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HEPATOTOXICOLOGICAL EVALUATION OF DANTROLENE SODIUM

THE UNIVERSITY OF ARIZONA

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HEPATOTOXICOLOGICAL EVALUATION OF DANTROLENE SODIUM

by

Janet Anne Durham

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A Thesis Submitted to the Faculty of the  
COMMITTEE ON TOXICOLOGY (GRADUATE)

In Partial Fulfillment of the Requirements  
For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

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## DEDICATION

To my parents, Norman and Jane Durham, for all the love, support and guidance they have provided me in all my endeavors.

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## ABSTRACT

The toxicity of D in Swiss-Webster mice and Sprague-Dawley rats was characterized. Animals were treated (po) with single or multiple doses of up to 400 mg/kg of D without any increases in SGPT or alterations in hepato-cellular architecture. To possibly enhance the hepatotoxicity of D, its biotransformation was altered by inhibiting acetylation, depleting glutathione, inducing biotransformation, and promoting reductive metabolism. None of the metabolic alterations elicited any toxicity of D. However, D was found to be readily absorbed, and available for biotransformation. Thus hepatic microsomal incubations were used to detect the possible bioactivation and covalent binding of  $^{14}\text{C}$ -D. Analysis for covalent adducts found only 20 pmol bound/mg protein. The suspected hepatotoxicity of D does not appear to be linked to its biotransformation or bioactivation. Additional studies will be necessary to clarify if other parameters are necessary for D to be a hepatotoxin.

## INTRODUCTION

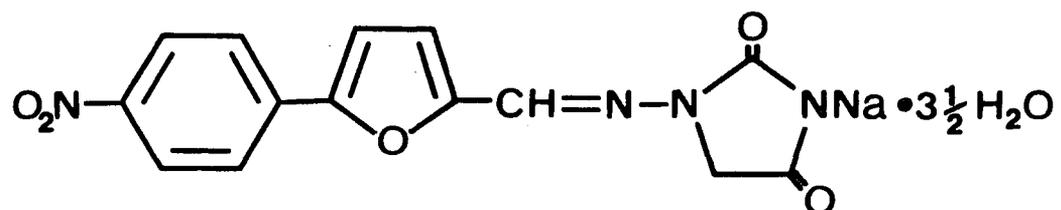
Dantrolene sodium, a hyantoin-furan derivative, was approved in 1974 for use in the management of skeletal muscle spasticity in patients with various neurological disorders (Snyder, Davis, Bickerton et al., 1967). In 1975, the Federal Drug Administration reported six deaths related to liver damage associated with this drug. Although excellent for the treatment of chronic spasticity since no central nervous system or respiratory depression occurs (Van Winkle, 1976; Ellis and Carpenter, 1974), sporadic incidences of overt hepatocellular injury and fulminant hepatic failure soon became associated with the chronic administration of dantrolene (Utili, Boitnott and Zimmerman, 1977). The incidence of hepatic injury during long-term dantrolene therapy is reported at 1.8% (Utili, Boitnott and Zimmerman, 1977), with symptomatic hepatitis in approximately 0.35-0.5% of patients (FDA, 1975), and fatalities in 0.2-0.3% of the cases (Utili, Boitnott and Zimmerman, 1977; Pinder, Brogden, Speight et al., 1977). However, transient elevations of SGOT are seen in about 10% of patients on dantrolene for 60 days or longer (Chyatte and Basmajian, 1973; Mayer, Mecomber and Herman, 1973). The risk of hepatic damage seems to be greater in females (Pinder Brogden, Speight et al., 1977; Utili, Boitnott and Zimmerman, 1977) and in patients over the age of 30 years (Utili, Boitnott and Zimmerman, 1977). All reported fatalities

occurred in patients after at least 8 weeks of therapy. A dosage of at least 300 mg/day was associated with a higher incidence of hepatotoxic reactions and a majority (85%) of the fatalities (Utili, Boitnott and Zimmerman, 1977). Unfortunately, it is not possible to even estimate the number of sub-clinical cases of mild liver damage which are probably much higher in incidence. Attempts to duplicate this dantrolene-associated hepatic injury and necrosis in laboratory animals has proven only minimally successful. Thus, the development of an animal model capable of reproducing the toxicity would lead to further understanding of dantrolene and its associated hepatotoxicity.

#### Physical, Chemical, and Pharmacological Characteristics

Dantrolene sodium (Dantrium, Norwich Eaton Pharmaceuticals, Inc.) is 1-((5-(p-nitrophenyl)-furfurylidene) amino) hydantoin sodium (Figure 1). It is a peripherally acting skeletal muscle relaxant indicated for use in spastic neurological disorders (Pinder, Brogden, Speight et al., 1977; Petusevsky, Faling, Rocklin et al., 1979; Utili, Boitnott and Zimmerman, 1977). There is also experimental evidence that dantrolene may be a therapeutic agent against malignant hyperthermia (Pandit, Kothary and Cohen, 1979; Meyler, Mols-Thurkow, Scaf et al., 1979).

The anhydrous salt has a molecular weight of 336, while the hydrated salt has a molecular weight of 399. It is slightly soluble



## Dantrolene Sodium

Figure 1. Chemical structure of dantrolene sodium (hydrated salt)

in water, however it readily hydrolyzes and precipitates the very insoluble free acid, dantrolene. Addition of dantrolene sodium to an alkaline solution helps increase slightly its solubility and decrease slightly its hydrolysis. Solubility data obtained from Norwich Eaton Pharmaceuticals report dantrolene sodium to be most soluble in polyethylene glycol 400 (80 mg/ml). Its solubility in chloroform or acetone is 20-25 mg/ml. Dantrolene, the free acid of dantrolene sodium, is a weak acid with a pKa of about 7.5 and exhibits extremely low solubility properties (1 mg/ml) in water.

#### Mechanism of Action

The site of action of dantrolene in the muscle has not yet been completely resolved. It is a peripherally acting agent that exerts its effect directly on skeletal muscle (Van Winkle, 1976), unlike other pharmacological agents which also produce central nervous system depression, motor incoordination, and respiratory depression (Ellis and Carpenter, 1974; Van Winkle, 1976). Numerous experimental studies have been performed in both mammalian and non-mammalian systems to determine the site of action of this drug. The consensus is that dantrolene does not act on the neuromuscular junction, the muscle sarcolemma, or the central nervous system (Van Winkle, 1976). It is also agreed upon that dantrolene exerts its action by dissociating the excitation-contraction coupling process necessary for muscle contraction. While researchers

still argue over which specific step or steps of the excitation-contraction coupling process dantrolene interrupts, they agree its effect is due to a decrease in calcium release from the sarcoplasmic reticulum (Morgan and Bryant, 1977; Putney and Bianca, 1974; Statham and Duncan, 1976; Hainaut, Brunko and Desmedt, 1975).

Although the exact mechanism of this drug's action on skeletal muscle is not known, the studies have limited its action to the skeletal muscle, except possibly at high doses. A dose-dependent decrease in cardiac muscle contractility (Meyler, Wesseling and Agoston, 1976; Bowman and Khan, 1977) and smooth muscle contractility (Graves, Dretchen and Kruger, 1977; Bowman and Khan, 1977) has been observed during in vitro studies. Even though these studies report decreases in contractility at concentrations higher than the therapeutic range, one must consider the possible effect on the heart if a patient with malignant hyperthermia were to be treated with high doses of dantrolene intravenously (Meyler, Wesseling and Agoston, 1976). Also to be considered is the interaction on the heart which may occur in patients concurrently being treated with drugs known to impair the activities of the sympathetic nervous system that interfere with calcium flux across cardiac cell membranes (Bowman and Khan, 1977).

#### General Hepatotoxicity

The reported 72 hr intraperitoneal LD<sub>50</sub> of dantrolene in mice is 1390 mg/kg (Ellis and Carpenter, 1974) and the oral LD<sub>50</sub> in rats is 1600

mg/kg (Francis and Hamrick, 1979). Animal studies have shown dantrolene to cause a dose-related inhibition of bile secretion and sulfobromophthalein excretion in the perfused liver (Abernathy, Utili, Zimmerman et al., 1978). A dose-dependent depression of the hepatic and adrenal cortex mixed function oxidase systems has also been reported (Francis and Hamrick, 1979, 1980). Chronic studies in pigeons by Silverman and Hikida (1977) have shown an initial body weight loss and discoloration and enlargement of the liver, which progresses to architectural changes and loss of some hepatic enzyme activities.

In vitro studies in nonpretreated rats have shown a dose-related inhibition of bile secretion and sulfobromophthalein (BSP) excretion upon addition of dantrolene to an isolated perfused rat liver system. The liver's ability to concentrate BSP in the bile also showed a dose-dependent decrease (Abernathy, Utili, Zimmerman et al., 1978). Pretreatment of rats with phenobarbital enhanced these adverse effects while 3-methylcholanthrene pretreatment had no effect on bile flow or excretion of BSP in the isolated perfused rat liver. A slight but significant increase in hepatic GOT release was seen only at the highest concentration of dantrolene studied. Studies performed using indocyanine green (ICG) as a marker for bile secretion also showed a significant decrease in bile flow, as well as in ICG excretion. ICG is a dye which does not require conjugation prior to excretion (Combes and Schenker, 1969), thus, the dantrolene effect does not seem to be medi-

ated by the inhibition of hepatic conjugation mechanisms (Abernathy, Utili, Zimmerman et al., 1978).

#### Effects on Hepatic MFO System

The effect of dantrolene on the hepatic mixed function oxidase (MFO) system was investigated by Francis and Hamrick (1979). Dantrolene was shown to prolong the pentobarbital sleeping time in rats. This prolongation was dose-related and was greater after five days treatment than after one day. A single dose of 100 mg/kg and accumulative doses up to 125 mg/kg (25 mg/kg for 5 days) decreased the rate of aminopyrine N-demethylase activity by 55% and 46%, respectively. No alteration in cytochrome P-450 content was found at these levels. Five days dosing at 100 mg/kg produced decreases in the rate of ethylmorphine N-demethylation and cytochrome P-450 content of 63% and 50%, respectively, while a single dose of 400 mg/kg resulted in a 53% decrease in cytochrome P-450 content. From these results, the authors concluded that dantrolene produces a dose-related inhibition of the MFO system.

Induction with phenobarbital and 3-methylcholanthrene resulted in an increase in cytochrome P-450 content in the presence and absence of dantrolene. Francis and Hamrick (1979) concluded that dantrolene does not block cytochrome P-450 synthesis since an increase in cytochrome P-450 content was detected. In 1980, Roy, Francis, Born et al. (1980) found phenobarbital pretreatment to decrease the ability of dantrolene to inhibit the mixed function oxide system.

All rats receiving 100 mg/kg for 5 days showed marked and consistent weight loss. No change was seen in the liver to body weight ratio and histological examination of the liver revealed no evidence of injury or necrosis (Francis and Hamrick, 1979).

#### Effects on Adrenal Cortex Function

The adverse effects of dantrolene on hepatic MFO activity have been reported (Francis and Hamrick, 1979; Roy, Francis, Burn et al., 1980). Due to its depressive effect upon the hepatic MFO system and drug metabolism, it was hypothesized that dantrolene would depress the adrenal cortex cytochrome P-450, thereby altering adrenocortical function (Francis and Hamrick, 1980). A dose-dependent decrease in serum glucocorticoids and serum glucose was reported in rats treated with dantrolene (25, 50, or 100 mg/kg for 5 days). A dose-related increase in the adrenal wet weight to body weight ratio was also seen. After three days recovery, the serum glucocorticoids and adrenal wet weight to body weight ratio were 80% and 85% of the control values, respectively.

Urinary 17-ketosteroid excretion was significantly reduced after three days of dosing with 100 mg/kg dantrolene, and reduced by 64% after five days of the regimen. The urinary level of 17-ketosteroids returned to control levels three days after terminating the dantrolene treatment (Francis and Hamrick, 1980). Histological analysis of the adrenal gland showed vacuolization and hyperemia at 25 mg/kg for 5 days, while cell

necrosis was evident at the higher doses. Five days after cessation of the high dose, vacuolization and hyperemia were still present. The increase in the adrenal wet weight to body weight ratio was attributed to hyperplasia of the adrenal cortex. The authors concluded that dantrolene, a hydantoin derivative, might act like diphenylhydantoin which has been shown to cause adrenal atrophy, adrenal cortical degenerative lesions, and a decrease in urinary 17-ketosteroid secretion.

