHIP BETWEEN THEORETICAL DOSAGES OF N-ACETYL-L-CYSTEINE (NAC) AND ITS MOLAR CONCENTRATIONS CALCULATED USING A CONVERSION FACTOR (CF)

MW = 163.2

CF = 1000/163.2 = 6.12 μg/ml x CF = μmol/l

[1X] = 70 μg/ml = 428.4 μmol/L

[X]	μmol/L	$\log_e$ μmol/L
.01	4.28	1.45
.03	12.84	2.55
0.1	42.84	3.76
0.3	128.4	4.86
1.0	428.4	6.06
3.0	1284	7.16
10	4284	8.36
30	12840	9.46
100	42840	10.67

The commercial preparation was stable and easy to work with. But because it contained disodium EDTA, a calcium chelator and hence potential modifier of cellular metabolism, a cell culture approved source of NAC was obtained (SIGMA) for comparison to the commercial preparation. Similar dilutions as noted in Table 3 were used in an attempt to formulate a suitable solution of generic NAC. However, the pH could not be stabilized for more than a few minutes and the solution was unusable. After this experience, the pH of three lot numbers of AirbronTM: 87L227 exp 2-91; 1B049 exp 4-94; 9M139 exp 1-93 (open for 28 days); and 9M139 exp 1-93 (open immediately prior to experiment) were compared. The pHs were 6.9, 7.0, 7.03, and 7.05 respectively which was consistent with manufacturer claims. The manufacturer did not reply when questioned about its method of stabilizing the product.

A commercial source of EDTA was obtained (S311, Fischer Scientific, Fair Lawn, New Jersey, USA). This was used to determine potential cytoprotective or cytotoxic effects in conjunction with NAC (with regards to its effect on the cytotoxicity of thiacetarsamide) (156). The concentration of EDTA was calculated to approximate that in the estimated ECF that would be provided by AirbronTM. The solution of EDTA was made by addition of 0.875 mg of EDTA in 8 ml of 0.9% saline, warmed to 37°C, then titrating the pH with 0.1N NaOH until a pH of 7.3 remained stable at room temperature.

Dilutions of this compound were used as reported (Table IV).

2.2.4 Development of cytotoxicity model for the effects of thiacetarsamide on NCTC 1469 mouse hepatoma cells

2.2.4.1 Comparison of viability of suspended versus adhered NCTC 1469 cells from individual test wells

Three wells from a multiwell plate, set up as previously described, were examined. An adjustable pipetter (PipetmanTM, P200, Gilson, France) was used to add the appropriate dilution of T to obtain 43.7 µmol/L of T in each 1 ml of medium in each well. Media from these wells were removed, and cellular viability was determined. One ml of fresh media was then placed in each sampled well. These wells were then gently scraped with a rubber policeman, and mixed gently by repeated pipetting. The viability of the cells was assessed, using Trypan blue as described above, by making wet mounts on microscope slides and counting 100 cells from six different fields for a total count of 600 cells from each well.

TABLE IV

RELATIONSHIPS BETWEEN THEORETICAL DOSAGE OF DISODIUM ETHYLENEDIAMINE (EDTA) AND ITS MOLAR CONCENTRATION CALCULATED USING A CONVERSION FACTOR (CF)

MW = 372.24

CF = 1000/372.24 = 2.69 μg/ml x CF = μmol/l

[1X] = 50 μg/ml = 134.5 μmol/L

[X]	μmol/L	$\log_e$ μmol/L
1.0	134.5	4.90
3.0	403.5	6.00
10	1345.0	7.20

The mean viable cell count of each 100 cells (total of 6 counts of 100 from each well) was then used to calculate a mean of the means. Comparison of viability counts between the suspended and adhered cells was then performed using paired t tests (157).

2.2.4.2 Preliminary investigation of cytotoxicity of thiacetarsamide on NCTC 1469 cell culture: 43.7 μmol/L T with variable exposure time

A 24 well cell culture plate prepared, as previously described in part **2.1**, was used. Thiacetarsamide was added to give a 43.7 μmol/L concentration in the medium. Cells were counted as stated above in part **2.3.1.** This procedure was performed at 0, 1, 2, 3, 4, and 5 hours, with four wells at each time period. Cells were collected by scraping the bottom of the wells, then pipetting the contents of each well to a separate test tube. This resulted in a mixture of the original suspended and adhered cells. This protocol was followed for the remainder of experiments. The mean viable cell count for each well was divided by the mean viable count of the control wells to provide an adjusted cell viability. Subtracting this value from 100 was then used to arrive at the "adjusted % dead" values. This procedure was then followed for the remainder of all experiments. The

benefit of using the adjusted % dead system of values was in providing a method of comparison between experiments when multiwell plates may have had different control viabilities.

2.2.4.3 Preliminary investigation of the effect of variable concentration of T with exposure time of one hour

Groups of six wells were exposed to each of 4 different concentrations of T for 1 hour. Concentrations used were 0, 43.7, 87.4, 218.5, and 437.0 μmol/L. Cells were collected and counted as described above in parts **2.3.1 and 2.3.2.**

2.2.4.4 Cytotoxicity of thiacetarsamide: comparison of direct exposure time versus exposure time plus 24 hours of post exposure incubation

Two multiwell plates (48 wells) were prepared and T was added to give a concentration of 43.7 μmol/L to all wells. Control wells were examined at the start and end of each experiment. Thiacetarsamide was added, to all but control wells, at the same time and each multiwell plate was handled as a matched control to the other. Suspended cells were collected from wells at .08, .16, .32, .64, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24, and 48 hours after addition of T and

counted to obtain the adjusted % dead. The matched wells had the media with T removed and 1 ml of fresh media added. These were incubated an additional 24 hours and then counted. Routine analysis of variance between plates (with or without 24 hours of incubation) and within plates (between concentrations) was performed with paired Bonferroni t-tests done to confirm the location of significant differences (152,157).

2.2.4.5 Effect of benzyl alcohol on NCTC 1469 cell culture

Serial dilutions of B-OH were made and 20 µl of each dilution added to test wells of prepared multiwell plates in order to create dilutions in media ranging from .00001 to 1.0% The estimated ECF concentration of B-OH resulting from the 1X dosage of T in dogs is .0001%. The time of exposure was 24 hours. All cells were collected and viability assessed.

2.2.4.6 Cytotoxicity of thiacetarsamide on NCTC 1469 cell culture with variable concentration of T, exposure of 4 hours, and 24 hours post exposure incubation

A prepared multiwell test plate was labelled to provide both pre and post control wells. Concentrations of .437, 1.31, 4.37, 13.11, 43.7, 131.1, 437, 1311, and 4370 µmol/L

of T were added to individual test wells. After 4 hours of exposure, the medium containing T was removed, and 1 ml of fresh medium was added. Following 24 hours of incubation, the cells were collected, processed, and the adjusted % dead cells determined. Statistical analysis was by routine analysis of variance (ANOVA) between wells (concentrations). Paired t tests were done to confirm the location of significant difference (152,157). Probit, logit/probit, $\log_e$, inverse log, cos and quadratic transformations were attempted in order to identify, with confidence, the best fit LC50 (SAS 6.03, SAS Institute Cary, N.Carolina, USA) (BMDP PC90, BMDP Statistical Software Inc., Los Angeles, California, USA).

2.2.4.7 Comparison of thiacetarsamide (as arsenamide) and Caparsolate™ with variable concentration of each agent, exposures of 4 hours, and 24 hours post exposure incubations

Two prepared multiwell test plates were labelled to provide both pre and post control wells. Concentrations of 1.31, 4.37, 13.11, 43.7, 131.1, and 437 µmol/L of T or Caparsolate™ were added to paired test wells on different multiwell plates. After 4 hours of exposure, the medium containing T or Caparsolate™ was removed, and 1 ml of fresh media was added.

Following 24 hours of incubation, the cells were collected, processed, and the adjusted % dead cells determined. Statistical analysis was by routine analysis of variance between wells (concentrations). Paired t tests were done to evaluate for significant differences between cytotoxic effects of the two compounds (152,157). Following this experiment, T always referred to the trade name product Caparsolate™.

2.2.5 Evaluation of the effects of NAC on NCTC 1469 mouse hepatoma cell culture

2.2.5.1 Effects of Airbron™ on NCTC 1469 cell culture with 24 hour exposure and variable concentration

A prepared multiwell test plate was labelled to provide both pre and post control wells. Airbron™ was added to wells to produce concentrations of 4.28, 12.8, 42.8, 128.4, 428.4, 1284, 4284, 12840, and 42840 µmol/L of Airbron™. After 24 hours of exposure, the cells were collected, processed, and the adjusted % dead cells determined. Statistical analysis used was analysis of variance between wells (concentrations). Paired Bonferroni t-tests were done to confirm the location of significant differences (152,157).

2.2.5.2 Effect of disodium EDTA on NCTC 1469 cell culture when concurrently exposed to thiacetarsamide

One six-well plate was prepared appropriately. Thiacetarsamide was added to the media to create concentrations of 0, 131.1 and 437 μmol/L. In a crossing grid design, EDTA was added to the same wells to create concentrations of 0, 134.5, 403.5, and 1345 μmol/L. The well receiving neither compound was the control. The T was removed after 4 hours, as in previous experiments, and the fresh medium with new EDTA was added to the wells. Statistical analysis consisted of analysis of variance with paired t tests to identify significant differences between wells.

2.3 Results

2.3.1 Comparison of viability of suspended versus adhered NCTC 1469 cells from individual test wells

Data presented in Table V clearly demonstrate that cells suspended in medium had the same viability as those adhered to the bottom of the wells ($p = 0.27$) and thus represented a similar population. It was therefore assumed that the use of both suspended and adhered cells would not

Table V

Cytotoxicity of Thiacetarsamide (T) on NCTC 1469 Cell Culture
Comparison of Suspended and Adhered Cells
exposure 4 hours incubation 24 hours
[T 43.7 µmol/L]

Test Well	Suspended % dead	(stdev)	Adhered % dead	(stdev)
A	51.0	(3.7)	48	(4.5)
B	55.3	(5.0)	53.8	(4.5)
C	53.5	(3.6)	49.3	(5.7)

influence the results of subsequent experiments and would facilitate manipulation of the cultures. Control plates from this and all subsequent experiments provided a range of 85 to 93% viable cells with a mean of 90.1%.

2.3.2 Preliminary investigation of cytotoxicity of thiacetarsamide on NCTC 1469 cell culture: 43.7 µmol/L T with variable exposure time

The intent of these preliminary experiments, was to obtain an approximate time of exposure to T that would permit calculation of an LC_{50}. Control cells had variable morphology, high mitotic index, variable nuclear to cytoplasmic ratios, variable degrees of vacuolation, anisokaryosis and anisocytosis (Figure 7). The LC_{50} was found to be at 4 hours for an exposure of 43.7 µmol/L of T (Figure 8).

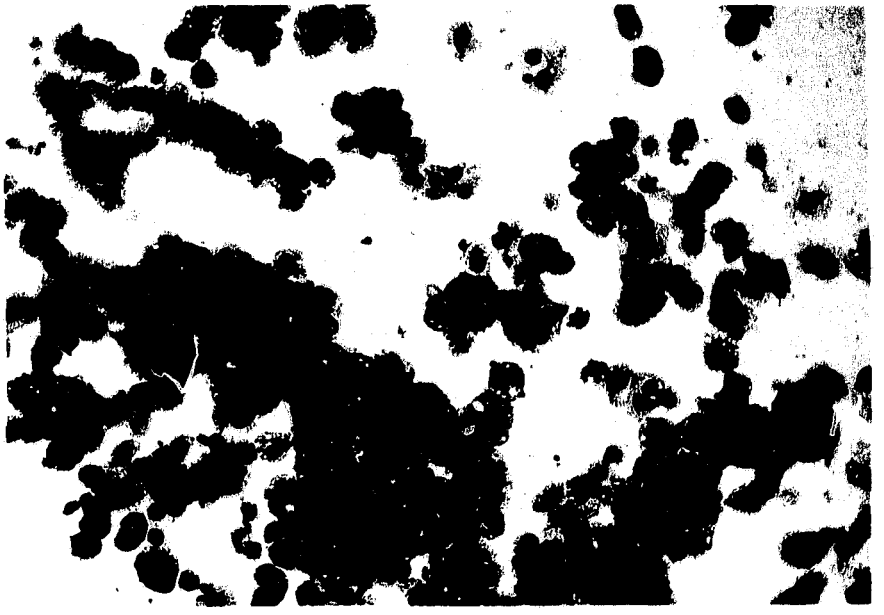

Figure 7. Photomicrograph of a cytospin preparation of NCTC 1469 mouse hepatoma cells after 3 feeding cycles (9 days after plating). (Wright's stain; magnification X150)

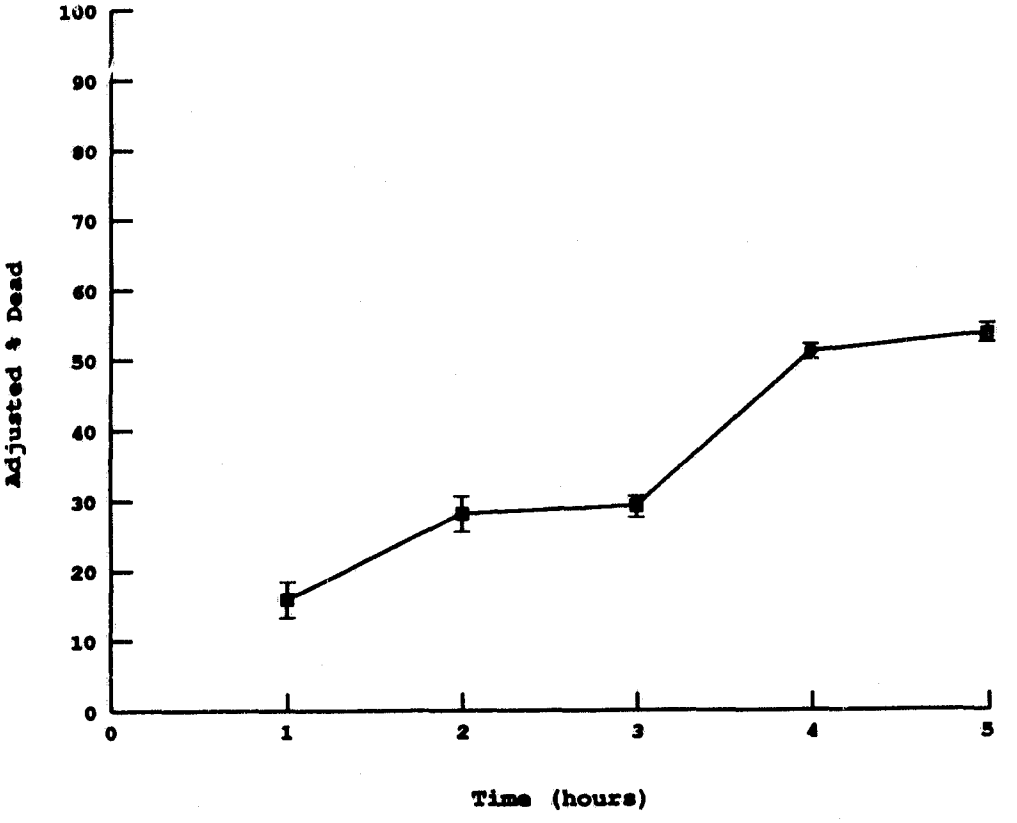

Figure 8. Cytotoxicity of thiacetarsamide (43.7 µmol/L) on NCTC 1469 cell culture using viable exposure times (hours).
Lethal Time 50 = 4 hr

Observations of the individual hepatocytes exposed to T, following cytospin preparation and light microscopy with Wright's stain, as well as observation with the TMS phase contrast microscope while the cells were suspended in media, indicated the presence of obvious morphologic deteriorations of the cells following exposure to T. Cells, in general were less rounded, more vacuolated, and had evidence of obvious blebbing of the cytoplasmic membrane even without uptake of Trypan blue dye (still viable). These morphologic changes were consistent throughout the rest of the experiments with exposure to T.