#### Clinical Toxicity

In humans, dantrolene has been implicated as the causative agent in a variety of adverse responses including general gastrointestinal and neurological side effects (Pinder, Brogden, Speight et al., 1977; Joynt, 1976; Goodman, Beckman and Carlson, 1977), increased serum levels of hepatic enzymes (Utili, Boitnott and Zimmerman, 1977; Lundin, Uden and Hanson, 1977; Ogburn, Myers and Burdick, 1976; Wilkinson, Portmann and Williams, 1979), mild to severe hepatic necrosis (Lundin, Uden and Hanson, 1977; Wilkinson, Portmann and Williams, 1979; Donegan, Donegan and Cohen, 1978), and death (Utili, Boitnott and Zimmerman, 1977; Goodman, Beckman and Carlson, 1977). It has also been implicated in the development of lymphoproliferative diseases (Wan and Tucker, 1980) and pleuropericardial reactions (Petusevsky, Faling, Rocklin et al., 1979).

The incidence of general side effects such as malaise and fatigue has been reported by Joynt (1976) to be as high as 92%, with

drowsiness and dizziness occurring in 30% and 14% of the patients, respectively (Dykes, 1975). These general effects are usually seen within the first week of therapy and then either subside or the patient adapts to coping with them (Joynt, 1976). Nausea and vomiting have been the most common gastrointestinal reactions reported, occurring in 9% of the patients, with diarrhea occurring in 2.5% of the patients. Other minor side effects reported include photosensitivity, skin rashes, and tachycardia. In a study by Joynt (1976), 14 of 18 patients withdrew due to adverse side effects; however, Dykes (1975) reports this withdrawal rate as low as 2.5%.

Dantrolene is known to cause minor abnormalities of hepatic function, which usually arise after the patient has been on dantrolene therapy for at least 8 weeks and on a dose no less than 200 mg/day (Utili, Boitnott and Zimmerman, 1977). Transient elevations of liver enzymes, specifically serum glutamic oxaloacetic transaminase (SGOT) is seen in about 10% of the patients on chronic dantrolene therapy (Utili, Boitnott and Zimmerman, 1977; Wilkinson, Portmann and Williams, 1979; Ogburn, Myers and Burdick, 1976). The elevation in SGOT can increase to over 1400 Wroblewski-LaDue units (W-L units), however, it is usually between 100 and 600 W-L units (normal < 40 W-L units). Other hepatic enzymes reported to be increased include alkaline phosphatase, glutamic pyruvic transaminase, and bilirubin (Chyatte and Basmajian, 1973; Gelenberg and Poskanzer, 1973).

Histological examination of liver biopsy specimens from patients exhibiting dantrolene-associated hepatic dysfunction produced a wide variety of architectural abnormalities ranging from mild focal non-specific inflammation and edema to massive necrosis and complete loss of lobular pattern. The inflammatory infiltrate was predominantly found in the portal triads and consisted of lymphocytes and/or granulocytes with a few eosinophils (Schneider and Mitchell, 1976; Donegan, Donegan and Cohen, 1978; Ogburn, Myers and Burdick, 1976). Edema was also seen primarily in the portal areas (Schneider and Mitchell, 1976; Wilkinson, Portmann and Williams, 1979). Bridging necrosis (Lundin, Uden and Hanson, 1977; Wilkinson, Portmann and Williams, 1979) and piecemeal necrosis with fibrotic invasion of the portal areas (Utili, Boitnott and Zimmerman, 1977) have been reported. A few of the lesions were of an acute nature and showed massive necrosis, often centrilobular, with extremely elevated liver enzymes (SGOT > 1000 W-L units). However, the majority of the lesions appeared to be subacute or chronic (Utili, Boitnott and Zimmerman, 1977; Wilkinson, Portmann and Williams, 1979) showing many characteristics of chronic active hepatitis.

#### Temporal Relationship of Toxicity

A temporal relationship and clinical course consistent with hepatic injury secondary to dantrolene has been proposed, i.e., a drug-induced idiosyncratic reaction, but a definite cause and effect rela-

tionship cannot be established without rechallenge. Many patients have been rechallenged (Ogburn, Myers and Burdick, 1976; Goodman, Beckman and Carlson, 1977; Utili, Boitnott and Zimmerman, 1977), and in all cases, the symptoms of hepatic injury returned. Some of the patients had to be removed from the drug, and one died (Goodman, Beckman and Carlson, 1977).

Nitrofurantoin, an agent used extensively in chronic urinary tract infections, and diphenylhydantoin, an anti-convulsive agent, are rarely incriminated as hepatotoxic agents. However, both have been implicated as the causative agent in reactions assumed to be idiosyncratic (Hatoff, Cohen, Schweigert et al., 1979; Harinasuta and Zimmerman, 1968). If one compares the structures of dantrolene, nitrofurantoin, and diphenylhydantoin, numerous similarities are seen, especially between dantrolene and nitrofurantoin. Both of these two compounds contain a hydantoin and furan ring. Another common aspect is in their metabolic pathways. Both undergo an initial reduction followed by acetylation of their nitro group. Hydroxylation of the hydantoin ring appears to be restricted to dantrolene.

Documented exacerbations of chronic liver disease due to nitrofurantoin include a spectrum of symptoms very similar to those seen with dantrolene. Hatoff, Cohen, Schweigert et al. (1979) report on a female who developed chronic active hepatitis after exposure to nitrofurantoin. Symptoms seen included an increase in transaminase levels,

which fluctuated as the patient was removed or rechallenged with the drug. No morphological abnormalities were found upon examination of the biopsy specimens. Diphenylhydantoin has been implicated in very few cases of hepatic injury, however Harinasuta and Zimmerman (1968) report a case of diphenylhydantoin drug-induced hepatitis. The clinical, biochemical, and histologic features were consistent with mixed hepatocellular type of hepatic injury due to drug hypersensitivity. These included increased transaminase levels, increased alkaline phosphatase levels, and diffuse parenchymal injury with areas of necrosis and infiltration of eosinophils into parenchymal and portal areas. Upon rechallenge, the liver functions remained normal, but blood studies revealed a rise in the number of eosinophils.

Both the cases mentioned above required an interval of at least five weeks between beginning administration of the drug and recognition of hepatic injury. This is close to the one- to four-week latent period observed with sulfonamides and many other drugs. This latent period and the above clinical symptoms provide additional support that the hepatic injury seen with dantrolene could possibly be a drug-induced idiosyncratic hypersensitivity reaction.

#### Absorption, Distribution, Excretion

There has been very little work published concerning the pharmacokinetic behavior of dantrolene. Results of studies in healthy sub-

jects and in patients with spasticity indicate that dantrolene is slowly and incompletely absorbed after oral administration. The work of Dykes (1975) suggests that only about 20% of an oral dose is absorbed, with the small intestine being the predominant site of absorption. Peak plasma levels are seen 3 - 6 hr after ingestion of an oral dose (Monster, Herman, Meeks, et al., 1973; Lietman, Haslam and Walcher, 1974). A definite dose-response relationship between muscle relaxation and plasma levels of dantrolene has been shown in both normal volunteers and in patients with chronic spasticity (Herman, Mayer and Mecomber, 1972; Monster, Herman, Meeks et al., 1973). Steady-state plasma levels can be maintained by administering the drug in divided doses.

The mean elimination half-life is reported as 8.7 hr in normal adults (Dykes, 1975) and 7.3 hr in children with chronic spasticity (Lietman, Haslam and Walcher, 1974). Elimination of dantrolene and its metabolites is principally through the urine and bile. Approximately 20-25% of an oral dose is excreted in the urine (Dykes, 1975), principally as 5-hydroxydantrolene (79%) and the acetylamino derivative (17%). Only 1 - 4% is excreted as unchanged dantrolene (Lietman, Haslam and Walcher, 1974). The acetylamino derivative has not been found in plasma, only dantrolene and its 5-hydroxy metabolite (Lietman, Haslam and Walcher, 1974).

#### Metabolism and Covalent Binding

The metabolism of dantrolene has been shown to proceed through both oxidative and reductive pathways, the former being the principal

route (Cox, Heotis, Polin et al., 1969; Hollifield and Conklin, 1973) (Figure 2). Oxidation of dantrolene (pathway A) results in the 5-hydroxylation of the hydantoin moiety producing a compound which has skeletal muscle relaxation activity (Dykes, 1975). Reduction of the nitro group (pathway B) to an amine produces aminodantrolene which, in some animals and man, is further acetylated (pathway C) to give acetyldantrolene or acetylaminodantrolene (Cox, Heotis, Polin, et al., 1969; Hollifield and Conklin, 1973). Dykes (1975) reports the identification of four dantrolene metabolites in human blood and urine. This fourth compound could be what had previously been referred to as "Metabolite A", an intermediate form in the oxidative pathway which spontaneously degrades to "Compound B" or 5-hydroxydantrolene (Cox, Heotis, Polin, et al., 1969).

The work of Ellis and Wessels (1978) compared the potential skeletal muscle relaxant properties of the identified metabolites with that of dantrolene under in vitro and in vivo conditions. The metabolite 5-hydroxydantrolene was the only metabolite to inhibit muscle contraction. Dykes (1975) reported 5-hydroxydantrolene to be ten times less potent than dantrolene.

An important result of drug metabolism studies in recent years has been the realization that many foreign compounds are metabolized by the liver to potent alkylating or arylating agents, which are then capable of binding to cellular macromolecules. Studies by Francis and

## Biotransformation of Dantrolene

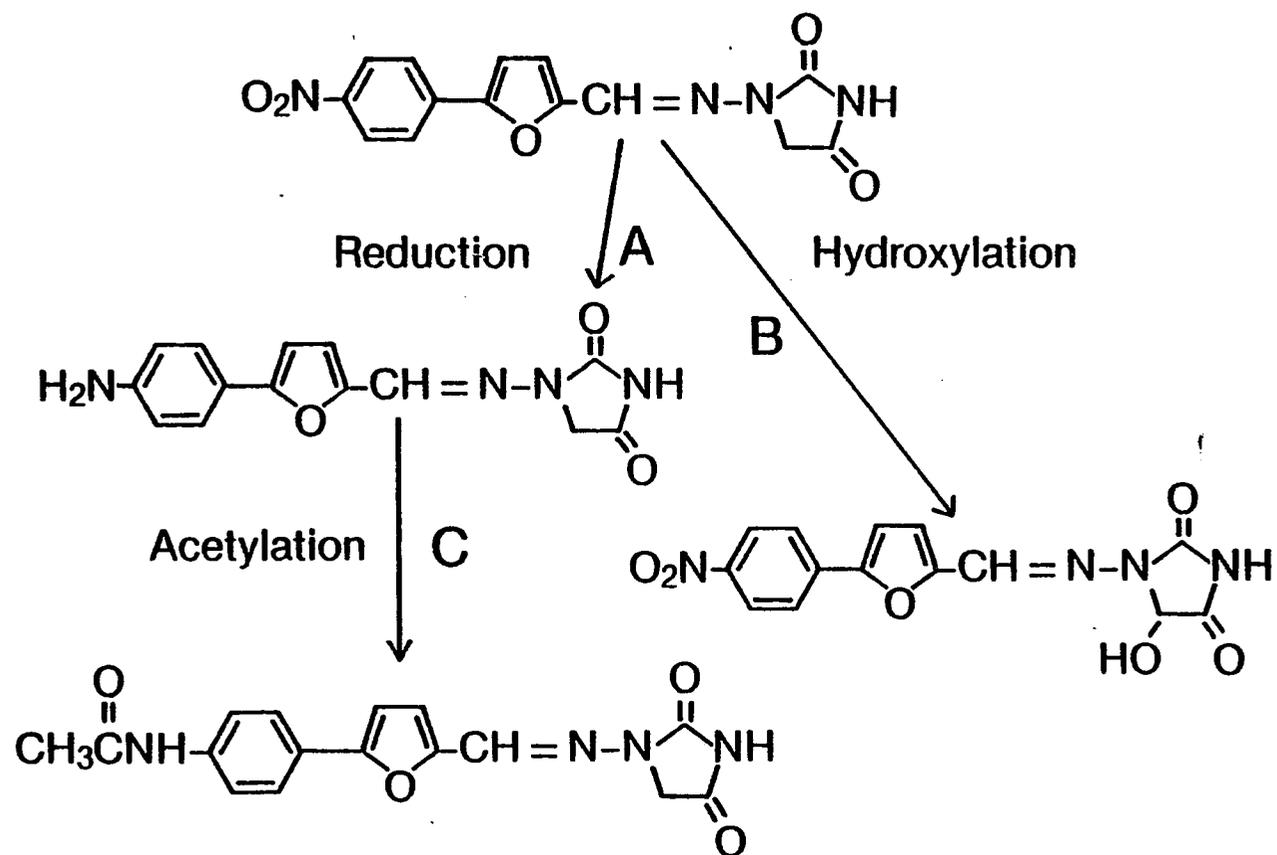


Figure 2. Proposed scheme of dantrolene biotransformation

Hamrick (1979) indicated that dantrolene treatment results in an alteration of the mixed function oxidase system and a decrease in hepatic cytochrome P-450. Further studies (Arnold, Epps, Cook et al., 1983) report the ability of aminodantrolene to inhibit the hepatic MFO system to a greater extent than the other metabolites. Aminodantrolene was also found to decrease hepatic cytochrome P-450 levels. Due to these results, binding studies of  $^{14}\text{C}$ -dantrolene to hepatic microsomal and cytosolic fraction were carried out to gain further information on the biotransformation of dantrolene and potential binding to hepatic proteins.

In vivo studies utilizing  $^{14}\text{C}$ -dantrolene (10  $\mu\text{C}/\text{mg}$ ) investigated the covalent binding of dantrolene to hepatic proteins in nonpretreated, diethylmaleate- and phenobarbital-pretreated rats. Eighteen hours after an intravenous injection of 1 mg/kg  $^{14}\text{C}$ -dantrolene, all rats were terminated. Examination of hepatic binding showed approximately equal amounts of radiolabeled dantrolene bound to microsomal (1.0 nmoles/mg protein) and 78,000 x g supernatant proteins (0.9 nmoles/mg protein). Pretreatment with diethylmaleate resulted in a significant increase in binding (3.9 nmoles/mg protein), while phenobarbital pretreatment produced a significant decrease in hepatic binding (0.6 nmoles/mg protein).

Concentrations of  $^{14}\text{C}$ -dantrolene ranging from 0.1 - 0.5 mM were used to study its in vitro binding to hepatic proteins. Results showed that binding of dantrolene was not dependent upon the presence of a

NADPH generating system. Binding was, however, dependent upon the concentration of dantrolene. The increase in binding observed with glutathione depletion (diethylmaleate pretreatment) would lead to the assumption that dantrolene or one of its metabolites may be acting as an electrophilic agent, thus forming a complex with hepatic proteins. However, phenobarbital pretreatment resulted in a decrease in binding, suggesting the parent compound may be the reactive agent. This idea is substantiated by the observed inhibition of the hepatic MFO system by dantrolene after phenobarbital pretreatment. Further support comes from the in vitro studies which showed equivalent binding of dantrolene in the presence or absence of a NADPH generating system. Thus, hepatic MFO activity does not seem to be required for the in vitro binding to cellular proteins.

#### Statement of the Problem

Reports from many sources propose a dantrolene-associated hepatic injury. This drug-induced hepatotoxicity is not unique to dantrolene. Nitrofurantoin, although usually associated with lung toxicity, has been implicated as a causative agent in liver toxicity. Metabolism studies of nitrofurantoin have indicated it is biotransformed to a reactive metabolite, probably a hydroxylamine derivative, which then binds to hepatic proteins leading to liver damage (McCalla, Reuvers and Kaiser, 1971). Due to structural similarities, it is possible to assume

that dantrolene may undergo this same type of biotransformation. Based on the premise that altering the biotransformation of dantrolene will produce reactive metabolites capable of binding to hepatic proteins, this study will attempt to develop an animal model capable of reproducing the hepatotoxicity seen in humans.

## MATERIALS AND METHODS

### Chemicals

Dantrolene sodium (D) and  $^{14}\text{C}$ -dantrolene sodium ( $^{14}\text{C-D}$ )(14 uC/mg) were generous gifts from Norwich Eaton Pharmaceuticals, Inc., Norwich, NY. Radiochemical purity, as determined by HPLC analysis, was 99%.

Para-chloroaniline (PCA) was purchased from Aldrich Chemical Company, Inc., Milwaukee, WI. Sulfamethazine (SMZ), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, bovine serum albumin, triethanolamine, glutathione (reduced form-GSH), and B-nicotinamide adenine dinucleotide phosphate (B-NADP) were all obtained from Sigma Chemical Company, St. Louis, MO. Tris(hydroxymethyl)aminomethane (THAM) was purchased from Fisher Scientific Company, Fair Lawn, NJ. Phenobarbital sodium (PB) was obtained from Mallinckrodt, St. Louis, MO. Beta-Phase was obtained from West Chem, San Diego, CA. Methanol and acetonitrile (Chromatography grade) were purchased from Burdick and Jackson Laboratories, Inc., Muskegon, MI. Ethanol, 200 proof, anhydrous was obtained from U.S. Industrial Chemicals Co., Anaheim, CA.

Distilled, deionized water was used to prepare all buffers and to dilute all acids and bases to the desired concentrations.

### Animals

Male, Swiss Webster mice (CD-1) weighing 25-40 g were obtained from Charles River Laboratories, Boston, MA. All mice were housed 5

to 6 animals per stainless steel cage, which contained pine shavings as bedding.

Male, Sprague-Dawley rats weighing 300-350 g were purchased from Hilltop Laboratories, Chatsworth, CA. They were housed 2 per stainless steel wire cage.

Wayne Lab Blox were fed to the mice and rats. Food and tap water was available ad libitum unless otherwise stated. Lighting was controlled to provide a 12 hr light-dark cycle; room humidity and temperature were set and maintained at 55% and 22<sup>0</sup>, respectively. After arrival all animals were allowed at least 5 days for environmental adaptation before experiments were undertaken.

#### Dosing, Blood collection, Histology

Dantrolene was suspended in corn oil and dosed per os, by gavage. Mice involved in acute toxicological studies employing a single dose concentration of dantrolene received a per os dose of 400 mg/kg. This dose was chosen based on the results of Francis and Hamrick (1979) who reported a single dose of 400 mg/kg to be the lowest dose to cause a decrease in hepatic cytochrome P-450 content. Carbon dioxide asphyxiation was the method of termination for all animals. After termination, blood was immediately withdrawn from the inferior vena cava into a heparin-coated syringe and transferred to a plastic tube. Serum was obtained by spinning the blood for 10 min at 3000 rpm then drawing off the top layer. The serum samples were stored at 4<sup>0</sup>C for a maximum of 48 hr

and analyzed for serum glutamic pyruvic transaminase activity. The liver was removed, weighed and a small section cut out from the right median lobe which was immediately placed in 10% buffered formalin for at least 48 hr. All histological processing was done by the Department of Surgical Biology, University of Arizona. The sections were 7 microns thick, slide mounted, stained with hematoxylin and eosin (H and E) and assessed for hepatic damage.

Evaluation of hepatic damage in each histological slide was carried out by examining five randomly chosen fields for cellular necrosis. The average value was reported as the percent necrosis.

#### Subcellular Isolation

Microsomes were prepared from male mice by the method of Gandolfi, White, Sipes, et al., (1980). Immediately after termination, the liver was perfused with ice cold 0.05 M Tris-1.15% KCl, pH 7.4. The liver was then excised and homogenized in a Kontes homogenizer in 3 volumes of the Tris-KCl buffer. The homogenate was centrifuged for 10 min at 3000 rpm (1100 x g), 10 min at 10,000 rpm (12,000 x g), and 10 min at 15,000 rpm (28,000 x g) in an SS-34 rotor, Sorvall Superspeed RC2-B, DuPont Company, Newtown, CT. The supernatant was poured through glass wool to remove visible liquid residues and the pellet was discarded. The supernatant was then centrifuged at 50,000 rpm (162,000 x g) for 40 min at 4°C in a TI-50.2 fixed angle rotor on a Beckman L8-55 Ultra-centrifuge, Beckman Instruments, Palo Alto, CA., to sediment the microsomal membranes. The microsomal pellet was resuspended in ice

cold 0.5 M Tris-HCl buffer and centrifuged again at 50,000 rpm for 40 min at 4°C. The resulting pellet containing washed microsomes was re-suspended in Tris-HCl buffer, and stored in 2 ml aliquots at -80°C until needed. Cytochrome P-450 content was determined by the method of Omura and Sato (1964).

### Assays

#### Protein

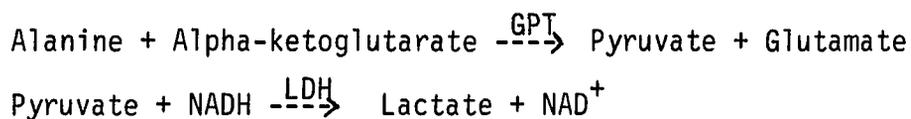
Protein concentrations of liver microsomal fractions were determined using the biuret method (Gornall, Bardawill and David, 1949). An aliquot (100-200  $\mu$ l) of the appropriate fraction was brought to a final volume of 0.5 ml with 0.05 M Tris-HCl buffer. Biuret reagent (2.5 ml) was added to each tube. The tubes were vortexed and allowed to sit at room temperature for 30 min. An absorbance reading of the sample was taken at 540 nm on a Gilford 300 Spectrophotometer. Absorbance values were compared with those of bovine serum albumin standards to obtain concentrations for samples in mg/ml. A color blank comprised of 0.5 ml buffer plus 2.5 ml biuret reagent was subtracted from each value prior to quantification.

Protein concentration of the exhaustively washed microsomal pellet dissolved in 1.0 N NaOH was determined using the Lowry method (Lowry, Rosenbrough, Farr et al., 1951). An aliquot (250  $\mu$ l) of the dissolved protein solution was diluted to a final volume of 0.5 ml with 1.0 N NaOH. Five ml of Lowry reagent was added and after 30 min, Folin-Ciocalteu Phenol reagent (0.5 ml) was added to each tube. The tubes

were vortexed and allowed to stand at room temperature for 30 min. An absorbance of the sample was taken at 660 nm on a Gilford 300 Spectrophotometer. Samples were quantified in mg/ml by comparing absorbance readings with those of bovine serum albumin standards.

### SGPT Activity

Serum glutamic pyruvic transaminase (SGPT) activity was determined on serum samples by the method of Wroblewski and LaDue (1956) as described in Sigma Technical Bulletin No. 55-UV. The reaction sequence is:



The overall reaction is measured by the rate of decrease in absorbance of NADH at 340 nm. When GPT activity is the limiting factor, the rate of decrease in absorbance becomes a measure of GPT activity.