2.3.3 Preliminary investigation of the effect of variable concentration of T with exposure time of one hour

The concentration of T did not appear to contribute as significantly to cytotoxicity as did the time of exposure (Table VI and Figure 8). Even at a 10 fold increase above the expected *in vivo* ECF, the level of cytotoxicity did not reach an LC_{50}. The only significant difference ($p = .03$) occurred between the 43.7 μmol/L concentration and the other higher ones. Because some quantity of As would presumably remain intracellular, and because of the numerous mechanisms for cellular injury as a result of As (as stated in chapter 1), it was determined that a post exposure incubation

Table VI

Cytotoxic effect of Thiacetarsamide (T) on NCTC 1469 cell culture with an exposure time of one hour

T µmol/L	adjusted % dead	SEM
43.7	35.6	2.9
87.4	43.1 *	5.5
218.5	40.5 *	5.1
437.0	43.1 *	1.8

* Significantly greater than 43.7 µmol/L T

period/time should be evaluated.

2.3.4 Cytotoxicity of thiacetarsamide: comparison of direct exposure time and exposure time plus 24 hours of post exposure incubation

The results of this experiment provided a sigmoid curve for both the direct exposure and the post exposure incubation groups (Figure 9). However, it is obvious that marked cell death continued after the removal of the T from the incubating media. The shorter times (less than 3 hours) ($p > .1$) and the longer times (greater than 8 hours) ($p > .1$) were not significantly different from the next respective time point. But all the time periods between 3 and 8 hours were significantly different ($p = .04$ to 0.0001). The LC_{50} for direct exposure only was 9.0 hours, while that with a post exposure incubation of 24 hours was 3.6 hours. Based upon this experiment the exposure period of 4 hours and the post exposure incubation period of 24 hours (28 hours per experiment) were chosen as the parameters for the model.

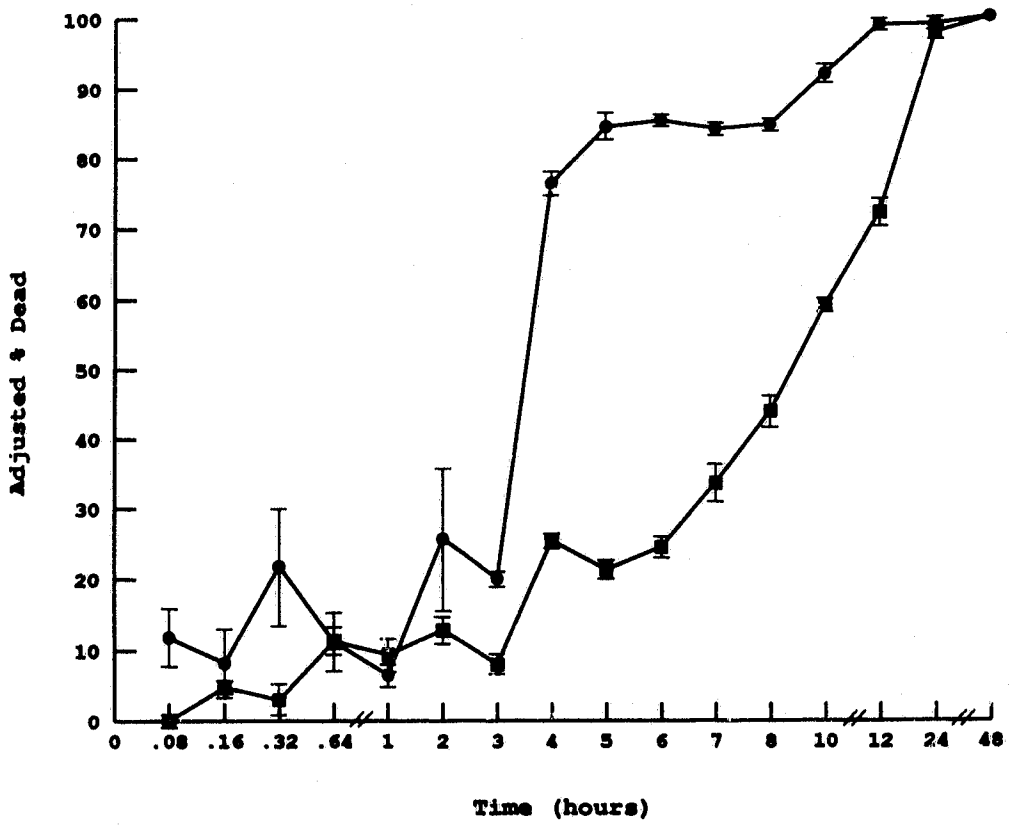

Figure 9. Comparison of the cytotoxicity of thiacetarsamide (43.7 µmuo/L) on NCTC 1469 cell culture exposed for a specified period of time or exposed to the drug for the same time and cultured for an additional 24 hours without the drug

■ = Time

● = Time followed by 24 hours of incubation

2.3.5 Effect of benzyl alcohol on NCTC 1469 cell culture

Because B-OH is a potentially toxic drug additive in Caparsolate™, it was evaluated for toxicity. This experiment was performed at this point in case it was significant, thereby negating further experiments using Caparsolate™ as the source of T. The ECF 1X concentration was previously estimated at .00925 μmol/L (Table II). Benzyl alcohol showed no significant ($p = .06$) cytotoxic effects until the 1 μmol/L level, and no marked toxicity until reaching the 100 μmol/L level (Figure 10).

2.3.6 Cytotoxicity of thiacetarsamide on NCTC 1469 cell culture with variable concentration of T, exposure of 4 hours, and 24 hours post exposure incubation

The concentration of T which would provide an estimate of the LC_{50} was obtained by varying the concentration and using an initial four hour incubation with T, then removal of T followed by an additional 24 hour incubation. A sigmoid curve was obtained with the LC_{50} = 51.94 μmol/L of thiacetarsamide (Figure 11). The original estimate for an ECF LC_{50} of thiacetarsamide was 43.7 μmol/L as described in **2.2.2.** Cell morphology deteriorated with increasing concentrations of T. At the 437 μmol/L (10X) concentration, only minimal differences could be discerned.

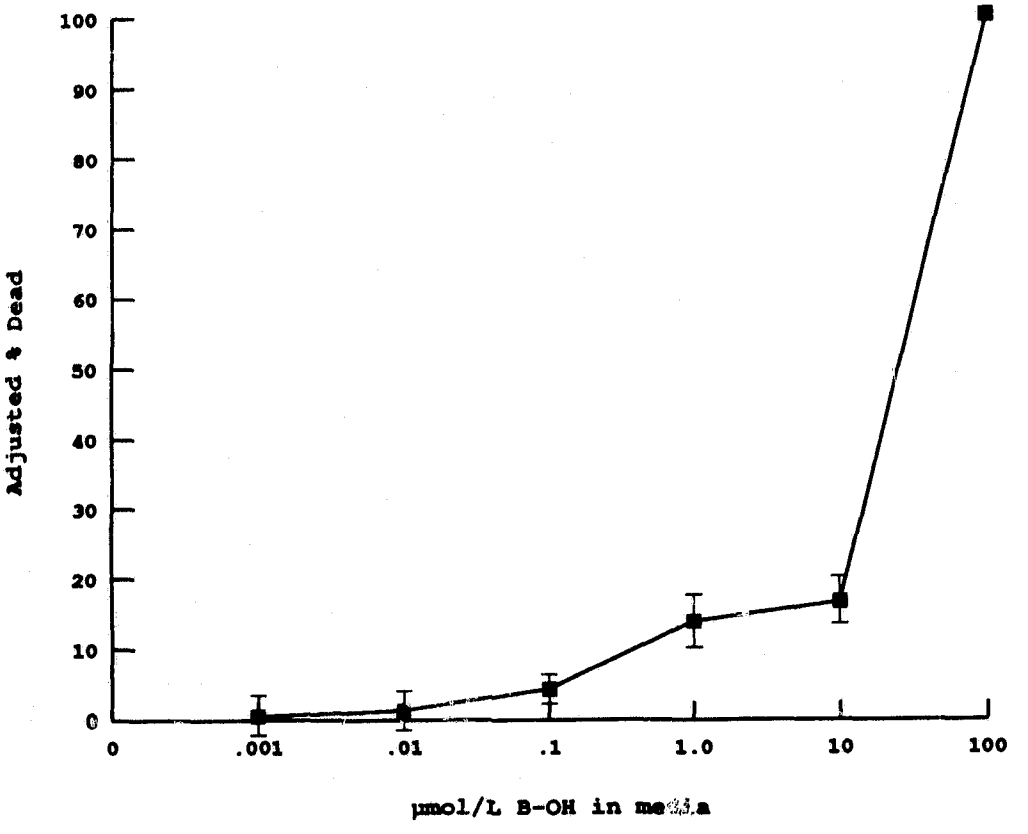

Figure 10. Cytotoxic effect of varying concentration of benzyl alcohol on NCTC 1469 cell culture

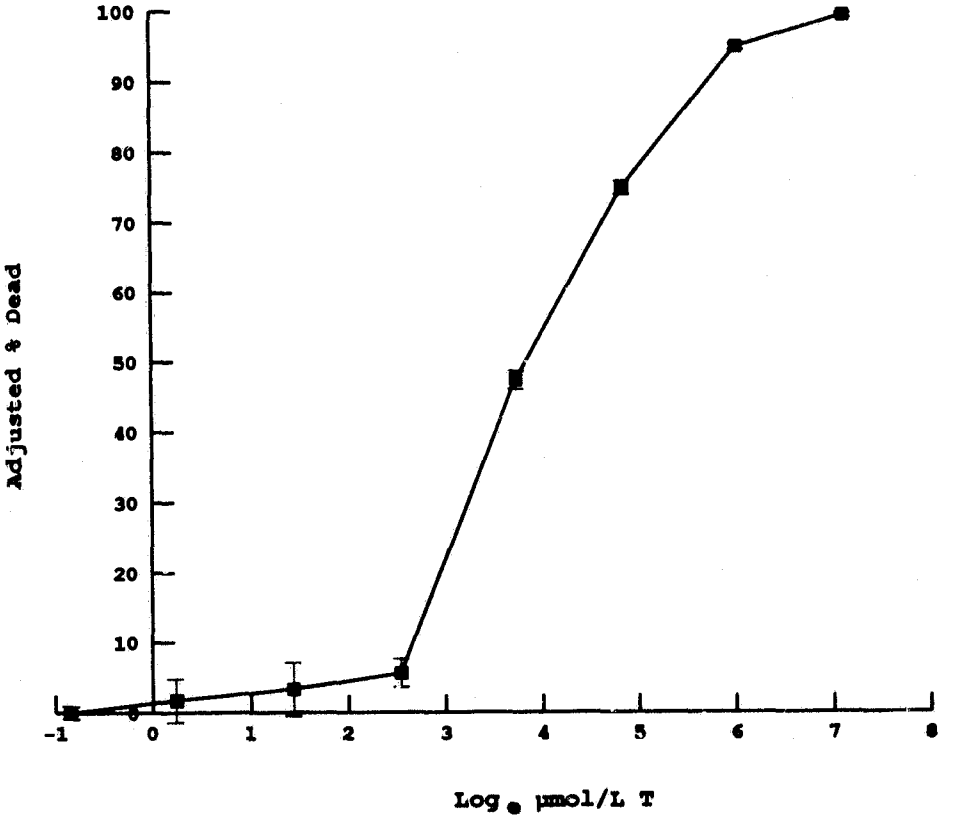

Figure 11. Cytotoxicity of thiacetarsamide on NCTC 1469 cell culture with exposure to the drug for 4 hours and with additional culture for 24 hours following removal of the drug
LC_{50} = 3.95 $\log_e$ µmol/L T LC_{50} = 51.94 µmol/L T

These included blebbing of cell membranes, increased vacuolation, and more pronounced variation in size (Figure 12) in cytospin preparations. As well, an increasing percentage of cells with uptake of Trypan blue stain were counted by phase microscopy. At 1311 μmol/L (30X) of T, marked alterations such as cell lysis and cytoplasmic swelling (Figure 13) were seen with cytospin preparations, and heavy uptake of Trypan blue was obvious. Probit transformation (SAS) was tried in order to transform the sigmoid curve into a straight line. This would provide a more accurate experimental value for the LC_{50} and a slope. This did not work. Other transformations such as $\log_e$, logit, negative reciprical, reciprocal, polynomial and square root all failed to straighten the line and to provide adequate confidence for the best fit line as determined by visual examination of the residuals following linear regression analysis of the transformed data.

2.3.7 Comparison of thiacetarsamide (as arsenamide) and Caparsolate™ with variable concentration of each agent, exposures of 4 hours, and 24 hours post exposure incubations

Since the decision to use the commercial product Caparsolate™ was one of convenience and this would be the agent used in experimental dogs, it was necessary to ensure

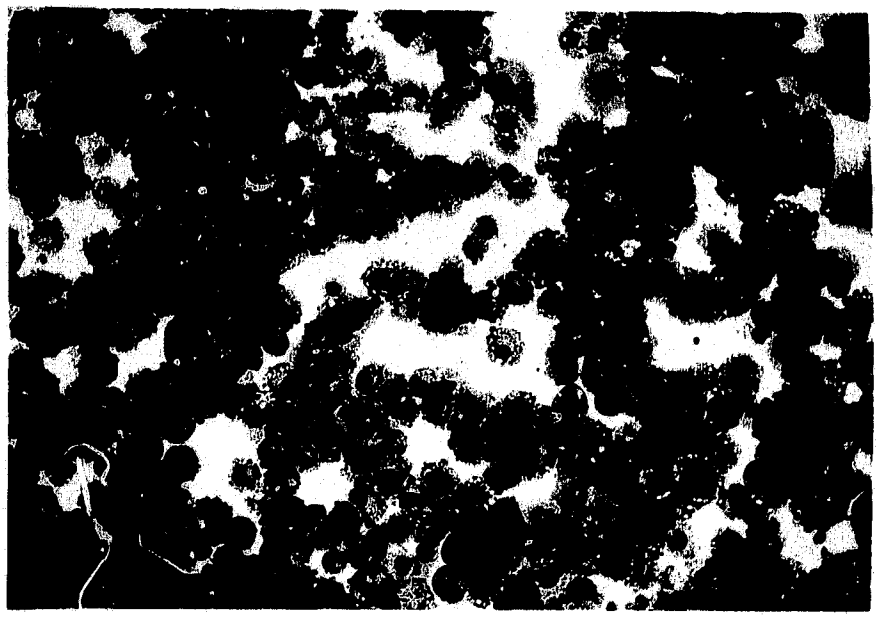

Figure 12. Photomicrograph of a cytospin preparation of NCTC 1469 mouse hepatoma cells showing mild cytotoxic alterations consisting of increased vacuolation and anisocytosis after 4 hours of exposure to 437 µmol/L of T and 24 hours of post exposure incubation (Wright's stain, magnification X150)

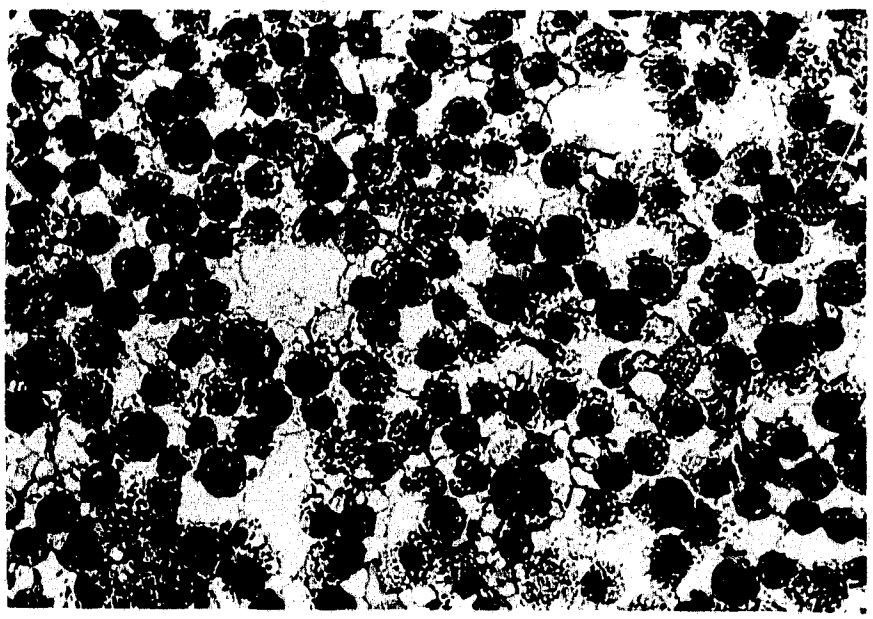

Figure 13. Photomicrograph of cytospin preparation of NCTC 1469 mouse hepatoma cells showing severe cytotoxic alterations after 4 hours of exposure to 1311 µmol/L of T and 24 hours of post exposure incubation (Wright's stain; magnification 150X)

that it did not vary significantly from the generic agent, arsenamide. Results of comparison of the two compounds showed no significant difference at any concentration ($p > .1$) (Figure 14).

2.3.8 Effects of AirbronTM on NCTC 1469 cell culture with 24 hour exposure and variable concentration

The results of this experiment did not suggest toxicity of NAC (as AirbronTM) on NCTC 1469 cell culture using the estimated ECF level of 428 μmol/L ($\log_e$ 6.06, refer to Table III) initially theorized (Figure 15). However, slight toxicity was noted at 128.4 μmol/L (.3X estimated ECF concentration). This was consistent in repeated experiments (not included in this thesis). At concentrations of 4284 μmol/L and greater, significant toxicity of NAC ($p < 0.01$) was noted. However this was beyond the ECF concentration anticipated for *in vivo* studies. The LC50 was 18034 μmol/L.

2.3.9 Effect of disodium EDTA on NCTC 1469 cell culture when concurrently exposed to thiacetarsamide

The results of this experiment indicate that EDTA at concentrations anticipated to be present in 1X ECF concentrations of Airbron™, whether in cell culture medium or ECF of dogs, should not influence effects of NAC against

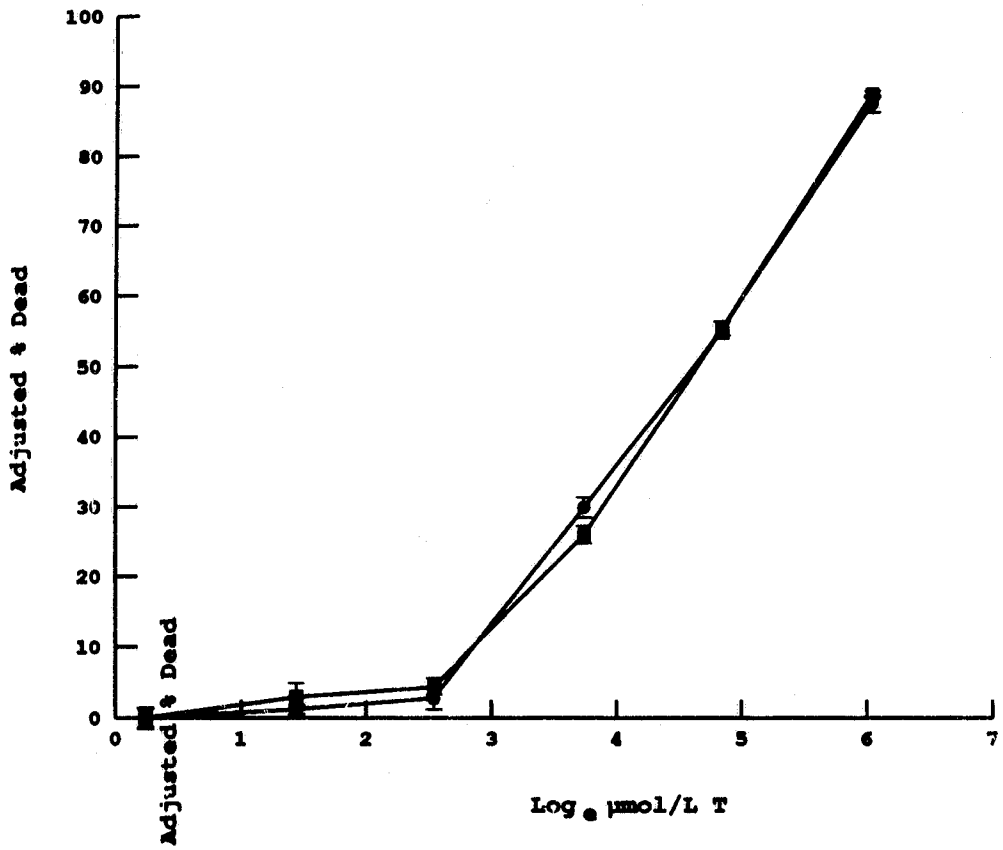

Figure 14. Comparison of cytotoxicity of thiacetarsamide (as arsenamide) and Caparsolate ™ on NCTC 1469 cell culture with an exposure to the drug of four hours and with an additional culture for 24 hours (incubation) following removal of the drug

● = Caparsolate ™
■ = Thiacetarsamide

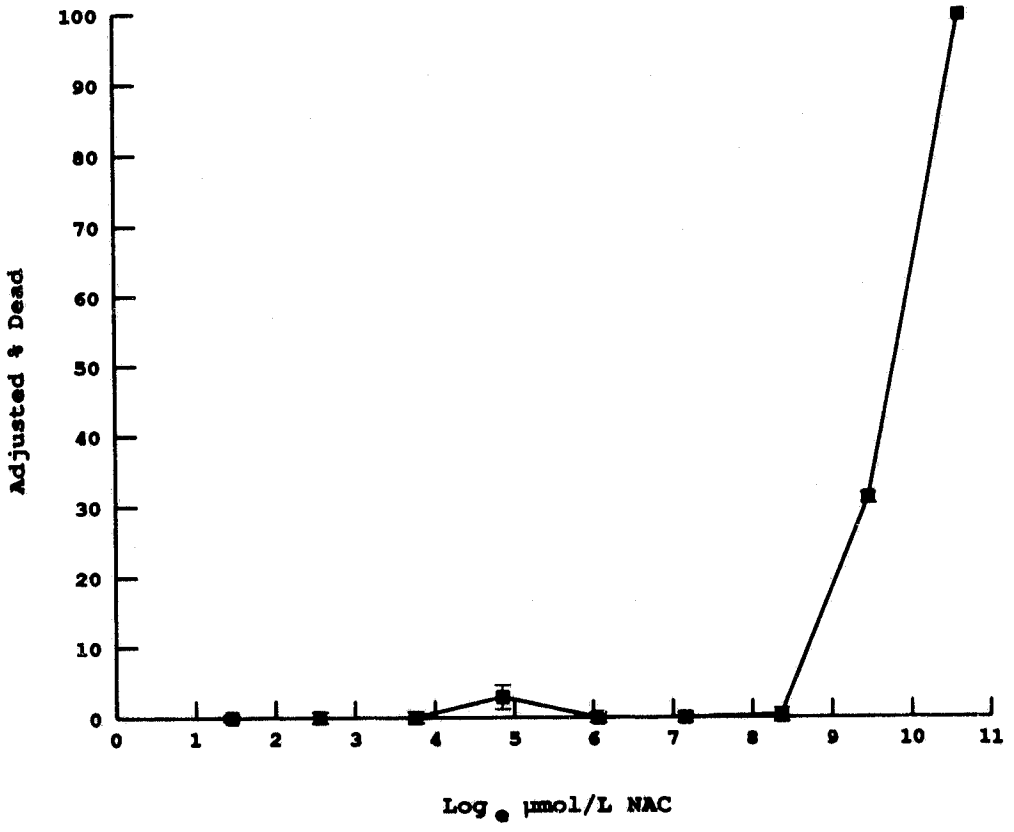

Figure 15. Effects of Airbron ™ on NCTC 1469 cell culture with a 24 hour exposure (incubation) to the drug LC_{50} = 18034 µmol/L NAC

T (Figure 16). Higher concentrations of EDTA are cytotoxic, however, the data suggest that the effects of increasing concentrations of T are of greater importance.

2.4 Discussion

In this chapter, an *in vitro* model for the toxic effects of T on NCTC 1469 mouse hepatoma cells has been developed. The time of exposure to T (4 hours), continued toxicopathic effects of As (24 incubation), and approximate concentration of T (51.94 μmol/L) to establish an LC_{50} for T on NCTC 1469 cell culture has been identified. In addition, this LC_{50} is sufficiently close to that hypothesized in chapter one and calculated in section **2.2.2** to continue its use *in vitro*. The results of the experiment with B-OH suggests that it is not interfering with the results of cytotoxic experiments using the commercial product Caparsolate™.

In considering the failure of Probit transformation for obtaining more refined estimates of the slope and LC_{50}, I rely upon the obvious visual changes to the cells and commentary by Salsburg 1986, in which he states that visual interpretation of toxicologic data is often necessary when complex interactions are occurring. Such is my assumption concerning the multiple possible toxic principles of As and thiacetarsamide as stated in Chapter One.

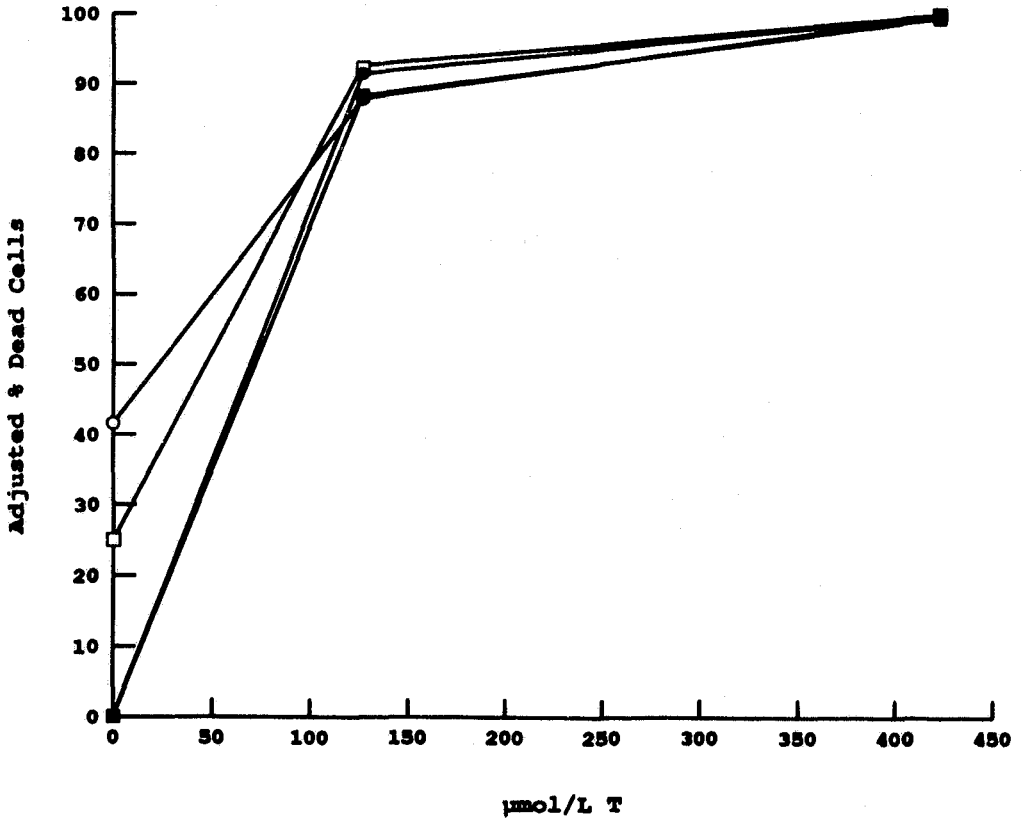

Figure 16. Effect of varying concentrations of disodium EDTA on NCTC 1469 cell culture when concurrently exposed to thiacetarsamide

■ = 0 µmol/L EDTA
□ = 131.1 µmol/L EDTA
○ = 437 µmol/L EDTA

The occurrence of marked structural changes (bleb formation) to the cell membrane has been reported in isolated fresh rat hepatocytes and has been assumed to represent significant alterations to the cytoskeleton. In addition, these experiments suggest that it is the effects of decreased thiol compounds and their effects on intracellular metabolism and functional structure that are the cause of these changes (146,158).