### In Vitro Covalent Binding of $^{14}\text{C}$ -Dantrolene

The in vitro metabolism of  $^{14}\text{C}$ -dantrolene to reactive intermediates capable of covalently binding to cellular macromolecules was assessed using techniques described by Gandolfi, MacDonald and Sipes (1979). All incubations were performed in 2 ml total incubation volumes in 12 ml glass screw cap incubation tubes. The reaction mixture contained: 1 mg/ml microsomal protein, 0.1-0.5 mM  $^{14}\text{C}$ -dantrolene (14 uC/mg), and a NADPH generating system (15 mg NADP, 30 mg glucose-6-phosphate, 50 u1 0.1 M  $\text{MgCl}_2$ , and 100 u1 glucose-6-phosphate dehydrogenase (100 units/ml) in 1.0 ml distilled water.  $^{14}\text{C}$ -dantrolene in a methanol

carrier was added to the appropriate tubes. The methanol was evaporated off under  $N_2$  gas. Tris-HCl (0.05 M, pH 7.4) buffer was then added and the  $^{14}C$ -dantrolene was resuspended by vortexing for 1 min. All other incubation components were added, and the tubes were flushed 3 times under pure  $N_2$ , while being kept on ice. Incubations were conducted at  $37^{\circ}C$  for 60 min in a Dubnoff Metabolic Shaking Incubator, Precision Scientific, Chicago, IL. All incubations were stopped by the addition of 8 ml ice cold 100% ethanol to precipitate the microsomal proteins and the isolation of the protein proceeded from this point. Control tubes contained all of the above components except for the NADPH generating system.

Following the addition of ethanol, the samples were centrifuged for 30 min in a Sorvall GLC-2B table top centrifuge at 3000 rpm. The supernatant was discarded, 4 ml of cold ethanol was added to the pellet and recentrifuged. This wash step was repeated 3 more times giving a total of 5 ethanol washes. This procedure was followed by sequential washes with 4 ml of chloroform:ethanol (1:3), 4 ml of cold 100% ethanol, 4 ml of cold 1% trichloroacetic acid, and 4 ml of methanol:ether (3:1). Centrifugation followed each of the above additions, with the resulting supernatant discarded each time. Following the methanol:ether wash, the samples evaporated to dryness overnight at room temperature. The resulting protein pellet was dissolved in 2 ml 1.0 N NaOH by incubating at  $37^{\circ}C$  for 3 hours. The dissolved protein was determined (Lowry, Rosenbrough, Farr et al., 1951) using

bovine serum albumin as the protein standard.  $^{14}\text{C}$ -radioactivity was determined by liquid scintillation counting. Covalent binding of  $^{14}\text{C}$ -dantrolene and/or its reactive intermediates to proteins was quantified as picomoles  $^{14}\text{C}$  label bound/mg protein.

#### Liquid Scintillation Counting

$^{14}\text{C}$ -radioactivity covalently bound to proteins was quantified using a Beckman 8100 liquid scintillation counter. Aliquots of protein dissolved in base were neutralized by an equal volume of 1.0 N perchloric acid to stop chemiluminescence, and then suspended in 15 ml Beta-Phase in a glass scintillation vial. A quench curve, which relates the extent of quenching in a sample to a counting efficiency, was prepared using  $^{14}\text{C}$ -toluene standards. The random coincidence monitoring feature of the Beckman ISC 8100 was employed to verify that recorded counts represented true disintegration events.

#### HPLC Assay for Dantrolene

Levels of dantrolene in serum from mice dosed with dantrolene (400 mg/kg po) were determined by high-performance liquid chromatography (HPLC) using a modification of a method reported by Hackett and Dusci (1979).

Analyses were performed on a Beckman, Model 110A high-performance liquid chromatograph equipped with a Gilson H M Holochrome variable wavelength detector integrated on a Hewlett-Packard 3380A Integrator. The column was an All-Tech 25 mm stainless steel packed with All-Tech  $\text{C}_{18}$  (10 microns). The mobile phase consisted of 40% acetonitrile and

60% distilled, deionized water containing 10 mM triethanolamine phosphate, and was filtered and degassed before use. The wavelength setting was 380 nm, and a flow rate of 2 ml/min produced an elution time of 5.1 min for dantrolene. The detection limit at this wavelength was 0.5 ug/ml.

One ml of acetonitrile was added to 100 ul of serum in a conical tube. Care was taken to ensure the production of two layers, as rapid addition may cause a lumpy precipitate leading to decreased recoveries (Hackett and Dusci, 1979). The mixture was vortexed at high speed for 1 min, centrifuged, and the acetonitrile then pipetted into a second tube. It was evaporated to dryness under N<sub>2</sub> gas in a 40<sup>o</sup> C waterbath. The residue was dissolved in 100 ul of mobile phase and 20 ul were injected onto the HPLC column.

A stock solution of 100 ug/ml was prepared by dissolving 10 mg of dantrolene in a very small amount of methanol and then bringing the solution to a final volume of 10 ml with plasma. Standards of 50, 25, 10, and 5 ug/ml were prepared by making serial dilutions from the stock solution. The standards were extracted following the procedure for the samples. Samples were quantified by comparing the sample peak area with those of the standard solutions. Sample concentrations were expressed as ug dantrolene/ml.

The efficiency of the method was determined by comparing the peak area of standard solutions prepared in mobile phase with those of

standard solutions which had been prepared in plasma and extracted. Recovery was found to be 85%.

#### Oral Pharmacokinetic Study in Mice

Mice were administered dantrolene, 400 mg/kg po, and then killed at 1, 2, 3, 4, 5, 6, 12, 17, 24, and 48 hr after dosing. Blood samples were taken from the inferior vena cava of each mouse. The blood samples were allowed to clot, and then centrifuged. The collected serum was stored at 4°C for 24 hr and then utilized in HPLC assay for the determination of dantrolene.

#### Toxicology Studies

##### Repeated Exposure to Dantrolene and Hypoxia

Reductive metabolism of some hepatoxins has been shown to potentiate their toxicity. Therefore, male mice were administered, by gavage, 150 mg/kg dantrolene every day between 8 and 10 AM for 3 weeks, and placed in a hypoxic atmosphere (12-14% O<sub>2</sub>) for 6 hr after each dose. Dantrolene was suspended in corn oil such that all animals received a constant volume to body weight ratio of 0.2 ml/40 g. Two control groups were included: vehicle and hypoxia, or vehicle and air only. After termination of the animals, blood was collected and a section of liver removed and placed in 10% buffered formalin.

##### Potentiation with Tri-iodothyronine

Tri-iodothyronine (T<sub>3</sub>) is known to potentiate the hepatotoxicity of carbon tetrachloride (Calvert and Brody, 1961) and halothane (Wood,

Berman, Harbison et al., 1980). Although the mechanism is not clear, it is thought to be related to an increase in oxygen utilization by hepatic cells. In an attempt to potentiate the hepatotoxicity of dantrolene, male mice were treated with 10 mg/kg sc  $T_3$  for 5 days. It was prepared in 5% Tween 80 in physiological saline (Table 1). Twenty-four hr after the last  $T_3$  dose, the mice were given dantrolene (50-400 mg/kg po). Twenty-four hr after dantrolene administration, the animals were terminated. Blood was collected for GPT determination, and a section of liver was removed and placed in phosphate buffered formalin (10%).

#### Sulfamethazine and p-Chloroaniline Pretreatment

Amino-dantrolene is rapidly acetylated, thus leaving very little time for the possibility of formation of a hydroxylamine from the amino-dantrolene. If acetylation is blocked, the chance of forming a hydroxylamine would be increased. Therefore, sulfamethazine (SMZ) and p-chloroaniline (PCA) were incorporated as acetylation competitors to dantrolene.

Mice received either a single intraperitoneal (ip) dose of para-chloroaniline (PCA) (100 gm/kg in corn oil) or were placed on PCA water (0.54%) for 3 days prior to dosing with dantrolene (400 mg/kg) (Table 1). Twenty-four hr after receiving dantrolene, all mice were killed. Blood was collected; the liver was removed, weighed, and a portion placed in 10% phosphate buffered formalin.

Mice were dosed with sulfamethazine (SMZ) (1.5-500 mg/kg ip) 30 min prior to receiving a single dose of dantrolene (400 mg/kg po) (Table 1). All animals receiving SMZ were dosed with it every 4 hr for the next 8 hr (3 dosing periods). All animals were killed 24 hr after the administration of dantrolene. Blood was collected for SGPT determination. The livers were removed, weighed, and a section placed in phosphate buffered formalin.

#### Glutathione Depletion

Previous studies with  $^{14}$ C-dantrolene showed that in vivo binding was increased in the presence of decreased glutathione levels (diethylmaleate pretreatment). However, preliminary studies during this project using diethylmaleate to deplete glutathione levels, revealed no adverse effects. Therefore, methyl iodide, a potent glutathione depletor (Johnson, 1965, 1966) was administered to mice in a single intraperitoneal injection (0.25-0.70 mmole/kg) (Table 1). Thirty min after receiving the methyl iodide, dantrolene (400 mg/kg po) was given. All mice were killed 24 hr later. Immediately after termination, blood was collected. The livers were removed, weighed, and a small section placed in phosphate buffered formalin (10%).

#### Phenobarbital Pretreatment

Previous studies with phenobarbital pretreatment produced results indicating dantrolene affects the hepatic mixed function oxidase system by decreasing levels of cytochrome P-450. Pretreatment with phenobarbital was studied in both mice and rats.

Mice were given a single ip injection (100 mg/kg) of phenobarbital, and placed on phenobarbital water (0.1%) for 5 days. They were then taken off phenobarbital water and placed on tap water for the remainder of the study. Twenty-four hr after being placed on tap water, the mice received, either once in 24 hr or 3 times in 12 hr, p-chloroaniline (100 mg/kg ip), given 30 min prior to each dantrolene dose (400 mg/kg).

Rats were subjected to the same induction regimen as explained above. They were then pretreated with sulfamethazine (SMZ) (75 mg/kg ip) and/or buthionine sulfoximine (0.38 mmole/kg ip) 30 min prior to receiving a single dose of dantrolene (400 mg/kg po). Half of these rats were exposed to a hypoxic atmosphere after receiving dantrolene. All animals were killed 24 hr after receiving dantrolene. Blood was collected and liver sections were preserved.

#### Multiple Dosing

Reports on human dantrolene therapy suggest that hepatic injury occurs more often after having taken the drug for several weeks on a daily basis. Thus, multiple doses of dantrolene (400 mg/kg po) were given in two different regimens. Mice received dantrolene either every 3 hr for 12 hr or every 6 hr for 48 hr, and were terminated either 12 hr or 6 hr after the last dose, respectively. Blood was drawn for GPT determination. The livers were removed, weighed, and a small section placed in buffered formalin.

### Statistics

Statistics were performed using one-way variance of analysis and Students' t-test (paired and unpaired) to determine whether the differences between two or more groups was significant. The groups were judged significant when a p value  $< 0.05$  was obtained.

Table 1: Summary of the pretreatment schedule for toxicological studies<sup>e</sup>.

	Dose	Dantrolene (mg/kg) <sup>a</sup>	Dosing Regimen <sup>d</sup>
Tri-iodothyronine (T <sub>3</sub> )	10 sc <sup>b</sup>	50-400	Five day pretreatment with T <sub>3</sub> , then single dose of D
Iodomethane (CH <sub>3</sub> I)	0.25-0.7 ip <sup>c</sup>	400	CH <sub>3</sub> I administered 20 min prior to single dose of D
Parachloroaniline (PCA)	100 ip <sup>b</sup>	400	PCA injected 30 min prior to single dose of D
Parachloroaniline Water	100 <sup>b,f</sup>	400	Three day exposure to PCA water before single dose of D
Sulfamethazine (SMZ)	1.5-500 ip <sup>b</sup>	400	SMZ dosed 30 min prior to single dose of D; SMZ dosed every 4 hr for next 8 hr (3 dosing periods)

a: Dantrolene (D) was suspended in corn oil and dosed per os.

b: mg/kg

c: mmole/kg

d: all animals were killed 24 hr after receiving dantrolene except in CCl<sub>4</sub> expt. when killed 24 hr after CCl<sub>4</sub> dose.

e: blood was drawn for SGPT determination and liver sections kept for histological assessment on all animals.

f: 100 mg/kg - 0.54% PCA water.

## RESULTS

### Oral Pharmacokinetic Study

Since the purpose of this project was to produce an animal model capable of reproducing the hepatotoxicity seen in humans, dantrolene was administered to all animals per os, by gavage. Earlier studies (Dykes, 1975) report oral absorption of dantrolene to be 20-30% of the dose. Therefore, a pharmacokinetic study was performed to insure drug absorption. From the graph (Figure 3), it can be seen that dantrolene is absorbed, thus any inability to produce overt toxicity cannot be attributed to lack of absorption.