The cytotoxicity of T is likely to be similar to those cited in the literature of Chapter One. The most prominent of these are sulfhydryl binding of As to enzymes and proteins within the cell which inhibit function and structure. The effect of T upon the lipoic acid portion of the pyruvate decarboxylase enzyme as well as numerous other enzymes of the cell is likely but unproven by these experiments. Also the marked blebbing of cell membranes suggests interference with the cytoskeleton as well. At this point, the only comment that can be made concerning the toxic mechanism of T is that it is probably predominantly intracellular since cell death continues after removal of T and concentration of T is more important than duration of exposure.

The hypothesis that NAC is not toxic at hypothesized dosages for use in dogs has been shown. The toxic effect of EDTA, at higher concentrations, may explain some of the

toxicity of Airbron™ as noted in section 2.3.8. The only significant difference between points is for concentrations of EDTA at the zero level of T ($p = .001$). These indicated not protection but a mild worsening of viability. Therefore, it is reasonable to utilize the commercial preparation of NAC for both the cell culture model and *in vivo* studies.

The additional compounds present in the commercial preparations of NAC and T have been examined, as discussed above; results indicate that these substances (EDTA and B-OH) will not influence results of future experiments. This provides the advantage of being able to use the same agents for *in vitro* studies that will, hopefully, have positive correlates with *in vivo* experiments.

3. EFFECTS OF N-ACETYL-L-CYSTEINE ON THE CYTOTOXICITY OF THIACETARSAMIDE IN MURINE HEPATOMA (NCTC 1469) CELL CULTURE

3.1 Introduction

In chapter 2, an *in vitro* model for cytotoxicity was established. This model indicated that cytotoxicity of T may be complex. Thiacetarsamide, as an arsenical compound, has a variety of cytotoxic mechanisms. Therefore, in considering possible protective effects of NAC, a number of factors have to be considered. If NAC is able to cytoprotect the cells, then what phase/time in the experiment is the addition of NAC most advantageous, and how will varying concentrations of both NAC and T contribute to any protective effects?

One hypothesis was that NAC should be least cytoprotective if administered at the time of exposure of the cells to T. This should be the case if presence of NAC, at the time of exposure to As, does not prevent all binding of As to -SH groups of important cellular molecules.

Further, -SH donor compounds may play a role in reversal of As toxicity. This would suggest that dissociation of the As-SH bond may occur due to simple kinetic chemical reactions. The intracellular availability

of -SH from NAC could facilitate this action and cytoprotection occur after exposure to T.

Finally, it was hypothesized that the continued presence of NAC should be most beneficial during the post T exposure incubation period in the cell culture model. This would occur because of the advantage of having available -SH donor substance (NAC as a glutathione precursor) present to assist recovery of potentially "reversibly" injured cells. This group of cells would be those represented by Figure 9, where there is evidence for continued cell death following removal of As from the cell medium.

If NAC acts as a hepatocellular cytoprotectant against the pathobiologic effects of thiacetarsamide in NCTC 1469 cell culture, when administered at concentrations which are consistent with estimated *in vivo* ECF concentrations, then it will be possible to proceed to *in vivo* experiments. These experiments would be based upon the higher clinical dosage range for T (3.3 mg/kg).

3.2 Materials and Methods

3.2.1 The cell culture model and use of NAC and T for these experiments were the same as those previously described in section **2.2** of this thesis. In each of the following experiments, two 24 multiwell plates were prepared. These were marked to indicate control wells (pre

and post) and concentrations of NAC horizontally and thiacetarsamide vertically in an overlapping grid (Figure 17). The last horizontal row was used for thiacetarsamide only. Statistical evaluation was performed with analysis of variance for all concentrations of T and NAC and with paired student's t tests to identify significant differences between wells (different concentrations of NAC, e.g., line one points compaired to line 2 points at the same concentrations of T) for each experiment. Probit, logit, $\log_e$, and polynomial transformations were also applied to the data in hope of applying t tests between slopes and LC50s for different concentrations of NAC.

3.2.2 Effect of concurrent NAC on the cytotoxicity of 4 hour exposure of thiacetarsamide in NCTC 1469 cell culture followed by 24 hours of incubation

Concentrations of NAC at 42.8, 128.4, 428.4, 1284, 4284, and 12840 µmol/L were added to the appropriate wells. This was followed as soon as possible (approximately 10 minutes) by administration of 4.37, 13.10, 43.7, 131.1, 437, and 1311 µmol/L of thiacetarsamide to provide for the overlapping grid of concentrations for each substance. Plates were agitated manually after each addition of

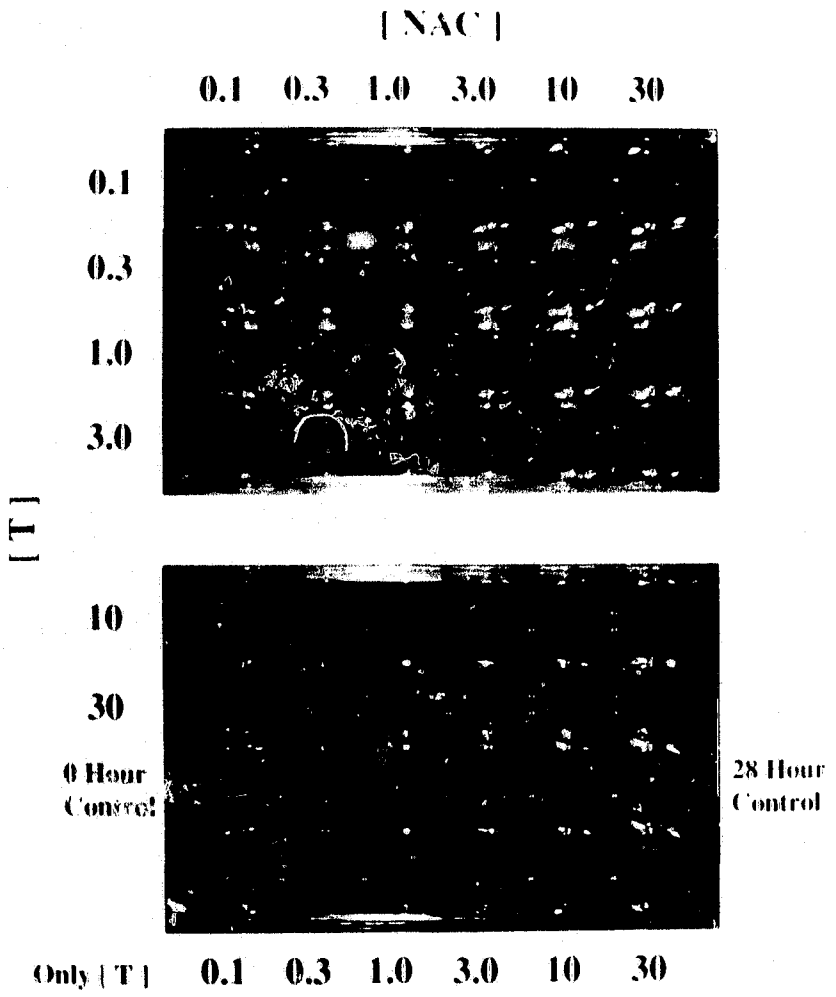

Figure 17. An overlapping grid design for varying concentrations of T and NAC was used. As well, a curve of T alone (0.0 μmol/L NAC) was provided for use as a comparison curve.

substance. The media was aspirated by Pasteur pipette at the end of the 4 hour exposure to both substances, without scraping the wells, and replaced with fresh media. After a 24 hour incubation period, cells were scraped from the bottoms of individual wells, collected, processed, and viability of cells counted as described previously.

3.2.3 Effect of 24 hour incubation with NAC following 4 hour exposure of NCTC 1469 cell culture to thiacetarsamide

Concentrations of 4.37, 13.11, 43.7, 131.1, 437, and 1311 μmol/L of thiacetarsamide were added to the appropriate wells. Plates were then agitated manually. The media was aspirated at the end of the 4 hour exposure to T, without scraping, and replaced with fresh media. This was followed by addition of NAC at concentrations of 42.8, 128.4, 428.4, 1284, 4284, and 12840 μmol/L in the described pattern. This provided the overlapping grid of concentrations considering the previous exposure to concentrations of T and the new addition of NAC. Plates were again agitated manually. After a 24 incubation period, cells were scraped, collected, processed, and viability counted as described previously.

3.2.4 Effect of concurrent exposure of NCTC 1469 cell culture to NAC and thiacetarsamide for 4 hours followed by replacement of NAC during 24 hour incubation time

Concentrations of NAC at 42.8, 128.4, 428.4, 1284, 4284, and 12840 μmol/L were added to the appropriate wells. This was followed in 10 minutes by administration of 4.37, 13.11, 43.7, 131.1, 437, and 1311 μmol/L of thiacetarsamide to provide for the overlapping grid of concentrations for both substances. Plates were then agitated manually. The media was aspirated at the end of the 4 hour exposure to both substances, without scraping, and replaced with fresh media. At this time, NAC was again added to the appropriate wells at the same concentrations as listed above. After completion of the 24 hour incubation period, cells were then scraped, collected, processed, and viability counted as described previously. Total exposure of cells to T was 4 hours (exposure) and to NAC was 28 hours (exposure + incubation).

3.3 Results

3.3.1 Effect of concurrent NAC on the cytotoxicity of 4 hour exposure of thiacetarsamide in NCTC 1469 cell culture

The results of this experiment include an analysis of variance with significant difference between concentrations of NAC and T ($p < 0.01$). Individual paired t tests were performed for paired data points (same concentrations of T), all concentrations of NAC and compared to the 0.0 μmol/L NAC line 1 (Figure 18). The failure of probit and like transformations to transform the sigmoid curve into a straight line precluded the desired ability of applying t tests (comparing) to LC_{50}s and slopes for different concentrations of NAC.

Using t tests, no significant differences between points for the 42.8 ($p = .2$), 128.4 ($p = .18$), 428.4 ($p = .08$) μmol/L [NAC] were noted. One data point from the 128.4 and one data point from the 12840 μmol/L were censored as outliers. Data points for 1284, 4284, and 12840 μmol/L of NAC, at points prior to T concentrations of 131 μmol/L ($\log_e$ 4.88), were not different statistically from those of the control curve at 0 μmol/L NAC (line 1). However, points after the 131 μmol/L ($\log_e$ 4.88) of T were different ($p < 0.01$). This indicated significant cytoprotective benefits

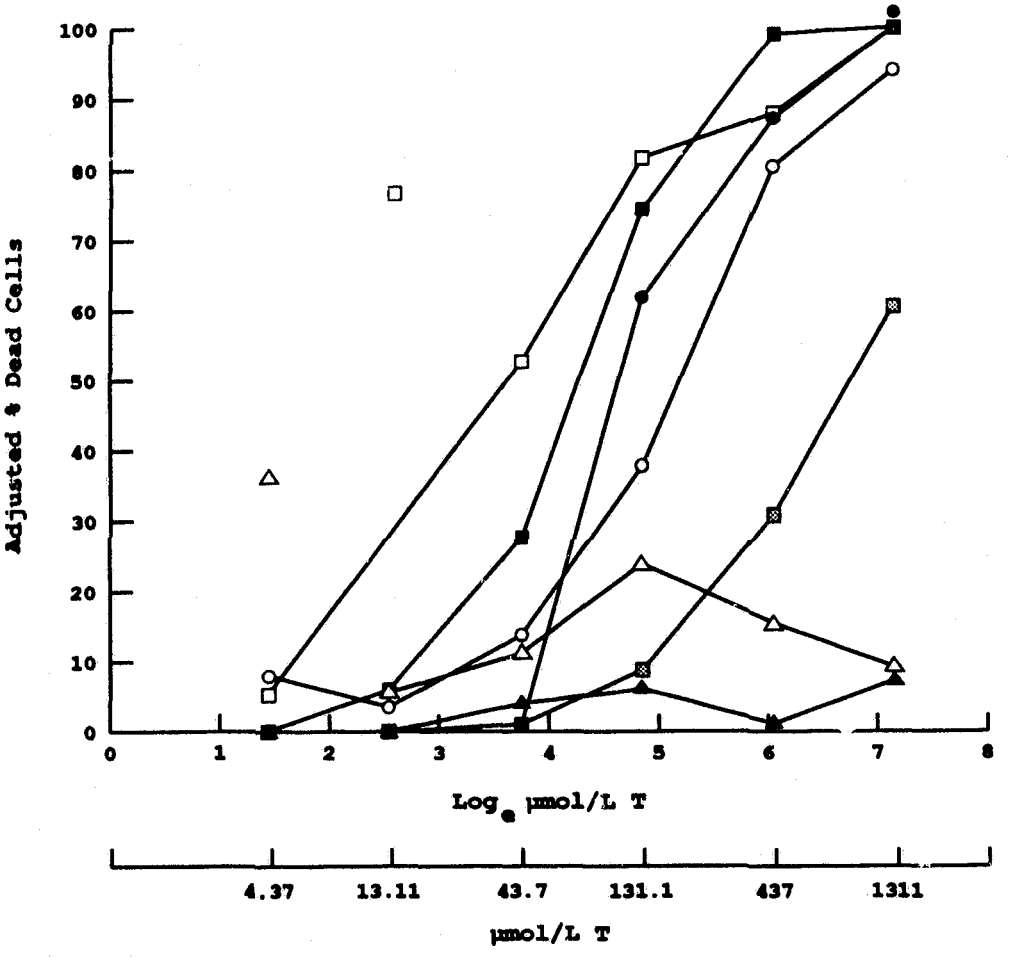

Figure 18. Effect of concurrent exposure to N-acetyl-L-cysteine on the cytotoxicity of thiacetarsamide in NCTC 1469 cell culture for 4 hours

■ Line one = 0.0 µmol/L of NAC
● Line Two = 42.8 µmol/L of NAC
□ Line Three = 128.4 µmol/L of NAC
○ Line Four = 428.4 µmol/L of NAC
▨ Line Five = 1284 µmol/L of NAC
▲ LIne Six = 4284 µmol/L of NAC
△ Line Seven = 12840 µmol/L of NAC

of higher concentrations of NAC against the toxicity of higher concentrations of T. Maximal cytoprotective benefits for NAC were noted at 1284, 4284, and 12840 μmol/L of NAC.

3.3.2 Effect of 24 hour incubation with NAC following 4 hour exposure of NCTC 1469 cell culture to thiacetarsamide

The results of this experiment were evaluated by analysis of variance with significant difference between concentrations of NAC and T ($p < 0.01$). Individual paired t tests were performed for all data points on all lines (varying concentrations of NAC) for same concentrations of T compared to the points on the 0.0 μmol/L NAC line 1 (Figure 19). The failure of probit and like transformations to transform sigmoid curves to straight lines precluded the desired ability to compare LC50s and slopes between varying concentrations of NAC.

Prior to a concentration of 13.11 μmol/L of T (Log_e 2.54) at the 0.0 μmol/L NAC, no significant differences were noted for points from lines of 42.8, 428.4, 1284, 4284 μmol/L. Concentrations of 128.4 and 12840 μmol/L of NAC were significantly different ($p < 0.01$). After the 43.7 μmol/L of T point all points on all lines were significantly different ($p < 0.01$) except the last point of 42.8 μmol/L (NAC). Maximal cytoprotective benefits for NAC were noted

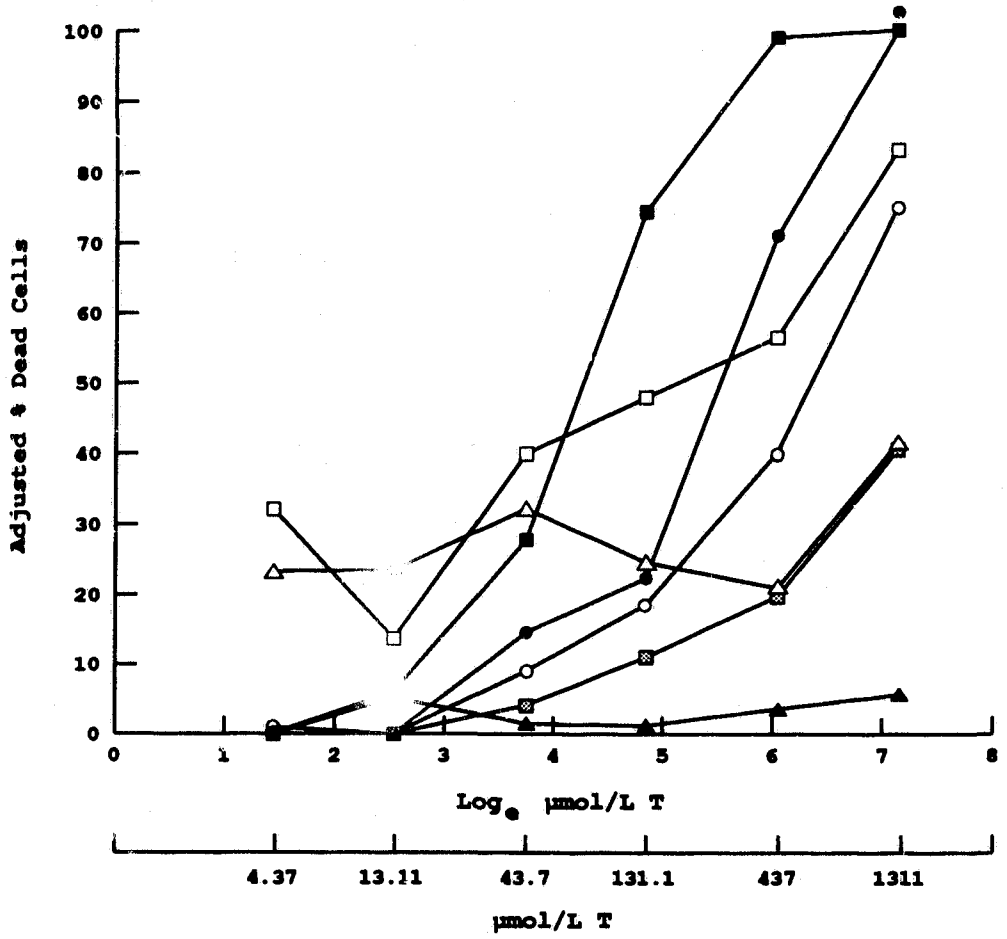

Figure 19. Effect of N-acetyl-L-cysteine on the cytotoxicity of thiacetarsamide when cultured with NCTC 1469 cell culture for 24 hours (incubation) after a 4 hour exposure to thiacetarsamide

■ Line one = 0.0 µmol/L of NAC
● Line Two = 42.8 µmol/L of NAC
□ Line Three = 128.4 µmol/L of NAC
○ Line Four = 428.4 µmol/L of NAC
▨ Line Five = 1284 µmol/L of NAC
▲ LIne Six = 4284 µmol/L of NAC
△ Line Seven = 12840 µmol/L of NAC

at 1284 and 4284 μmol/L. These results indicated marked cytoprotection by NAC in the post T exposure period.

3.3.3 Effect of concurrent exposure of NCTC 1469 cell culture to NAC and thiacetarsamide for 4 hours followed by replacement of NAC during 24 hour incubation time

The results of this experiment included an analysis of variance with significant difference between concentrations of NAC and T ($p = 0.0001$). Individual paired t tests were performed for all data points on all lines (varying concentrations of NAC) for the same concentrations of T compared to the 0.0 μmol/L NAC (Figure 20). The failure of probit and like transformations to transform the sigmoid curves to straight lines precluded the ability to compare LC50s and slopes of lines for different concentrations of NAC by t tests.

The 12,840 μmol/L of NAC resulted in 100% cell death in this experiment where the NAC was present the full 28 hours. As well, significant increases in the adjusted % dead counts were noted for 128.4 and 4284 μmol/L ($p = 0.001$) as compared to 0.0 μmol/L NAC (control). However, maximal cytoprotective benefits for NAC were noted by lines for 428.4 and 1284 μmol/L ($p = 0.0001$). And, there was

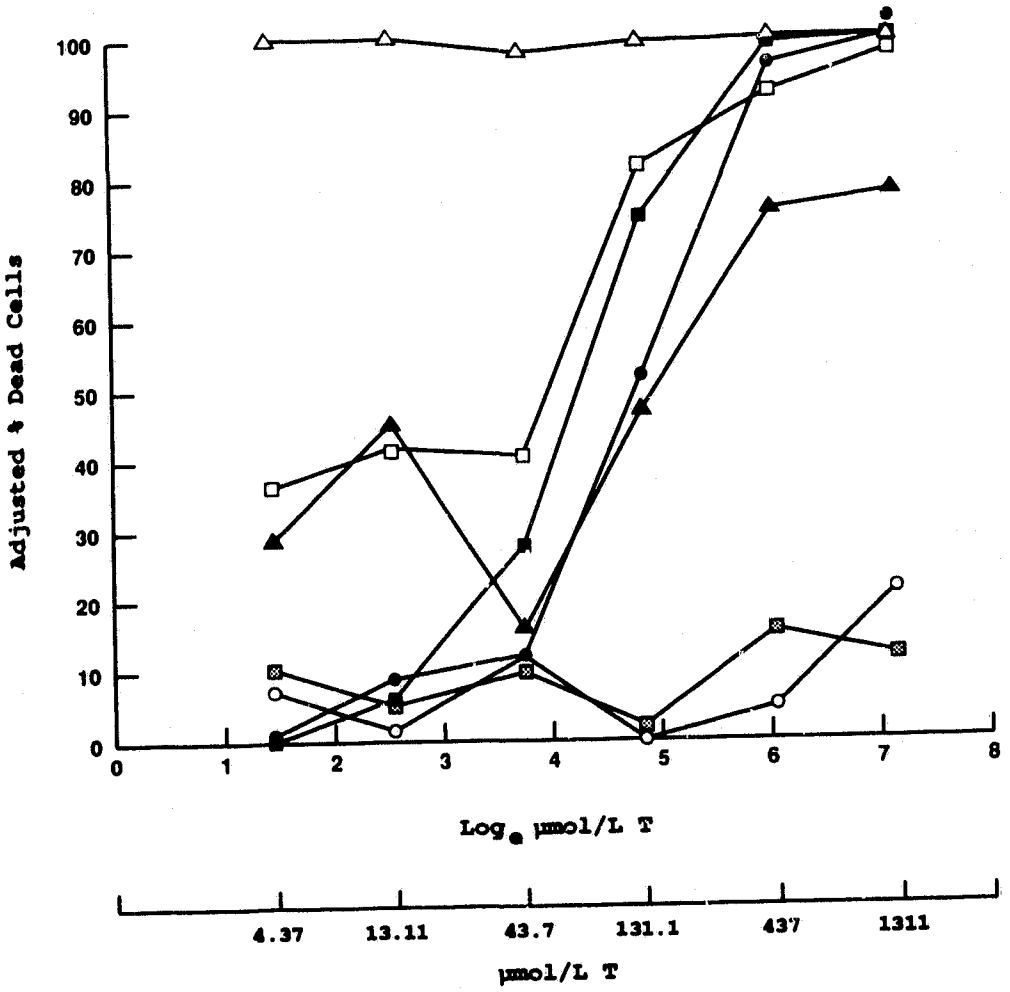

Figure 20 Effect of N-acetyl-L-cysteine on the cytotoxicity of thiacetarsamide when NCTC 1469 cell culture are concurrently exposed to NAC and T for 4 hours and incubated with NAC alone for an additional 24 hours

■ Line one = 0.0 µmol/L of NAC
● Line Two = 42.8 µmol/L of NAC
□ Line Three = 128.4 µmol/L of NAC
○ Line Four = 428.4 µmol/L of NAC
▧ Line Five = 1284 µmol/L of NAC
▲ LIne Six = 4284 µmol/L of NAC
△ Line Seven = 12840 µmol/L of NAC

significant protection for the 4284 μmol/L of NAC line at the 437 and 1311 μmol/L of T points ($p = 0.04$).

3.4 Discussion

All three experiments demonstrated marked cytoprotective benefit of NAC against the cytotoxic effects of T in the NCTC 1469 murine hepatoma *in vitro* cell culture model.

The optimal cytoprotective concentration of NAC varied within 3 log concentrations ([1X and 10X] from Table III) between the three experiments. This is very important since this range will allow utilization of accepted concentrations of NAC and T *in vivo* in dogs as discussed in the hypothesis at the end of chapter one.

With NAC present for the four hour exposure only, the optimal concentration was 12840 μmol/L. During 24 hour post exposure incubation with NAC, a lower concentration of 4284 μmol/L of NAC appeared to be optimal. And, with NAC present prior to exposure to T, and throughout the 28 hours of the experiment, the optimal cytoprotection occurred, once again, at lower concentrations of 1284 or 428.4 μmol/L of NAC (Figure 20).

The results indicate that the longer the exposure of NAC to cell cultures the more likely it is for itself to be cytotoxic. The reasons for the cytotoxicity of NAC may

include alterations in cellular metabolism that are substrate/product related and not identified, e.g., competition for sulfhydryl groups of cellular enzymes which are detrimental only at certain levels (128.4 μmol/L NAC). This has been reported and left unexplained by Hayes, et al 1986 (156). Direct alterations to the media which exceed the buffering capacity or osmolality for maintenance of homeostasis of the cells (12840 μmol/L of NAC) are other mechanisms which are possible causes of NAC cytotoxicity.

Thiacetarsamide, as stated in chapter one, has numerous potential mechanisms of toxicity including enzyme inhibition, interference with aerobic respiration, and potential alterations to intracellular structure. This has been directly seen with the interference of thiol metabolism in rat hepatocytes using thiol inhibitors (156).

The relationship of NAC and thiacetarsamide (As) may therefore undergo multiple complexities of kinetic reactions based upon concentration kinetics, media or intracellular pH influences on pKa enzyme kinetics, which proteins/enzymes are most interfered with at what concentrations, and variability between experiments for the relative degree of log phase of cell growth versus plateau phases of growth (57,77,104,109,123,128,131,134,137,145,148,156,158,159).

4. INFLUENCE OF N-ACETYL-L-CYSTEINE AND DEXTROSE ON THE HEPATOTOXIC EFFECTS OF THIACETARSAMIDE IN THE DOG

4.1 Introduction

4.1.1 NAC as a cytoprotectant against T

The results of the previous experiments *in vitro* indicated clearly that NAC could protect against the cytotoxic effects of T. This data is supported by literature which reports, *in vivo*, that NAC can be cytoprotective against the hepatotoxic effects of other compounds such as acetominophen (113,127,128). Therefore studies were conducted *in vivo* to determine if a correlation could be established.

4.1.2 Dextrose as a cytoprotectant against T

Dextrose was chosen as a second substance to be investigated. It has been suggested because of its general cytoprotective ability with regards to numerous non-specific cell stressors (80,81,101,111). Administration of dextrose has been shown to spare hepatocellular glycogen stores by

stimulation of portal release of insulin and hepatic glycogenesis (111). This glycogen is thought to act as a "sink" for free radicals generated by release of free electrons from impaired intracellular and mono-oxidase metabolic reactions (160). An excess of glycogen may also support continued aerobic respiration of the cell via the Kreb's cycle (111).

4.1.3 Quantification of hepatotoxicity *in vivo*

Numerous traditional methods of assessing *in vivo* hepatocellular damage have been utilized. The monitoring of serum hepatic enzymes (both leakage, e.g., ALT, and inducible, e.g., serum alkaline phosphatase); metabolism and elimination of substances (bile acids, albumin, urea, organic anions, bilirubin); gross, light, and ultrastructural pathologic alterations; and determination of lethal dose (LD_{50}) curves have all been described (161,162,163).