### Toxicological Studies

#### Repeated Exposure to Dantrolene and Hypoxia

Mice were administered a daily dose of dantrolene (150 mg/kg po) for 3 weeks, and exposed to a hypoxic atmosphere (12-14% O<sub>2</sub>) for 6 hr after every dose in order to produce elevated levels of dantrolene in the liver, and to change the preferential pathway of dantrolene from oxidative to reductive.

This 3-week dosing regimen of dantrolene followed by hypoxia failed to produce any elevation in serum GPT values (Table 2) or liver histology as compared to the two control groups. There was no time-related (cumulative dose) toxicity seen in the dantrolene + hypoxia mice as measured by GPT levels (Figure 4); nor was there any loss in mean body weight (Figure 5).

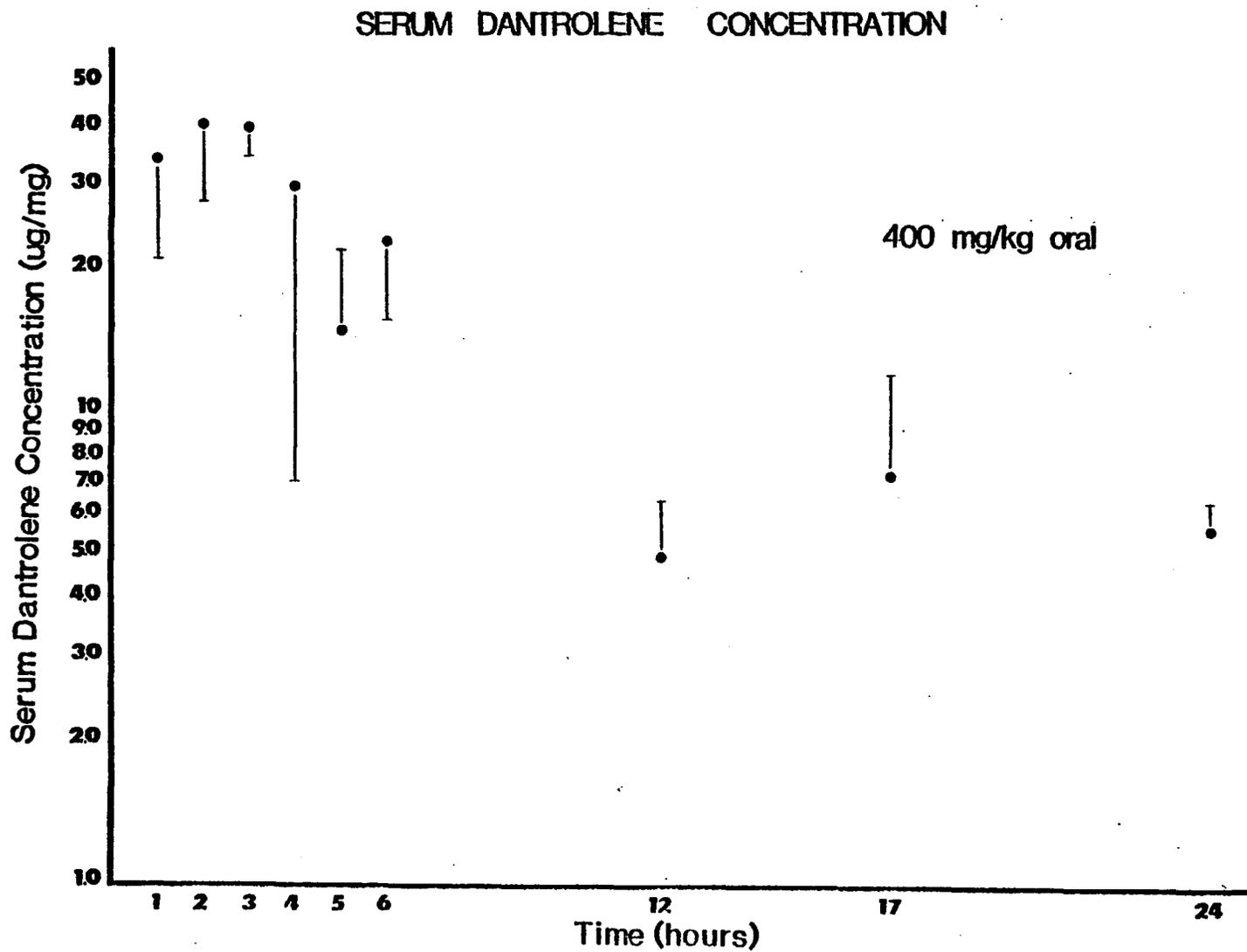


Figure 3. Time course of serum dantrolene concentration after a single oral dose of dantrolene (400 mg/kg) in mice

Table 2: Serum GPT levels after repeated exposure to dantrolene and hypoxia.

<u>Treatment</u> <sup>a</sup>	<u>SGPT</u> <sup>b</sup>	<u>N</u>
Dantrolene + Hypoxia	25 <sub>±</sub> 2	23
Control + Hypoxia	29 <sub>±</sub> 5	9
Control	26	2

a: Dantrolene: 400 gm/kg, po; every day for 21 days  
 Control: Corn Oil Vehicle, po; every day for 21 days  
 Hypoxia: 12-14% Oxygen Atmosphere, 6hr/day for 21 days

b: Mean <sub>±</sub> SEM (W-L Units/ml) 24 hr after Dantrolene dose.

No significant differences at  $p < 0.05$

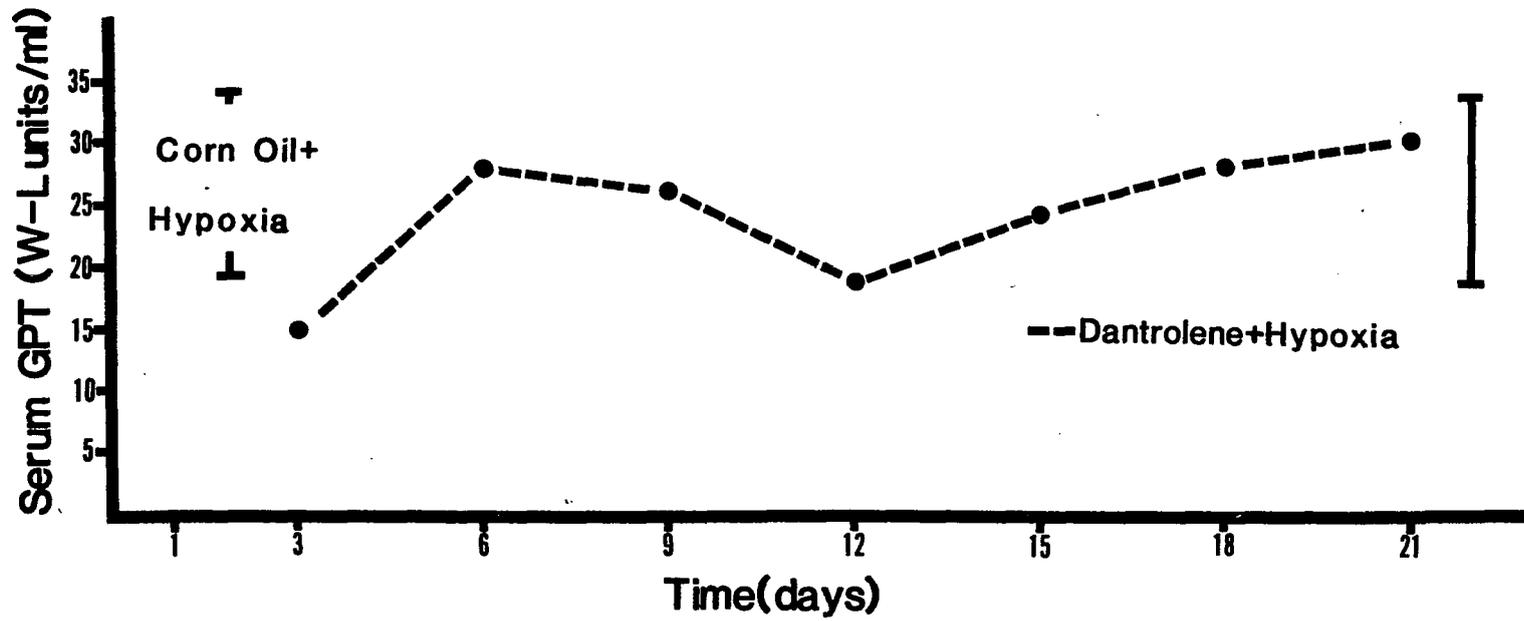


Figure 4. Time course of serum GPT over 21 days in dantrolene (150 mg/kg) and hypoxia (12-14% O<sub>2</sub>) exposed mice (----).

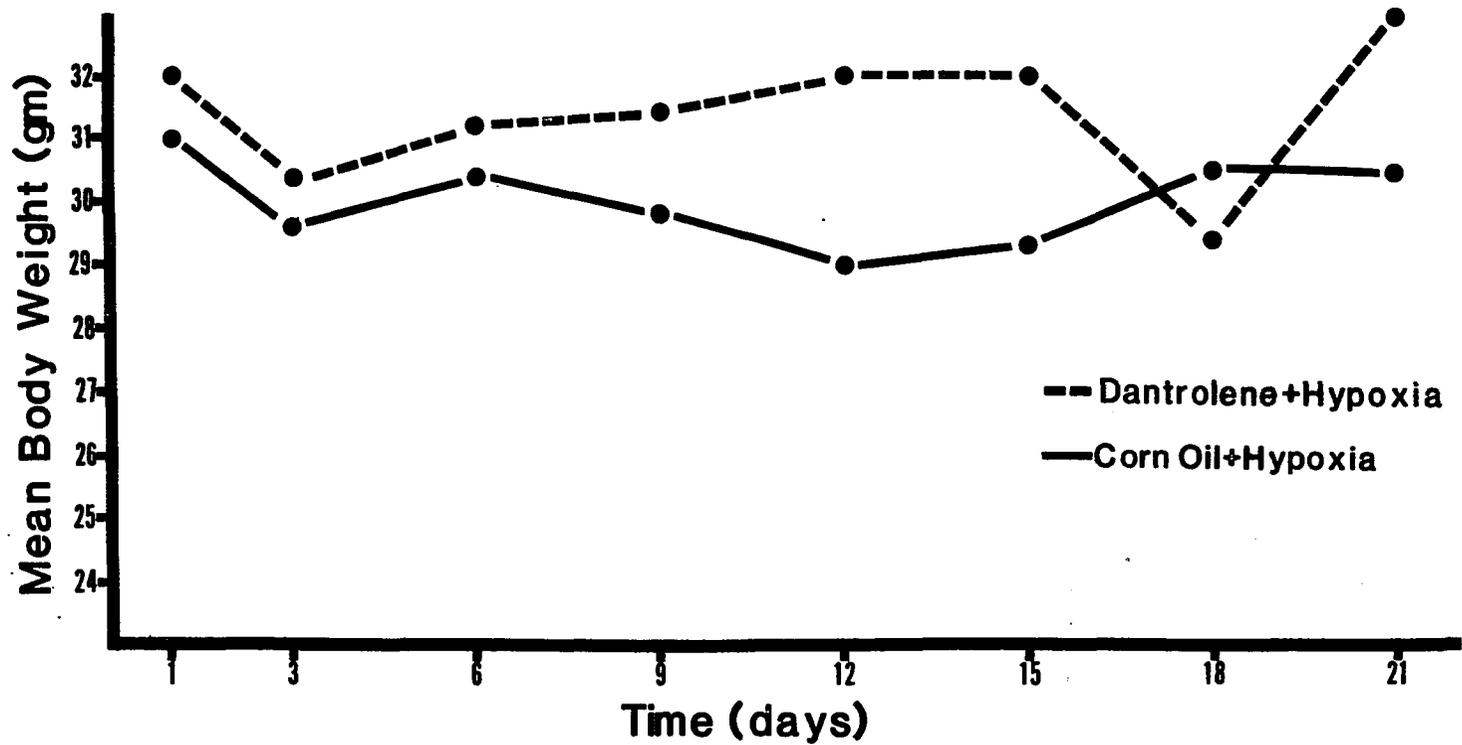


Figure 5. Time course of mean body weight over 21 days in dantrolene (150 mg/kg po) and hypoxia (12-14% O<sub>2</sub>) exposed mice (----) and corn oil vehicle and hypoxia (12-14% O<sub>2</sub>) exposed mice (----).