It was also a purpose of this experiment to utilize sequential percutaneous liver biopsies during a toxicologic trial. This experiment would therefore be a survival one. This is in accordance with current desires for reduction in use of laboratory animals. Hepatic biopsy is often the most effective diagnostic test available to identify the cause and histologic pattern of hepatic disease and injury

(161,164,165,166). Percutaneous hepatic biopsies are performed in humans without general anesthesia or sedation, but with the use of local anesthesia only. Percutaneous-transabdominal needle biopsy techniques have been previously recommended in the veterinary literature. This has been utilized primarily in clinical cases with normal platelet count and normal coagulation function, for which diffuse hepatic disease and a normal-to-enlarged hepatic mass are strongly suspected or have been experimentally induced. Laparoscopy, laparotomy, and ultrasonography-guided hepatic biopsy are recommended in cases not fulfilling these criteria (161,164,166).

If the lesions created by hepatotoxicants can be identified by gross or light microscopic pathological evaluation, then morphometric or simple descriptive scoring systems are appropriate for biopsy samples (167). Morphometric quantification via computer/video assisted measurements was considered for this project. Because of the small size of biopsy material (needle biopsy) (163), it was decided that a traditional scoring system for assessment of morphologic hepatocellular damage would be appropriate. Additional data consisting of serum chemistries and hemograms were collected for analysis independently or in correlation with biopsy data.

4.1.4 Hypothesis

The hypothesis of this experiment was that NAC and dextrose would be hepatocellular cytoprotectants against the effects of thiacetarsamide in the dog, and that sequential percutaneous transabdominal hepatic needle biopsies could be used in a survival experiment to assess diffuse toxic hepatic damage.

4.2 Materials and Methods

4.2.1 Dogs used for *in vivo* studies

Twenty-four dogs (18 mixed breed and 6 beagles) were obtained from commercial sources. There were 9 males and 15 females weighing between 10 and 24 kg. Housing and all procedures performed on the dogs complied with the guidelines of the Animal Care Committee, University of Prince Edward Island and those of the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals. The dogs were housed for a minimum of 21 days prior to commencing experimental procedures and 3 blood samples were drawn from jugular veins at 7-day intervals to acclimate them to the future procedures.

4.2.2 Sampling Procedures for experimental dogs

The day of the first biopsy procedure (72 hours prior to treatment), a complete blood count (hemogram), platelet count, serum chemical analysis, and activated clotting time were performed. Hemograms and serum chemical analyses were performed by the AVC Diagnostic Laboratory (AVC-UPEI, Charlottetown, PEI, Canada). These tests included ALT, ALP, bilirubin, blood glucose, albumin, urea, creatinine, electrolytes, total protein and GGT. Activated clotting times were performed by the operator prior to biopsy (Hemochron 400, International Technidyne Corp., and CA510 Celite, Hemochron Blood Coagulation Tubes, Edison, New Jersey, USA). Samples were obtained from each dog before any drugs were administered. The obtained values were in normal reference ranges for the laboratory.

Dogs were randomly assigned to one of 3 groups. However, each group underwent the experiment as a group without blinding to the operator. All biopsy samples were coded (blinded), so as to preclude bias upon histopathologic examination. All pretreatments (saline, NAC, or dextrose) were given 15 minutes before each injection of thiacetarsamide.

Thiacetarsamide was given to all groups intravenously at 3.3 mg/kg at 7 AM and 3 PM for 2 days. Blood tests were repeated each day during and for 2 days after the

treatments. The second hepatic biopsy sample was obtained from each dog 24 hours after the last treatment.

4.2.2.1 Transabdominal percutaneous hepatic biopsy technique

The percutaneous-transabdominal biopsy procedure was modified from reported techniques (161,164). Food was withheld from the dogs for 12 hours prior to each procedure. Chemical restraint consisted of acetylpromazine 0.25 to 0.50 mg/kg given orally 60 minutes prior to IV administration of 2% solution of thiamylal, which was administered to attain a light surgical level of anesthesia. This was assessed on the basis of suitable central nervous system depression for the procedure involved (globes rotated, no withdrawl to toe pinch, no alteration of cardiac or respiratory rate to the subsequent 1 mm incision). The dogs were positioned and held in oblique dorsal recumbency at a 30 to 45° angle with the right side toward the table. This position was chosen to present the left lateral liver lobe closest to the operator and maximize the likelihood that the gallbladder would be to the right of the abdominal midline (in the right hemiabdomen). An area between the tip of the xiphoid, the left costal arch and the umbilicus was clipped and surgically prepared. A site within this area was chosen for percutaneous puncture after digital palpation for the liver

margins was attempted. An 1 to 2 mm incision was made through the skin to the hypodermis. The tip of the cutting biopsy needle (Tru-Cut Biopsy Needle. Travenol Laboratories. Deerfield, Illinois, USA) was placed through the skin incision and abdominal muscles to the approximate level of the parietal peritoneum. At this time respiratory movement of the dog was assessed and the needle was advanced on inspiration. The depth of the first pass of the needle was 1 to 2 cm. If this shallow attempt was unsuccessful, then deeper penetrations were made to the 5 cm level. Advancement of the needle by the operator was gently continued in synchrony with inspiration. The intrahepatic and cutting phases of the needle biopsy were performed at apparent peak inspiration of the animal. The direction of needle advancement was initially left, dorsad toward the vertebral column, and craniad. The biopsy needle was redirected if the initial attempt was unsuccessful and only after the needle was withdrawn to the level of the abdominal muscles. This was done to reduce the risk of hepatic parenchymal laceration. Selection of the angle for redirection of the needle was based on the perceived location of the liver, body type, previous tactile sensation (i.e., resistance of the diaphragm if too far craniad) and tissue recovery (i.e., fat from the falciform ligament). Deep-chested dogs generally required a more vertical angle of needle advancement and deeper penetration.

Once the first biopsy specimen was obtained, the same direction was used for the second one. The hepatic biopsy specimens were gently teased from the needles with a portion of the specimen placed in formalin and a second in 2% glutaraldehyde. A 1 mm ribbon of nitrofurazone ointment (Furacin Soluble Dressing, Austin Laboratories Canada Ltd. Joliette, Quebec) was placed over the incision. The dogs were positioned in sternal recumbency so as to place the weight of the liver and abdomen over the biopsy site. The dogs were returned to their cages when they were able to stand. Each dog underwent the described biopsy procedure twice during the experimental protocol. The first pair of biopsy specimens served as a control, and the second set was collected five days later (ie, 24 hours after the last dose of thiacetarsamide).

Heart rate, respiratory rate, mucous membrane color, and oral mucous membrane capillary perfusion (as assessed visually following digital pressure) were monitored for each dog at 10 minute intervals for the first hour following the procedure in order to detect clinically significant changes. All dogs were observed daily in their kennel for four weeks after biopsy.

4.2.3 Statistical methods

Data (difference between pre and post biopsy scores, post biopsy scores, as well as clinical pathologic values) were evaluated using one-way analysis of variance (ANOVA), Two Sample t tests [Bonferroni] both pooled and non-pooled, and Newman-Keuls t tests. Biopsy score differences (pre - post), post biopsy scores, and sum ALT (from 0, 24, 48, and 72 hours) and 72-hour ALT values were rank correlated (Spearman rank correlation) (Minitab 7.0,Minitab Inc., State College, Pennyslvania, USA) (BMDP) (PCS) (Pharmacologic Calculation System 4.1, Microcomputer Specialists, Philadelphia, PA, USA). Mann-Whitney two sample rank tests were performed on post biopsy and biopsy score differences (Minitab 7.0). Repeated measures analysis of variance for linear models was also performed for hematologic and chemical values (BMDP).

4.2.4 Scoring system for hepatic biopsy pathology

The hepatic scoring system had to be reproducible and to take into consideration the potential forms of hepatotoxicity which might be evidenced, since different compounds result in different patterns or types of hepatocellular injury (117,162,168,169,170). Hematoxylin

and eosin (H&E) was the stain used for evaluation of formalin fixed hepatic tissues. The parameters which were included in the scoring evaluations were: focal aggregates of inflammation due to individual hepatocellular death; induction of biliary hyperplasia; inflammation; and presence and pattern of hepatocellular injury (necrosis). A minimum of 5 portal areas were examined for each biopsy specimen. An overall assessment was made and a score given for each specimen using the previously described criteria which are given in the following pages.

Random focal aggregates in hepatic parenchyma - A

predominantly neutrophilic - (N)

predominantly monocytic - (M)

lobular distribution

Grade 0 - no focal aggregates observed

Grade 1 - few observed (1-2 per 5 portal tracts/acini)

Grade 2 - mild/present (3-5 per 5 portal tracts/acini)

Grade 3 - moderate/marked (5-8 per 5 portal tracts/acini)

Grade 4 - severe (>8 per 5 portal tracts/acini)

Biliary Ductular Hyperplasia - B

Biliary ductular (epithelial hyperplasia) was recorded when an increased presence of biliary epithelium or ductules in acinar areas or increased prominence in portal tracts was noted

Grade 0 - normal

Grade 1 - suspect increase of biliary structures per acinus

Grade 2 - mild/present (every acini or portal tract involved although not prominent)

Grade 3 - moderate/marked (prominent in all acini and portal tracts, as well as noted in parenchyma)

Grade 4 - severe (all portal tracts and acini with severe proliferation)

Inflammation - I

predominantly neutrophilic - (N)

predominantly monocytic - (M)

lobular distribution

Grade 0 - none observed/normal

Grade 1 - few areas with small numbers of inflammatory cells

Grade 2 - mild (obvious but mild increase in inflammatory cell numbers)

Grade 3 - moderate (heavier infiltration but without alteration of normal architecture)

Grade 4 - severe (heavy cellular infiltrate in multiple lobules with disruption of architecture)

Necrosis - N / Hepatocellular Cytopathic Effect

Evidence for hepatocellular injury included acidophilia, hydropic degeneration, acidophils, necrosis (piecemeal, bridging, massive, submassive), and lobular distribution or pattern.

Grade 0 - none observed/normal

Grade 1 - few areas of hydropic change or altered staining with 1-4 affected hepatocytes per area observed

Grade 2 - mild (obvious necrosis of hepatocytes but limited numbers in specific areas)

Grade 3 - moderate (mild piecemeal necrosis, greater numbers necrotic hepatocytes consistently present in specific lobular areas)

Grade 4 - severe (bridging necrosis, piecemeal necrosis)

4.2.5 Experimental *in vivo* groups

4.2.5.1 Experimental *in vivo* group I

Group I (n = 9, 7 mixed breed and 2 beagles) were pretreated with .7 ml/kg of normal saline given IV 15 minutes before each injection of T. Samples were collected as previously described.

4.2.5.2 Experimental *in vivo* group II

Group II (n = 6, 4 mixed breed and 2 beagles) were pretreated with 10 ml/kg of 10% dextrose given IV 15 minutes before each injection of T. This dosage was selected because it is routinely used in glucose tolerance testing in canines with normal response being return to normal blood glucose values within 15-30 minutes while under the influence of barbiturate compounds (171). Samples were collected as previously described.

4.2.5.3 Experimental *in vivo* group III

Group III (n = 9, 7 mixed breed and 2 beagles) were pretreated with .7 ml/kg of NAC as a 20% solution given IV 15 minutes before each injection of T. Samples were

collected as previously described.

4.3 Results

4.3.1 General

All dogs survived the procedures and treatments. Each biopsy specimen was evaluated for the presence of hepatic tissue and determined visually to be of adequate quantity. Prior to fixation, liver biopsy specimens measured 0.5 to 1 mm in diameter by 10 to 15 mm in length. Hepatic parenchyma was obtained in all 96 specimens (24 dogs x 2 pre samples x 2 post samples) and each contained multiple complete acini. All biopsy specimens were of suitable quality for the purpose of histologic evaluation as evidenced by lack of confounding (processing or biopsy) artifact, presence of good staining characteristics, and identifiable cellular and organelle morphologic features. Although samples were processed in glutaraldehyde for ultrastructural studies, pathologic results were of such a severe magnitude that they precluded the value of ultrastructural evaluations (75,162,163). Hepatic biopsy samples were coded as described and scored by one person (MEH) using the predetermined system described above in section 4.2.4. Results for samples are reported by coded and then uncoded (animal) numbers as well as by the suffix

of -1 or -2 to identify pre and post treatment specimens respectively (Appendix A).

Serum chemistries and hemogram data were recorded and entered in datafiles (Minitab 7.0).

4.3.2 Results for *in vivo* groups

4.3.2.1 Serum chemistries and hemogram results

The results of the treatment groups are presented (Tables VII, VIII, IX). Only the data considered pertinent due to statistical significance, for chemistries and hematologic values, are presented.

Mild to marked variation for chemistry and hematology values was present within groups. Presentation of the 72 hour data for ALT was chosen because it represented the peak of ALT values following treatment with T and was sampled immediately prior to the post treatment hepatic biopsy.

Mean 72 hour ALT and sum ALT values were significantly different ($p < .01$) (PCS, Minitab 7.0). However the site of significance was predominantly between the dextrose and NAC groups. Between groups I and II, there was no consistent worsening effect of dextrose on the hepatic pathology as measured by ALT leakage.

TABLE VII

GROUP I (SALINE) N = 9

Biopsy scores and ALT levels in dogs exposed to thiacetarsamide with prior treatment with saline.

animal ID	PRE-N-POST[a]		Pre-I-Post[b]		ALT 72 hr[c]
1	1	4	1	4	1338
3	1	4	1	4	3761
5	0	1	0	0	148
14	0	4	0	3	360
12	1	0	0	0	63
10	0	4	0	3	2880
20	1	3	1	2	216
23	0	2	0	0	615
7	0	0	0	3	123
Total	4	22	3	19	6504
Σ/n	.44	2.4	.3	2.1	723

[a] pre and post treatment biopsy scores for necrosis

[b] pre and post treatment biopsy scores for inflammation

[c] alanine amino transferase values at 72 hours from first injection of thiacetarsamide

TABLE VIII

IN VIVO RESULTS GROUP II (DEXTROSE) N = 6

Biopsy scores and ALT levels in dogs exposed to thiacetarsamide with prior treatment of dextrose.

animal ID	PRE-N-POST[a]		Pre-I-Post[b]		ALT 72 hr[c]
19	0	3	1	3	10091
22	0	4	0	3	7231
18	0	4	0	4	2376
25	0	3	1	2	1563
16	0	3	1	3	671
8	0	4	1	4	1294
Total	0	21	4	19	23226
Σ/n =	0	3.5	.6	3.16	3871

[a] pre and post treatment biopsy scores for necrosis

[b] pre and post treatment biopsy scores for inflammation

[c] alanine amino transferase values at 72 hours from first injection of thiacetarsamide

TABLE IX

IN VIVO RESULTS GROUP III (N-ACETYL-L-CYSTEINE) N = 9

Biopsy scores and ALT levels in dogs exposed to thiacetarsamide with prior treatment of NAC.

animal ID	PRE-N-POST[a]		Pre-I-Post[b]		ALT 72 hr[c]
21	1	0	1	0	316
11	0	1	1	2	54
6	0	2	0	2	875
17	0	0	1	0	75
24	0	1	0	1	31
2	0	0	1	0	93
9	0	0	0	1	561
15	0	3	1	3	2871
4	0	1	0	1	44
Total	1	8	5	10	4920
Σ/n =	.11	.8	.55	1.1	546

[a] pre and post treatment biopsy scores for necrosis

[b] pre and post treatment biopsy scores for inflammation

[c] alanine amino transferase values at 72 hours from first injection of thiacetarsamide

However, this comparison (saline versus dextrose) was close to standard significance levels ($p = 0.06$) by Bonferroni test. The comparison of groups I and III was not significant ($p > 0.1$) using Bonferroni tests but was with Newman-Keuls test ($p < 0.05$). Utilizing the Mann-Whitney two sample rank procedure (based upon median values to help compensate for large variance), a significant difference for ALT levels at 72 hours was found, again, for the dextrose (higher) versus NAC (lower) treatment groups ($\alpha = .0113$). Comparisons of groups I and III and I and II were not significant by this test. No other hematologic or biochemical test demonstrated adequate significance when examined by ANOVA, repeated measures analysis, or paired t tests.

4.3.2.2 Biopsy results

Proper identification of pre-treatment biopsy categories for necrosis and inflammation was evident by the prevalence of 0's and 1's in these biopsy specimens' scores. Inflammation and necrosis scores of 0 and 1 could have been considered equivalent to normal, because of the presence of 1's in the pre-treatment biopsy samples (Figure 21 and 22). However, this was not done since the scoring system was predetermined.

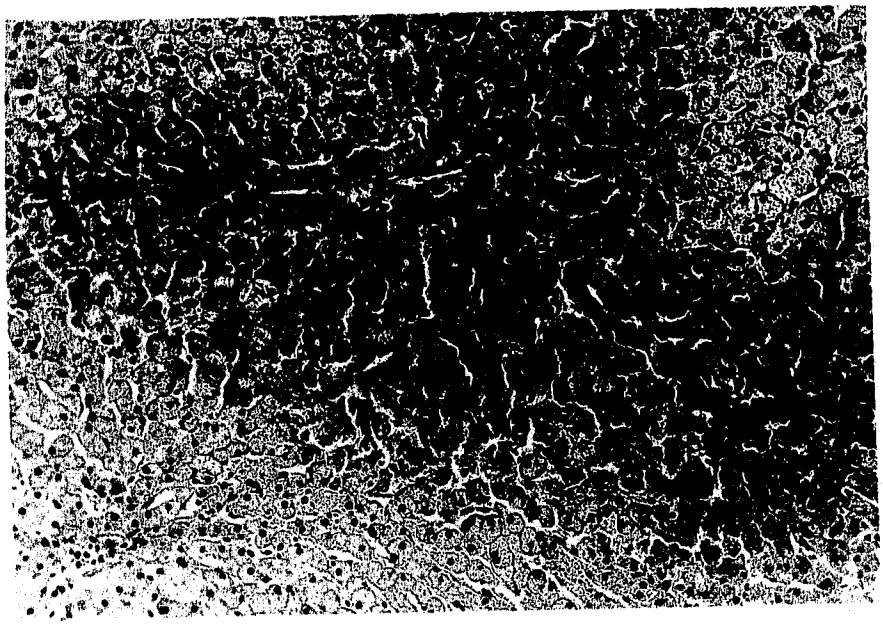

Figure 21. Normal biopsy - score 0 (H&E; magnification X150)

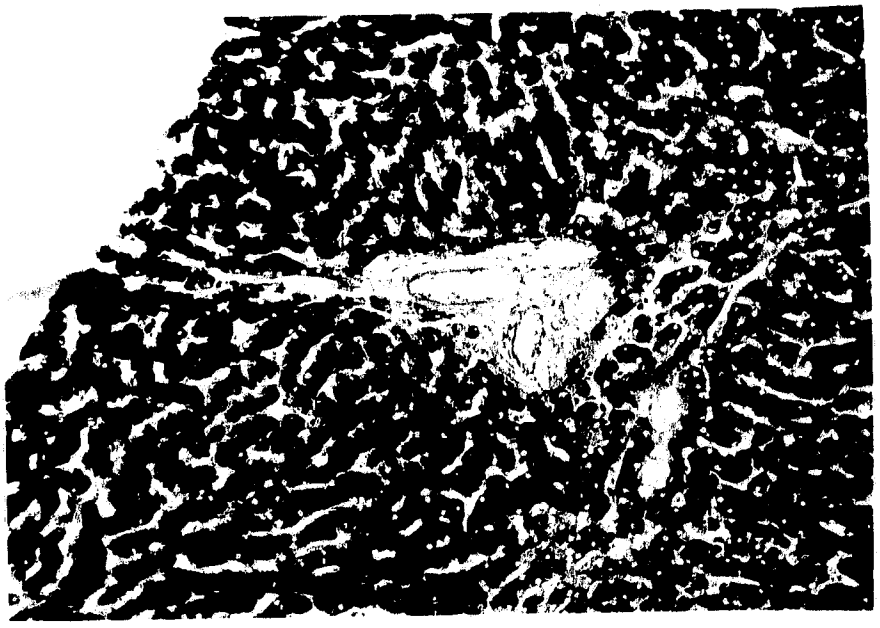

Figure 22. Normal biopsy - score 0 (PAS; magnification X150)

The pattern of necrosis and inflammation in post-treatment samples was predominantly periportal (zone 1). Occasional focal or pericentral accumulations of inflammatory cells with simple acidophilic hepatocytes were seen but these were not consistent or considered significant since they were equally present in pretreatment biopsies.

Examination of periodic acid Schiff (PAS) stained slides was performed after scoring was completed. The advantage of PAS staining was the marked demonstration of glycogen depletion and evidence of hydropic degeneration noted in those areas already assessed as being necrotic and inflamed with H & E stains (Figures 23 and 24).

Analysis of variance for the three groups, analyzing both biopsy score differences and then post biopsy scores alone, indicated significant difference between groups ($p < 0.01$ for difference between pre and post scores and $p = 0.003$ for post biopsy scores). Two sample t tests were then performed on the post biopsy values to identify where the significance was. These procedures were performed with both pooled standard deviation (assuming a comparative standard deviation between groups) and then without pooling (since differences for values of the standard deviations appeared large) (PCS, Minitab 7.0).

Saline versus dextrose was not significantly different ($p = 0.12$). Saline versus NAC was significantly different ($p = 0.036$) with results showing lower scores.

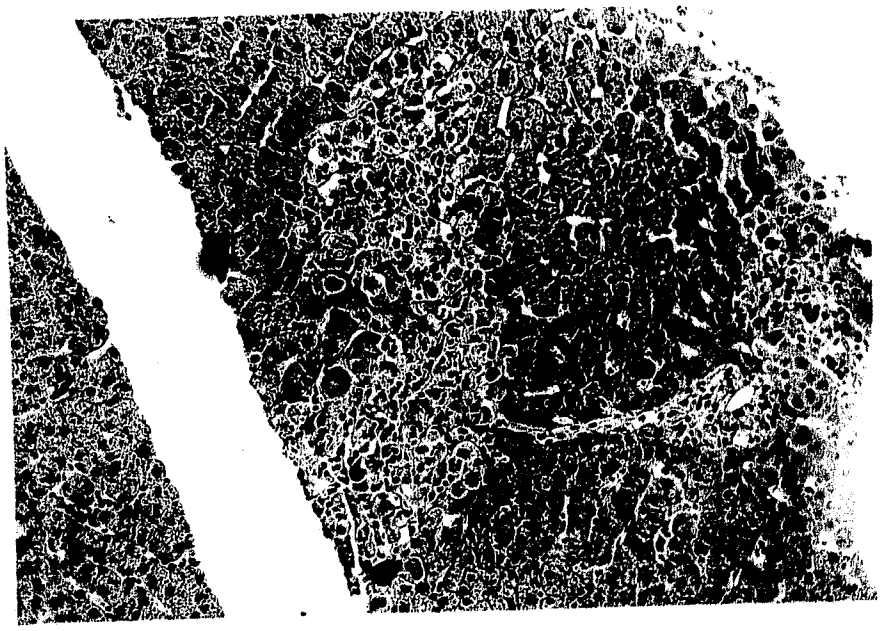

Figure 23. Abnormal biopsy - score 4 for inflammation with piecemeal and early bridging necrosis (H&E; magnification X150)

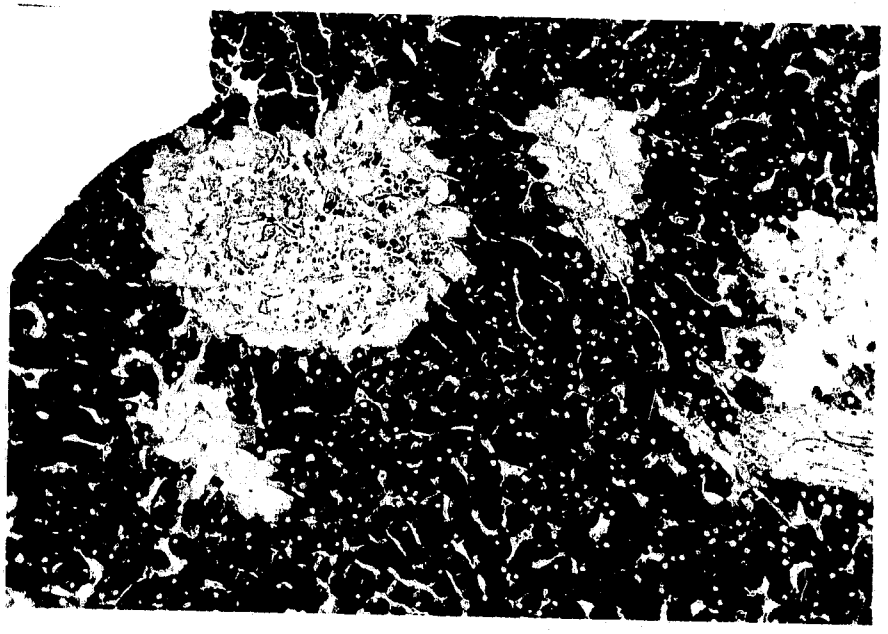

Figure 24. Abnormal biopsy - score 4 inflammation and necrosis; note the glycogen depleted periportal hepatocytes which correlate with acidophilic and degenerate ones seen with H&E stain (PAS; magnification X150)

Dextrose versus NAC was highly significant ($p = 0.0001$) with dextrose worse and NAC improved.

Due to wide variance within groups, the Mann-Whitney test was performed in order to evaluate for significant differences of the median values of the post biopsy values (as compared to the means as used in ANOVA) as a non-parametric test. Utilizing the median values would reduce the effect of a few outliers of data. Results of this series of two sample comparative tests rejected α as $> .05$ for comparison of saline versus dextrose and saline versus NAC. However, α was < 0.0025 for dextrose versus NAC and hence significant (Minitab 7.0).

Spearman rank correlation results are suggestive of a strong positive correlation between inflammation and necrosis scores ($r = 0.94$). Comparisons of inflammation scores between groups, by ANOVA, were similar to those for necrosis scores ($p = 0.002$). Inflammation, inflammation + necrosis, and inflammation + necrosis + sum ALT were also examined but found to be of no additional benefit.

Statistical analysis of focal aggregates and biliary ductule hyperplasia was not significant by ANOVA between and within groups (pre and post treatment) for biopsies of all groups. Therefore, these criteria were not considered further in the biopsy scoring system.

4.3.2.3 Comparison of biopsy and ALT results

In comparing the mean difference between groups I and III, with a Bonferroni test, a significant cytoprotective effect of NAC was demonstrated with biopsy scores ($p = 0.036$). But a p value of 0.06 was found for the ALT values between these two groups at 72 hours and as a sum ALT of all days. The use of Newman-Keuls tests for post biopsy data (less stringent than Bonferroni tests) revealed that the majority of significant difference for ALT and post biopsy scores ($p < 0.01$ as found by ANOVA), resided between groups II (dextrose was worse) and III (NAC was improved) (Table X). It did not indicate that dextrose worsened the effect of T on hepatocellular pathology except for the improved p value between I and III ($p < 0.05$) and between II and III ($p < 0.01$) (PCS).

Spearman rank correlation revealed positive correlation between post biopsy scores and sum ALT enzyme levels for individual dogs ($r = .694$) when not examined by group. Two sample rank correlation tests, linear regression analysis, and repeated measures analysis for individual days between groups and within groups failed to reveal a correlation or association between one chemistry or hematologic value versus any other test value or biopsy, e.g., one test could not be used as a correlative or predictor of another.

TABLE X

STATISTICAL SUMMARY OF IN VIVO BIOPSY SCORE AND ALT LEVEL RESULTS FOR GROUPS I, II, AND III

Group	Post Bx a	St.Dev.	Confidence Value	72 hr ALT
I Saline (n=9)	2.4	1.7	F = 5.8 ANOVA (99% c.i.) (I, II, III)	1056
II Dextrose (n=6)	3.5	0.5	p < 0.06 (Bonferroni) (I vs. II)	3871
III NAC (n=9)	0.9	0.8	p < 0.036 (Bonferroni) (I vs. III)	547

4.4 Discussion

4.4.1 Biopsy and ALT data

A number of features of the results indicate that T is likely a direct hepatotoxin. There was a predominance of periportal (acinar zone 1) hepatocellular damage. The only significant chemistry or hematologic parameter was an increase in serum ALT. This is an enzyme whose presence indicates cell membrane leakage. Thiacetarsamide in serum would first come in contact with, and be at its highest intrahepatic fluid concentration, where the hepatocytes are in closest proximity to the hepatic arterial flow (portal triad and acinar zone 1). At this location, its toxicity would not likely be significantly affected by mixed function oxidase activity or toxic intermediaries more commonly associated with acinar zone 3 (centrilobular - periacinar) toxic hepatopathies. These findings are suggestive of classification of T as an acute direct hepatocellular liver toxicant (117,172). This weakly implies that *in vivo* it is T, and not a metabolic transformation of T, that is responsible for the periportal hepatocellular injuries.

The results of the above study show a positive influence via sample biopsies for cytoprotection in group III (NAC) over group I (saline) and group II (dextrose). A negative effect is mildly suggested when comparing the data

for samples of group II against group I.