This is in contrast to the observation seen by Silverman and Hikida (1977) in studies with pigeons. They observed a 25% loss in body weight during the first 3 weeks of dantrolene administration. However, the pigeons were not concomitantly subjected to hypoxic conditions.

Histological evaluation of liver sections showed no evidence of necrosis or injury from either the dantrolene or hypoxia. No gross abnormalities were observed (Figures 6 and 7).

#### Potentialiation with Tri-iodothyronine

Tri-iodothyronine ( $T_3$ ) is known to potentiate other hepatotoxins and promote reductive metabolism. As dantrolene metabolism contains a reductive pathway, potentialiation of this pathway should lead to an increased production of amino-dantrolene, thus leading to a reactive intermediate and possible hepatotoxicity. Therefore, mice were subjected to 5 days pretreatment with  $T_3$  (10 mg/kg sc), and 24 hr after the last  $T_3$  dose were given a single dose of dantrolene (50-400 mg/kg po).

A significant difference was seen in the GPT values for the dantrolene (50 mg/kg)/ $T_3$  group versus dantrolene alone and  $T_3$  alone (Table 3). There was no significant difference in liver:body weight ratios between these groups. However, those mice receiving  $T_3$  had slightly increased liver:body weight ratios. An unusual aspect of the results was the decrease in the GPT values as the dantrolene dose was increased.

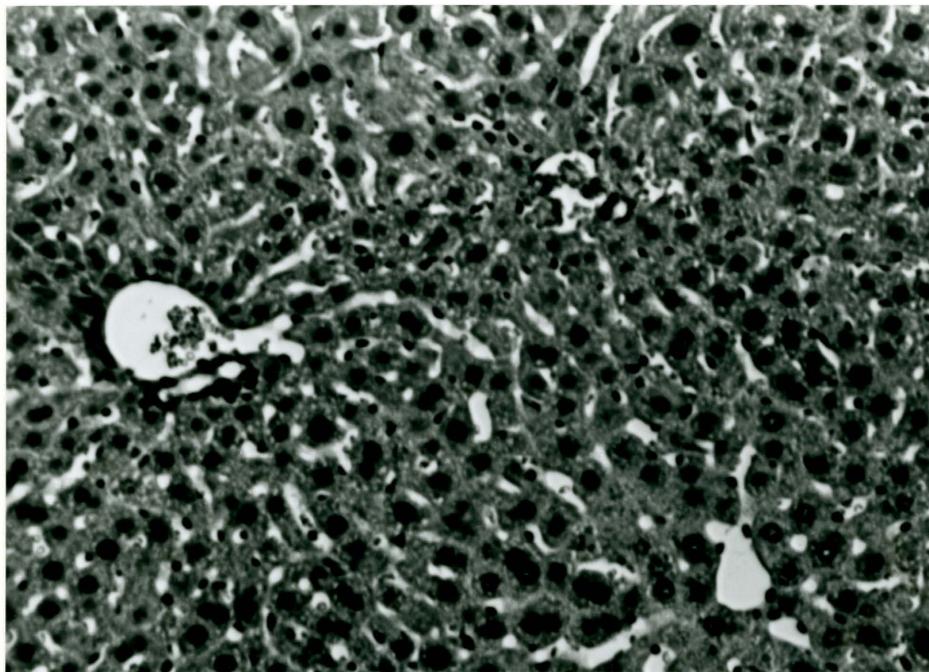


Figure 6. H & E stain of a liver section from a mouse given a single oral dose of dantrolene (400 mg/kg)

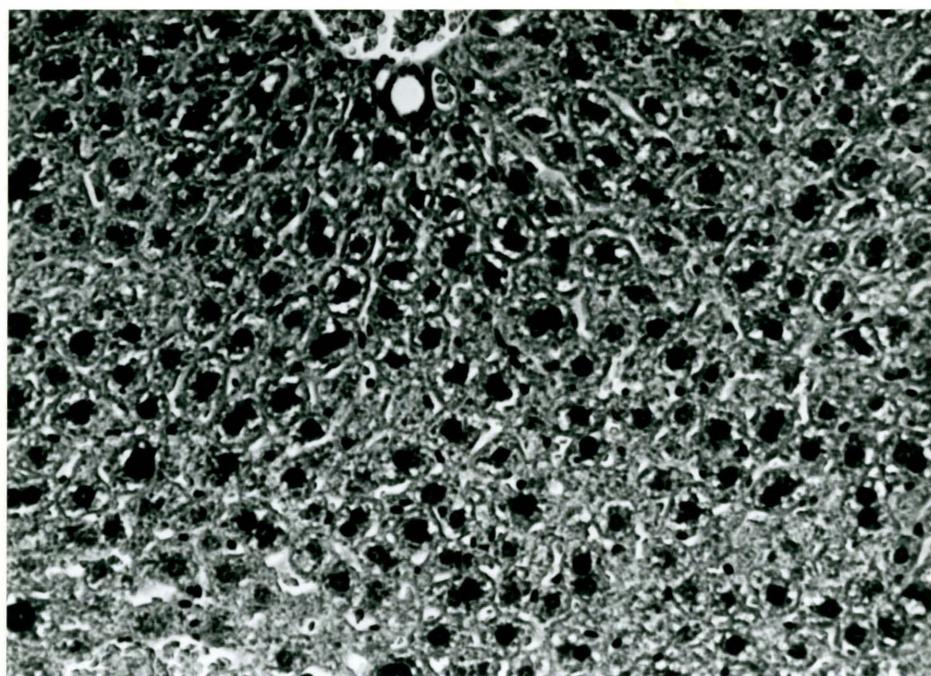


Figure 7. H & E stain of a liver section from a mouse exposed to dantrolene (400 mg/kg po) and hypoxia (12-14% O<sub>2</sub>) for 21 days--SGPT activity = 29 W-L units

Table 3: Serum GPT levels and liver:body weight ratios following dantrolene administration after tri-iodothyronine ( $T_3$ ) pretreatment.

<u>Treatment</u> <sup>a</sup>	<u>Dantrolene</u> <sup>b</sup>	<u>SGPT</u> <sup>c</sup>	<u>Liver: Body Weight</u> <sup>d</sup>	<u>N</u>
Dantrolene + $T_3$	50	162+40 <sup>e</sup>	6.34+.41	3
	100	86+26	6.12+.10	3
	200	106+29	6.25+.04	3
	400	49+8	6.08+.28	3
$T_3$	---	44+6	6.87+.39	3
Dantrolene	400	29+6	5.99+.49	3

a:  $T_3$ : 10mg/kg, sc, every day for 5 days.

b: mg/kg, po, dantrolene.

c: Mean + SEM (W/L Units/ml), 24 hr after dantrolene dose.

d: Mean  $\bar{x}$  sd, 24 hr after dantrolene dose.

e:  $P < 0.05$  vs dantrolene alone or  $T_3$  alone.

Microscopic evaluation of liver sections revealed no architectural differences between the 50 mg/kg dantrolene/T<sub>3</sub> group and the 400 mg/kg dantrolene/T<sub>3</sub> group. The elevated levels of GPTs suggest a toxicological effect. However, it is very sporadic and there is no histological evidence of cellular injury (Figures 8 and 9).

#### Sulfamethazine and p-Chloroaniline Pretreatment

By inhibiting the acetylation of dantrolene, there should be an increase in the amount of the free amino-dantrolene metabolite. This metabolite could then be a substrate of N-hydroxylation, which may produce a reactive intermediate. In order to alter the acetylation of the dantrolene pathway, sulfamethazine (SMZ) and p-chloroaniline (PCA) were incorporated as acetylation competitors to dantrolene.

As can be seen in Table 4, there were no significant differences in GPT levels or liver:body weight ratios between any groups. There was no histological evidence of injury to support the increased levels of GPT seen in the dantrolene/SMZ (150 mg/kg) group.

Two regimens of PCA were given to mice. One group received a single ip dose of PCA just prior to dantrolene, while the other group was placed on PCA-water for 3 days prior to dantrolene. Exposure to PCA for 3 days would result in a more gradual and sustained decrease in acetyl CoA levels, while a single ip dose just prior to dantrolene would produce a short-term decrease in these levels. Neither PCA pretreatment regimen produced any significant increase in GPT levels or liver:body weight ratios (Table 5). However two mice which received the single PCA

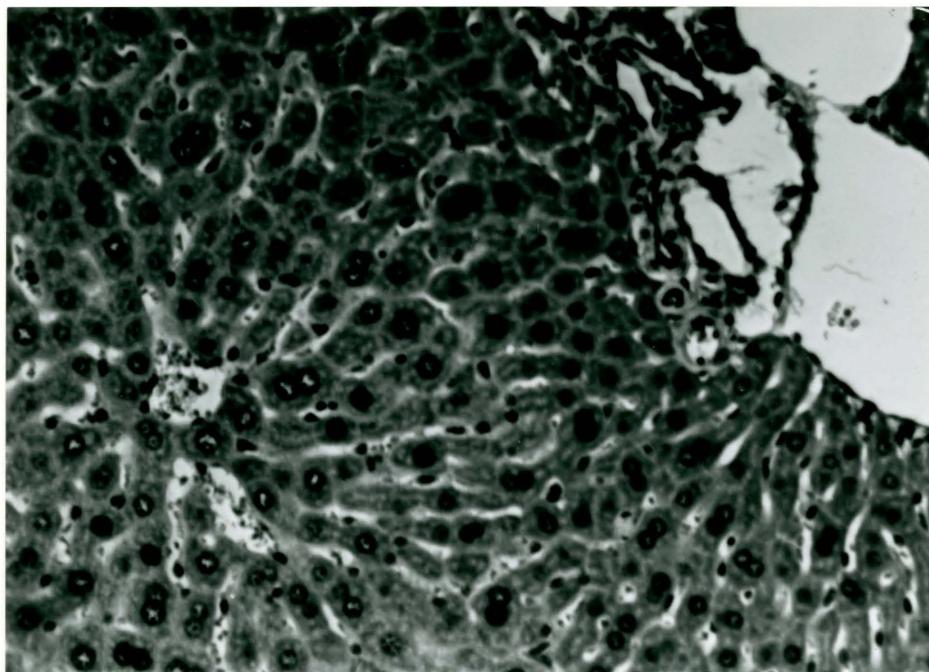


Figure 8. H & E stain of a liver section from a mouse pretreated with T<sub>2</sub> (10 mg/kg ip) then dosed with dantrolene<sup>2</sup> (50 mg/kg po)--SGPT activity = 138 W-L units

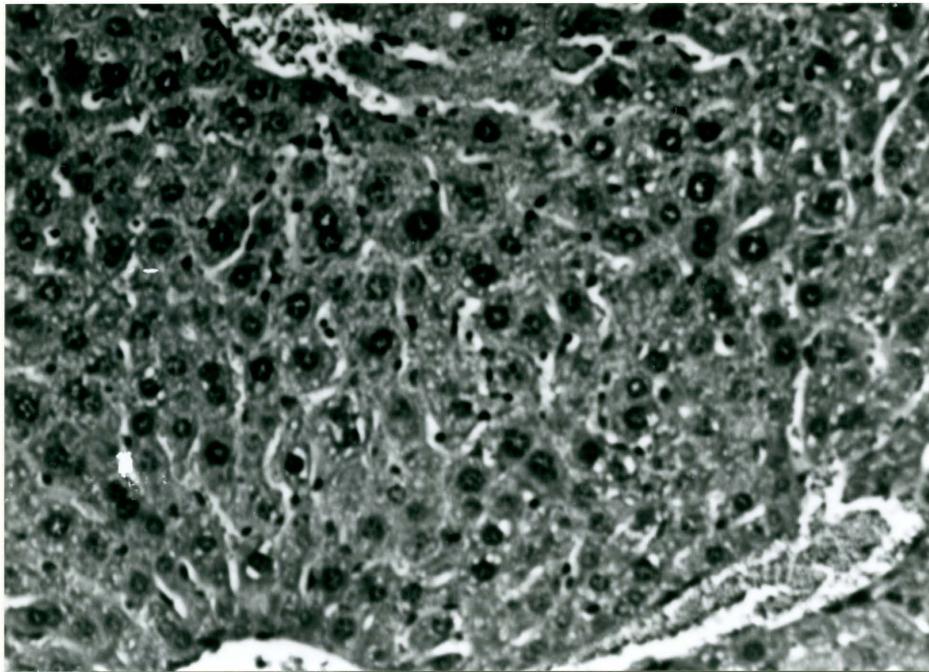


Figure 9. H & E stain of a liver section from a mouse pretreated with  $T_3$  (10 mg/kg ip) then dosed with dantrolene (400 mg/kg po)--SGPT activity = 63 W-L units

Table 4: Serum GPT levels and liver:body weight ratios following dantrolene administration after sulfamethazine (SMZ) pretreatment.

<u>Treatment</u> <sup>a</sup>	<u>SMZ</u> <sup>b</sup>	<u>SGPT</u> <sup>c</sup>	<u>Liver: Body Weight</u> <sup>d</sup>	<u>N</u>
Dantrolene <sup>a</sup> + SMZ <sup>b</sup>	1.5	45 <sub>±</sub> 10	5.64 <sub>±</sub> .77	5
	15	58 <sub>±</sub> 13	5.14 <sub>±</sub> .84	9
	150	100 <sub>±</sub> 51	5.77 <sub>±</sub> .48	7
	500	48 <sub>±</sub> 16	6.48 <sub>±</sub> .35	4
SMZ <sup>b</sup>	1.5	----	-----	-
	15	23 <sub>±</sub> 5	6.88 <sub>±</sub> .59	3
	150	49 <sub>±</sub> 13	5.76 <sub>±</sub> .98	7
	500	41 <sub>±</sub> 9	6.59 <sub>±</sub> .80	6
Dantrolene <sup>a</sup>	---	33 <sub>±</sub> 7	5.80 <sub>±</sub> .58	8

a: 400 mg/kg, po.

b: SMZ pretreatment, 30 min prior to dantrolene, mg/kg, ip.

c: Mean  $\pm$  SEM (W-L Units/ml), 24 hr after dantrolene dose.

d: Mean  $\pm$  sd, 24 hr after dantrolene dose.

No significant difference at  $p < 0.05$ .

Table 5: Serum GPT levels and liver:body weight ratios following dantrolene administration after p-chloroaniline (PCA) pretreatment.

<u>Treatment</u> <sup>a</sup>	<u>SGPT</u> <sup>b</sup>	<u>Liver: Body Weight</u> <sup>c</sup>	<u>N</u>
Dantrolene + PCA	58+15	6.28+.68	6
Dantrolene + PCA-water	19+1	5.56+.34	5
PCA	29+6	5.93+1.12	6
PCA-water	20+1	5.95+.45	4
Dantrolene	29+6	5.99+.49	6

a: Dantrolene: 400 mg/kg, po.

PCA: 100 mg/kg, ip, 30 min prior.

PCA-water: 0.54% PCA, 3 days prior.

b: Mean + SEM (W-L Units/ml), 24 hr after dantrolene dose.

c: Mean  $\pm$  sd, 24 hr after dantrolene dose.

No significant difference at  $p < 0.05$ .

dose did show increased GPT levels over the control (PCA only and dantrolene only) mice. Since mice on the PCA-water showed no indication of dantrolene-induced toxicity, histological evaluations were not done. However, they were cyanotic demonstrating the ability of PCA to induce methemoglobinemia.

Histological evaluation of the PCA and the PCA/dantrolene mice revealed some mild cellular disarray and hemorrhage. However, it was not possible to say that PCA pretreatment enhanced the hepatotoxicity of dantrolene, since there was no significant increase in the hepatic injury in the PCA/dantrolene group. PCA itself is a hepatotoxin, therefore, care must be taken in not mistaking damage from PCA with damage from dantrolene (Figure 10).

#### Glutathione Depletion

Methyl iodide is a potent glutathione depletor (Johnson, 1965, 1966), and previous studies have shown an increase in covalent binding of dantrolene with decreased levels of glutathione (Roy, Francis, Born et al., 1980). Therefore, mice were pretreated with methyl iodide to deplete glutathione levels prior to dantrolene administration. A significant difference in GPT levels and liver:body weight ratios was seen for both the dantrolene/methyl iodide (0.70 mmole/kg) and the control methyl iodide (0.70 mmole/kg) versus the dantrolene alone group (Table 6). Since both of these high level methyl iodide groups showed apparent toxicity, it is difficult to establish whether the increase in GPT is due to the formation of a dantrolene reactive intermediate or

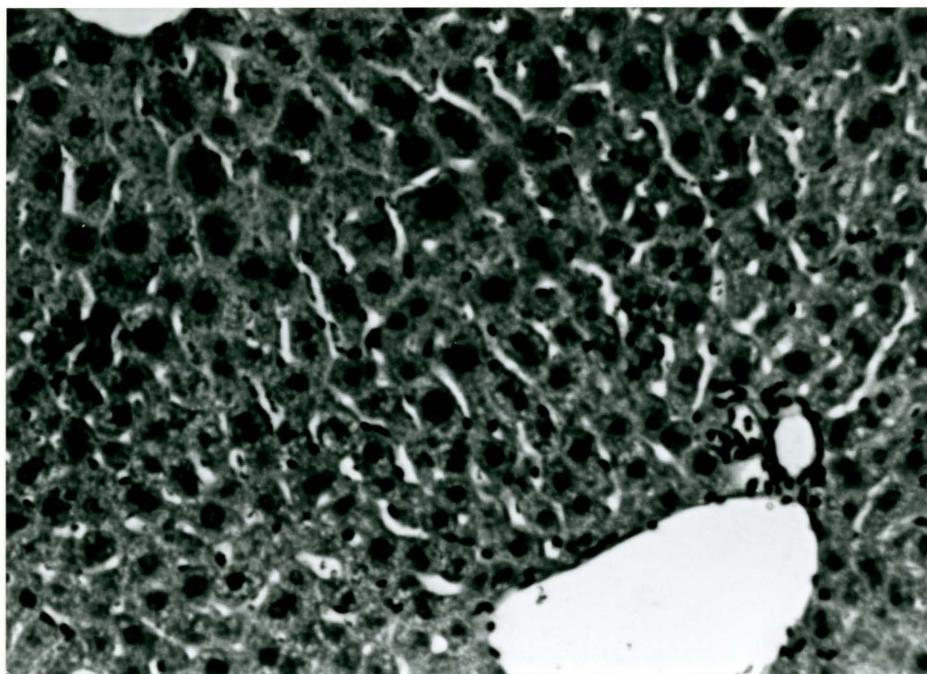


Figure 10. H & E stain of a liver section from a mouse pretreated with PCA (100 mg/kg ip) then dosed with dantrolene (400 mg/kg po)--SGPT activity = 150 W-L units

Table 6: Serum GPT levels and liver:body weight ratios following dantrolene administration after methyl iodide ( $\text{CH}_3\text{I}$ ) pretreatment.

<u>Treatment</u> <sup>a</sup>	<u>SGPT</u> <sup>d</sup>	<u>Liver Body Weight</u> <sup>e</sup>	<u>N</u>
Dantrolene + $\text{CH}_3\text{I}$	62 <sub>29</sub>	5.68 <sub>.54</sub>	4
Dantrolene + $\text{CH}_3\text{I}$	142 <sub>84</sub> <sup>f</sup>	7.35 <sub>.50</sub> <sup>f</sup>	3
$\text{CH}_3\text{I}$ <sup>b</sup>	20 <sub>2</sub>	5.72 <sub>.14</sub>	4
$\text{CH}_3\text{I}$ <sup>c</sup>	107	6.65	2
Dantrolene	31 <sub>5</sub>	6.07 <sub>.55</sub>	6

a: Dantrolene: 400 mg/kg, po.

b:  $\text{CH}_3\text{I}$ : 0.25 mmole/kg, ip, 30 min prior.

c:  $\text{CH}_3\text{I}$ : 0.70 mmole/kg, ip, 30 min prior.

d: Mean  $\pm$  SEM (W-L Units/ml), 24 hr after dantrolene dose.

e: Mean  $\pm$  sd, 24 hr after dantrolene dose.

f:  $P < 0.05$  vs dantrolene alone.

the methyl iodide itself. There was some sporadic increases in GPT levels for the dantrolene/methyl iodide (0.25 mmole/kg) group but these did not produce a significant increase over the methyl iodide (0.25 mmole/kg) or dantrolene control groups. Histological assessment of liver sections shows no overt hepatic damage (Figure 11).

#### Multiple Dosing

Clinical studies on dantrolene toxicity have suggested that hepatic injury occurs more often after having taken the drug for several weeks on a daily basis, and at a dose level of at least 300 mg daily. In order to mimic this regimen, multiple doses of dantrolene were used. Two multiple dosage regimens were employed. As seen in Figure 3, 3 hr after receiving dantrolene, serum levels were at their peak, and were still increased 6 hr after dosing. These results, along with previous reports stating peak plasma levels were reached 3-6 hr after an oral dose led to dosing mice every 3 hr for 12 hr or every 6 hr for 48 hr. By dosing at these intervals, a higher steady state level in the liver will be reached.

Neither of these regimens produced any significant increases in GPT values or liver:body weight ratios over the control mice (Table 7). There was no histological evidence of hepatic damage.

#### Phenobarbital Pretreatment

Previous studies with phenobarbital pretreatment in rats produced results indicating dantrolene affects the hepatic mixed function oxidase system by decreasing levels of cytochrome P-450 (Roy,

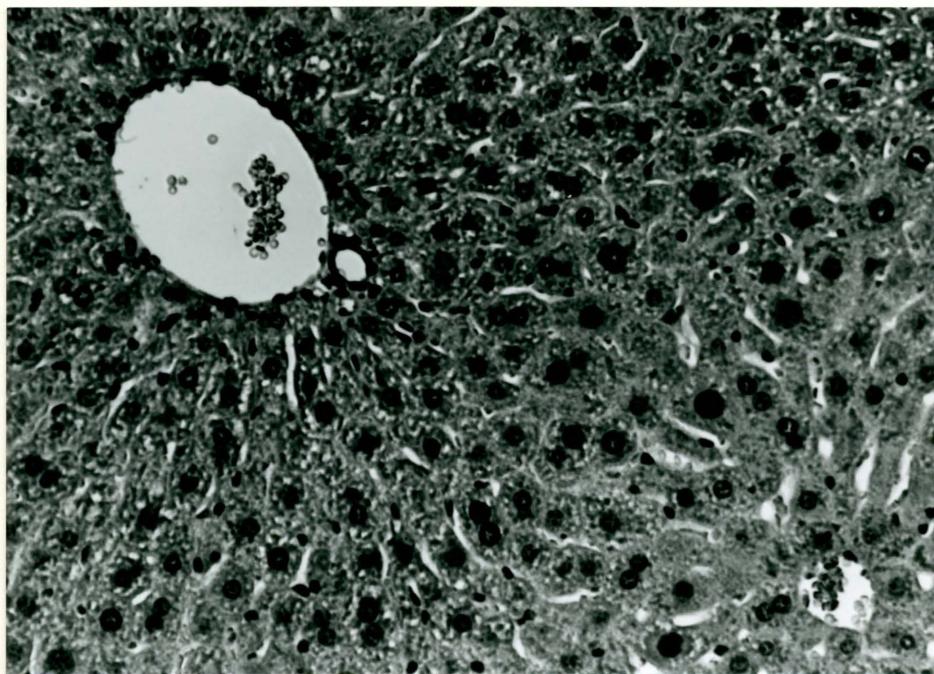


Figure 11. H & E stain of a liver section from a mouse pretreated with  $\text{CH}_3\text{I}$  (.25 mmole/kg ip) then dosed with dantrolene (400 mg/kg po)--SGPT activity = 146 W-L units

Table 7: Serum GPT levels and liver:body weight ratios following multiple doses of dantrolene.