Overall, a moderately positive correlation for both the sum of ALT values for the four days and the 72 hour ALT is shown with the degree of post treatment hepatopathology in individual animals. But because of marked variations of some individuals, I do not believe this to be a strong enough correlation to clinically assess degree of hepatic damage based upon serum ALT. Stronger statistical significance for the ALT enzyme values may have been evident if censoring of the data for "outliers" had been performed (Table XI).

4.4.2 General interpretation of data

NAC was a successful cytoprotectant against the pathobiologic effects of T. This occurred at a clinically useful dosage of NAC and with the desired therapeutic level of T (3.3 mg/kg) as described in chapter one. Based upon these results and those of chapter three, exploration of additional dosages of NAC *in vivo* may prove even more beneficial.

TABLE XI

ALANINE AMINO TRANSFERASE (ALT) VALUES AT 72 HOURS

Group	Mean	Median	SEM
Saline (N = 9)	1056	360	453
Dextrose (N = 6)	3871	1970	1575
NAC (N = 9)	547	93	306

The alternative of per os administration for NAC would also be a reasonable course to explore, since this might provide higher concentrations of NAC due to direct portal vein delivery to the liver.

An unexpected finding was the potential for negative effect of dextrose upon the hepatic pathology induced by T. This might have resulted from enhanced substitution for phosphorus by arsenic. The presence of intravenous glucose would result in rapid increase in hepatocellular glucose dependent energy metabolism and glycogen production. As stated previously in chapter one, the actions of As on lipoic acid and disruption of an active cytochrome oxidase system could lead to failure of cellular respiration and release of injurious free radicals. The effect of administering a hyperosmolar solution may also have acted as an osmotic diuretic. This theoretically might lead to a reduced extracellular fluid volume and hence a higher concentration of T in that fluid. It may have been preferable to administer the dextrose earlier, e.g., 2 hours, in order to more effectively "preload" the hepatocytes with glycogen and thereby more closely parallel the hypothesis for cytoprotection by glycogen.

5. SUMMARY OF RESULTS AND CONCLUSIONS

5.1 Review of the hypotheses

The general hypothesis for this series of *in vitro* and *in vivo* experiments was presented in section 1.6 in which it was postulated that NAC would protect against the toxic effect of T. This hypothesis was formulated after presentation of background information concerning arsenic toxicology and the use of arsenical compounds in various cell cultures, particulary transformed cell lines (57,93,118). As well, investigations of NAC in cell culture systems and aspects of cytoprotection resulting from its use were reported (126,128). The toxic mechanisms of As include binding of -SH groups present in prominent enzyme and organelle systems (cytochrome oxidases, lipoic acid of pyruvate decarboxylase, actin cytoskeletal filaments, etc.) (35). Previous knowledge of dithiol compound cytoprotection (BAL) for arsenic toxicoses was reported (49). NAC is thought to act primarily as a competitive substrate and as a precursor for intracellular thiol (-SH) compounds, e.g., GSH (156,158). Therefore, it was logical to consider evaluating the cytotoxicity of thiacetarsamide and the cytoprotective value of NAC in hepatoma cells *in vitro*. The lack of an available transformed canine hepatoma cell line precluded investigation in a cell line of the same species, which

might have enhanced the correlation of *in vitro* and *in vivo* data. However, as previously discussed, there are reported similarities between canine and murine metabolism of arsenic.

The hypothesis that the monothiol substance, NAC, would be a cytoprotectant against the hepatotoxic effects of the thiol binding arsenical compound, thiacetarsamide, was proven *in vitro* and *in vivo*.

5.2 Summary of *in vitro* results

Development of *in vitro* models for cytotoxicity testing, prior to testing of toxic substances in live animals, is desirable because it allows for rapid screening of large numbers and various concentrations of compounds (129). This can achieve a reduction in the number of laboratory animals needed. In particular, *in vitro* models are valuable when new variants of drugs or compounds need to be evaluated (134,137). In these situations, a particular target organ of toxicity will likely be known and an appropriate cell line selected. The basic cytotoxicity of new compounds can also be assessed in non organ specific cell lines such as fibroblasts or HeLa cells (132,134,137).

The use of techniques more adapted to rapid quantification of cell viability can be utilized. Those

techniques most often cited include automated colorimetric plate readers for vital dyes (neutral red) or reactive products (nitrotetrazolium reactions), cellular enzyme release to media (lactate dehydrogenase, ALT), and synthetic activity such as protein production or uptake of radiolabeled thymidine (130,131,159).

The *in vitro* model of NCTC 1469 mouse hepatoma cell culture was successful. The studies were reproducible and the characteristics of a non-adherent dependent cell line made the experimental protocol easier. The dosage range for both NAC and T were determined and cytoprotection by NAC could be demonstrated. The results indicated that T probably remains intracellular and cells undergo a form of metabolic injury, since cell death continues after removal of T from the media. The results of the 24 hour period of post exposure incubation demonstrated this. It also suggests that at least some of the intracellular injuries remained reversible since incubation with NAC during that 24 hours was shown to be cytoprotective. NAC was a successful cytoprotectant, in this *in vitro* model, against the toxic effect of T.

5.3 Summary of *in vivo* results

The hepatic biopsy scoring system worked well. It clearly separated the pre and post treatment groups. The

system of *sequential* transabdominal percutaneous hepatic needle biopsies was also effective in this model of diffuse hepatocellular toxicity. It reduced the need for "terminal" experiments, allowing for a lower total number of animals used in research since these dogs went on to other studies.

The comparative connection between development of an *in vitro* model and the *in vivo* model is important to note (136). The *in vitro* cytoprotection was evaluated at varying dosages for both compounds, including the one selected for *in vivo* study. This was particularly important since the future proposed clinical use of T is at the experimental dosage of 3.3 mg/kg of body weight, a 50% greater dosage then is currently in clinical use.

NAC was a successful cytoprotectant *in vivo*. Evidence was found that NAC provided cytoprotection against some of the pathobiologic effects of T. This conclusion was based predominantly upon the biopsy results. The weaker significance of ALT serum levels between treatment groups was probably due to the complexity of potential hepatocellular injuries that As exerts. Hepatocellular injury is not necessarily an all or none response with regards to cell death. This results in greater variance. The release of ALT into serum is only an indicator of leakage of the enzyme across a functionally or physically damaged cell membrane. The serum value for ALT rises when hepatocytes are reversibly or irreversibly injured (88).

Therefore, ALT does not correlate with cell death but cell membrane injury. The stronger p value between the dextrose and NAC treated groups may indicate that T is more injurious to cells which are metabolically active (pyruvate decarboxylase, glucose-6-phosphatase, cytochrome oxidases), or in which hepatotrophic hormones have increased metabolic activity, e.g., insulin release in response to IV glucose stimulation.

Another problem is the variable response of hepatocytes to T between different individual animals. Every zone 1 of every hepatic acinus does not necessarily receive the same exposure to T. Hepatocellular metabolism, preexisting storage of GSH, renal elimination of T, and shunting of blood flow from one hepatic lobule to another via hepatic arterial flow alterations are all possible explanations for the variation between individuals (75,101). The exact reasons for individual variation in response to T are unknown. As discussed in Chapter One, 15% of dogs have a clinically relevant hepatotoxic event at 2.2 mg/kg. For this study which used limited numbers of animals and had expected high individual variation in response to T, it is fortunate that it was possible to obtain p values less than 0.05.

5.4 Future directions

5.4.1 Clinical use

The potential clinical application of NAC in the treatment of CHD with T, may lay in two directions. First, it may allow for the use of a more effective heartworm adulticidal dose of T (3.3 mg/kg). However, the mechanism by which T kills heartworms has not been reported. If its killing action is also dependent upon reversible binding to -SH groups, then use of NAC concurrently with T could result in protection of the adult heartworm. The *in vivo* studies were performed with a single concentration of NAC. Further studies could use the higher concentrations of NAC suggested by the *in vitro* results of Chapter Three. The magnitude of hepatocellular cytoprotection might be enhanced by these higher concentrations of NAC.

A second clinical application of results from this study could be the use of NAC following toxic hepatocellular reaction to the administration of T. When it occurs, clinically relevant hepatotoxicity is seen (vomiting, anorexia, icterus) within a few hours after the first or second dose of T to the dog. Based upon the *in vitro* results in chapter three, some of the hepatotoxic effects could be reversed by use of NAC within this clinical "post

exposure incubation" period. Again, further studies are needed to prove or disprove this.

5.4.2 Mechanisms of thiacetarsamide hepatocellular toxicity

Thiacetarsamide apparently continues to injure cells after it has been removed from the extracellular fluid. Investigation of the predominant, or combination of, metabolic injuries created by T could be explored *in vitro*. By use of agents such as cimetidine (blockage of Cytochrome P450), it would be possible to assess the role of metabolic transformation of T by hepatocellular enzymes. Use of blockers of methylation or addition of methyl donors may shed light upon mechanisms of hepatic metabolism of T. Use of calcium channel blockers, calcium depletion, and identification of intracellular calcium fluxes in cells exposed to T may help identify the mechanism of cell death. Glutathione depletion or measurement of intracellular levels of GSH *in vivo*, by pre and post treatment biopsies, would help establish the role of GSH as a factor for potential individual variation. As well, measurement of intracellular pre and post T exposure GSH levels might be used to explain the induction of tolerance to T which was discussed in Chapter One. Utilizing HPLC and radiolabelling, assays for the intermediary arsenical metabolites of T, both

extracellularly and intracellularly at different times, and the location of As within the cells could also be performed. This information might help establish the As moiety of T which is predominantly responsible for cell injury (35,51,52,74,97).

5.5 Conclusion

The development of *in vitro* and *in vivo* models for testing hepatocellular cytoprotection of NAC against the pathobiologic effects of thiacetarsamide was performed.

NCTC 1469 mouse hepatoma cell culture system successfully predicted cytoprotection by NAC against the effects of T. Selection of dosage for use *in vivo* could be established *in vitro*. NAC was a successful cytoprotectant *in vivo* against T.

The hepatopathologic scoring system worked well, as did use of sequential transabdominal hepatic needle biopsies. Reduction in the use of laboratory animals was achieved by use of *in vitro* testing followed by sequential needle biopsies during *in vivo* experiments.

APPENDIX A
Hepatic Biopsy Scores

Coded Biopsy Sample Number	Score	Uncoded Biopsy Animal Number	Treatment Group
1	A(M)1 random B 2 I(M)1 portal and periportal N 0 periportal	K11-1	3
2	A(N)2 zone 1 B 1 I(N)3 portal and periportal N 3 zone 1 and periportal	S19-2	2
3	A 0 B 1 I 0 N 0	U21-2	3
4	A 0 B 1 I(N)1 perivenular N 1 perivenular	A1-1	1
5	A 1 B 2 I(N)1 periportal/sinusoidal N 1 periportal	C3-1	1
6	A(N)1 periportal B 1 I 0 N 1 periportal	E5-2	1
7	A 0 B 0 I(M)3 periportal/portal N 4 periportal	N14-2	1
8	A(M)1 random B 1 I 0 N 0	L12-2	1
9	A 0 B 0 I 0 N 0	J10-1	1

10	A 0 B 2 I 0 N 0	F6-1	3
11	A 0 B 2 I(M)1 perivenular N 0	Q17-1	3
12	A 0 B 3 I(MN)3 periportal/septal/zone 1 N 4 periportal/bridging zone 1/ piecemeal	V22-2	2
13	A 0 B 2 I 0 N 0	R18-1	2
14	A(M)2 zone 3 B 2 I(M)2 periportal/portal N 3 periportal	Y25-2	2
15	A 0 B 2 I(M)2 perivenular and periportal/portal N 1 periportal	K11-2	3
16	A 0 B 0 I 0 N 0	X24-1	3
17	A 0 B 0 I(M)1 perivenular N 1 periportal	T20-1	1
18	A(N)2 random B 2 I(M)1 periportal/portal N 1 periportal/zone 1	X24-2	3
19	A 0 B 1 I(M)1 portal N 1 periportal	U21-1	3

20	A(M)1 random B 0 I 0 N 1 periportal HD	L12-1	1
21	A 0 B 2 I(M)1 portal N 0	I9-2	3
22	A(N)2 midzonal B 1 I(M)4 portal/periportal and perivenular N 4 periportal and piecemeal necrosis	A1-2	1
23	A(M)1 random B 1 I 0 N 0	W23-1	1
24	A 0 B 2 I 0 N 0	B2-2	3
25	A 0 B 1 I(MN)4 periportal/portal N 4 periportal/bridging and piecemeal necrosis	C3-2	1
26	A 0 B 1 I(MN)3 portal N 3 periportal/zone 1/piecemeal necrosis	O15-2	3
27	A(N)2 random B 1 I 0 N 0	G7-2	1
28	A(MN)3 random/zone 1 B 2 I(M)2 portal N 2 periportal and perivenular	F6-2	3
29	A(M)1 B 2 I 0 N 0	E5-1	1

30	A 0 B 1 I(M)1 portal N 0	O15-1	3
31	A(M)1 random B 3 I(M)3 portal/periportal/zone 1 N 3 periportal/zone 1/bridging necrosis	P16-2	2
32	A 0 B 3 I(M)1 perivenular N 1 periportal/perivenular HD and acidophilia	D4-2	3
33	A 0 B 0 I 0 N 0	G7-1	1
34	A(E)1 perisinusoidal B 2 I(M)1 portal N 0	P16-1	2
35	A 0 B 2 I(MN)3 portal/periportal N 4 periportal/piecemeal	J10-2	1
36	A(M)1 random B 3 I 0 N 0	D4-1	3
37	A 0 B 0 I 0 N 0	I9-1	3
38	A 0 B 2 I 0 N 0	N14-1	1

39	A 0 B 1 I 0 N 0	Q17-2	3
40	A 0 B 0 I 0 N 0	V22-1	2
41	A(M)1 random B 3 I(M)2 perivenular N 3 periportal piecemeal necrosis	T20-2	1
42	A(M)1 random B 1 I(MN)4 portal/periportal/zone 1 N 4 periportal, piecemeal, bridging, zone	R18-2	2 1
43	A 0 B 1 I(M)1 portal N 0	B2-1	3
44	A 0 B 1 I(M)1 portal and perivenular N 0	Y25-1	2
45	A(M)3 random B 1 I(MN)4 portal/periportal N 4 periportal, bridging, piecemeal, zone 1	H8-2	2
46	A 0 B 3 I(M)1 perivenular N 0	S19-1	2
47	A 0 B 2 I 0 N 2 periportal HD	W23-2	1
48	A 0 B 0 I(M)1 perivenular N 0	H8-1	2

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