<u>Treatment</u>	<u>SGPT<sup>c</sup></u>	<u>Liver: Body Weight<sup>d</sup></u>	<u>N</u>
Dantrolene <sup>a</sup>	35 <sub>±</sub> 11	5.80 <sub>±</sub> .06	4
Dantrolene <sup>b</sup>	62 <sub>±</sub> 9	5.34 <sub>±</sub> .14	3
Control	60 <sub>±</sub> 7	5.78 <sub>±</sub> .63	7

a: Every 3 hr for 12 hr, killed 12 hr after last dose.

b: Every 3 hr for 48 hr, killed 6 hr after last dose.

c: Mean  $\pm$  SEM (W-L Units/ml).

d: Mean  $\bar{x}$   $\pm$  sd.

No significant difference at  $p < 0.05$ .

Francis, Born et al., 1980). Combination pretreatment studies were performed in phenobarbital pretreated mice and rats in order to encompass numerous processes by which the biotransformation of dantrolene could be altered. After pretreatment with phenobarbital, mice were administered, either once in 24 hr or 3 times in 12 hr, an ip injection of p-chloroaniline (100 mg/kg ip) given 30 min prior to each dantrolene dose. By exposing the mice to PCA, the acetylation of dantrolene will be decreased, as well as hepatic cytochrome P-450 levels due to phenobarbital pretreatments.

Rats were also subjected to phenobarbital pretreatment. They were then dosed with sulfamethazine (SMZ) and/or buthionine sulfoximine (BSO) in an attempt to alter dantrolene metabolism. Half of these rats were exposed to hypoxic conditions after receiving dantrolene. By pretreating rats with these two compounds and then subjecting them to hypoxia, the reductive pathway of dantrolene would be enhanced, creating more suitable conditions for the production of reactive metabolites.

Phenobarbital pretreatment did not enhance the ability of other previously employed methods used in altering metabolism to elicit any evidence of hepatic injury as measured by serum GPT levels and liver: body weight ratios. Histological assessment of hepatic sections showed no damage.

### Binding of $^{14}\text{C}$ -dantrolene to Hepatic Macromolecules

Many hepatotoxic agents require bioactivation to a reactive intermediate before exerting a toxic effect. Previous studies have shown that  $^{14}\text{C}$ -dantrolene covalently binds to rat hepatic microsomal protein in an atmosphere of ambient air (Roy, Francis, Born et al., 1980).  $^{14}\text{C}$ -radioactivity binding studies were undertaken during this study to provide further information on the requirement of biotransformation to dantrolene-associated hepatotoxicity.

Prior to use, mouse hepatic microsomes were assessed for hepatic cytochrome P-450 activity. This activity was found to be 0.6 nanomoles cytochrome P-450/mg microsomal protein. To assure these microsomes were active, they were used to bioactivate a known hepatotoxin, carbon tetrachloride, which undergoes a reductive microsomal metabolism, and were found to be capable of bioactivating this toxin (2.8 nmoles bound/mg protein).

From Table 8, it can be seen that in vitro binding of dantrolene to hepatic microsomal protein was not dependent on the concentration of dantrolene and the atmosphere used. Binding was actually enhanced in the absence of a NADPH generating system and in a  $\text{O}_2$  atmosphere. Major differences though, were seen in the quantity of  $^{14}\text{C}$ -dantrolene bound, where this study found picomoles bound/mg protein as compared to nanomoles bound/mg protein in an earlier report (Roy, Francis, Born et al., 1980).

Table 8: In vitro covalent binding of  $^{14}\text{C}$ -Dantrolene to hepatic microsomal protein.

$^{14}\text{C}$ -Dantrolene (mM)	Atmosphere	Picomoles $^{14}\text{C}$ -bound/mg protein <sup>a</sup>		N
		+NADPH	-NADPH	
0.1	O <sub>2</sub>	6.4	11.5	2
0.2	O <sub>2</sub>	26.1	18.0	2
0.5	O <sub>2</sub>	33.0	28.2	2
0.1	N <sub>2</sub>	6.0 <sub>±</sub> 1.3	5.5 <sub>±</sub> 1.4	4
0.2	N <sub>2</sub>	9.5 <sub>±</sub> 5.8	12.7 <sub>±</sub> 5.0	4
0.5	N <sub>2</sub>	13.7 <sub>±</sub> 7.7	18.4 <sub>±</sub> 7.6	4

a: Incubation for 60 min at 37°C.

## DISCUSSION

Dantrolene sodium, a peripheral skeletal muscle relaxant, has been implicated as the causative agent of dantrolene-associated liver damage in humans, ranging from transient mild hepatic dysfunction to fulminant hepatic failure. Attempts to reproduce this drug-induced hepatic injury in animals has proven only minimally successful. Therefore, the development of an animal model capable of duplicating this hepatic damage would further the understanding of the mechanism of dantrolene action and the associated drug-induced hepatotoxicity.

In creating an animal model for dantrolene-associated hepatotoxicity, several factors had to be considered: the dose of dantrolene, its route of administration, and mechanisms of altering its biotransformation. A dose of 400 mg/kg was chosen for the acute studies based upon the results of Francis and Hamrick (1979). Their studies found 400 mg/kg, intraperitoneally, to be the lowest dose to produce an effect on the hepatic mixed function oxidase system, i.e., a decrease in cytochrome P-450 content, with a single administration in rats. Dantrolene therapy in humans is administered orally, via capsules. Therefore, a per os route of administration was chosen in order to simulate as closely as possible the regimen of human therapy. The possibility of a time-related phenomenon for the production of toxicity as well as acute toxicological studies were performed.

Certain hepatotoxins appear to elicit a greater toxic effect when reductively metabolized. Thus, the possibility exists that hypoxic conditions may potentiate the hepatotoxicity of reductively metabolized compounds. Such is the case with halothane (McLain, Sipes and Brown, 1979) and carbon tetrachloride (Shen, Garry and Anders, 1982). Perhaps a better example of reductive metabolism potentiated hepatotoxicity is nitrofurantoin. Dantrolene and nitrofurantoin are structurally similar in that they both possess a furan ring and hydantoin ring connected by an azomethane linkage (Figure 12). The major difference is the substitution on the furan ring, where dantrolene has a nitrophenyl group and nitrofurantoin has only a nitro group. This minor structural difference allows for a more parallel reductive pathway between these two drugs, in which both undergo an initial reduction followed by acetylation of their nitro group.

Rat liver microsomes have been shown to catalyze the nitroreduction of nitrofurantoin resulting in a metabolite capable of binding to protein (Boyd, Stiko and Sasame, 1979). Due to its extreme lability, the specific structure of this reactive metabolite is not known. However, the nitroreduction of other 5-nitrofuran derivatives has produced metabolites capable of binding to macromolecules, and these metabolites are strongly suspected to be hydroxylamine derivatives (Wang, Behrens, Ichiwaka et al., 1974). It has been proposed recently that the nitroreduction of ronidazole, a 5-nitro imidazole derivative, leads to reactive metabolites possessing the ability to bind to hepatic proteins

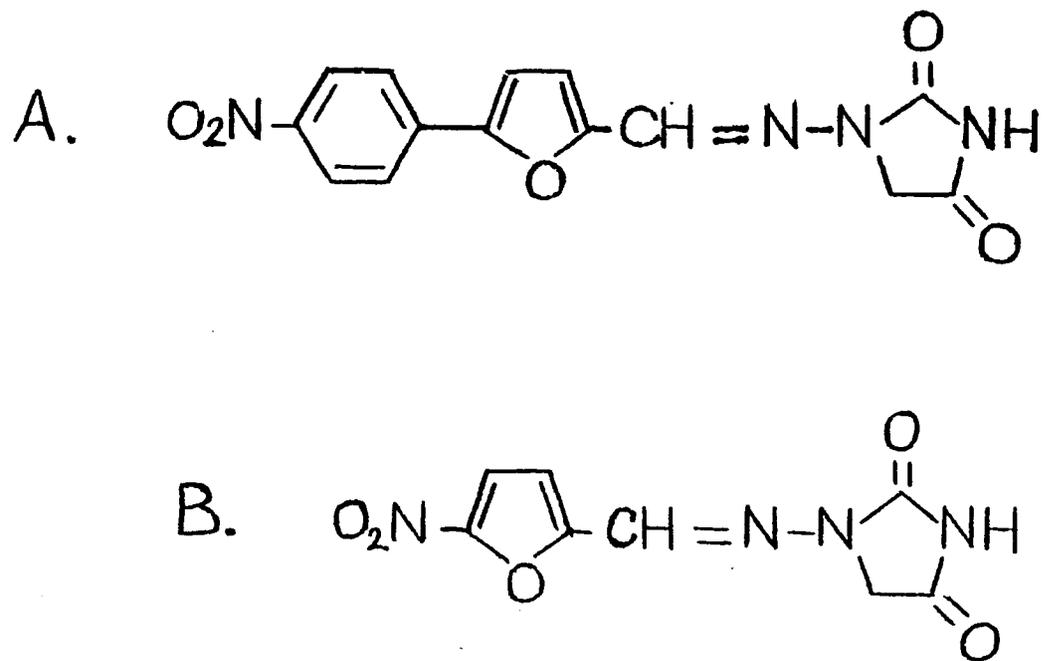


Figure 6. Comparative structures of dantrolene (A) and nitrofurantoin (B).

(Miwa, West, Walsh et al., 1982). Based on the evidence that hypoxia potentiates the hepatotoxicity of carbon tetrachloride and halothane, and that biotransformation of nitrofurantoin and other aromatic 5-nitro derivatives via reductive metabolism produces a species capable of binding proteins, it is reasonable to assume that hypoxia may promote the expression of dantrolene hepatotoxicity. This is because dantrolene biotransformation includes a reductive pathway and structurally has a nitro group which can be activated.

Mice were subjected to repeated exposures of dantrolene and hypoxia in an effort to create higher levels of dantrolene presented to the liver and enhance reductive metabolism. No adverse effects were observed due to treatment with either dantrolene or hypoxia as measured by serum GPT levels and histological assessment of liver sections. Along with the three animal groups studied in this experiment, another group which received dantrolene and room air, should have been incorporated. This would have served as the dantrolene control for the dantrolene + hypoxia group, and also to monitor the effect of dantrolene as the parent compound.

Tri-iodothyronine ( $T_3$ ) has been proposed to induce an intracellular hypoxia. This condition could favor metabolism of some hepatotoxins down a reductive pathway (Wood, Berman, Harbison et al., 1980). The specific mechanism of action is not known, however, there is an increased cellular usage and requirement for oxygen (Calvert and Brody, 1961).  $T_3$  is known to potentiate the hepatic damage caused by carbon tetrachloride (Calvert and Brody, 1961) and halothane (Wood, Berman,

Harbison et al., 1980). Although the exact mechanism of action is not completely understood, it is thought to be related to the existence of intracellular hypoxia and an increase in oxygen utilization.  $T_3$  has also been reported to decrease hepatic glutathione and cytochrome P-450 levels (Wood, Berman, Harbison et al., 1980). Since the conjugation of glutathione with a reactive species is often a protective mechanism, a decrease in glutathione levels and a proposed intracellular hypoxia could lead to deleterious effects produced by hepatotoxins.

The presence of a reductive pathway in the biotransformation of dantrolene allows the assumption that pretreatment with  $T_3$  prior to dantrolene administration would shift metabolism down the reductive pathway, thereby creating higher levels of amino-dantrolene. This metabolite, which is usually rapidly acetylated, would then be available for N-hydroxylation. Although not definitely proven, the biotransformation of nitrofurantoin to reactive metabolites is thought to also proceed through a N-hydroxylation step producing hydroxylamine derivatives which then bind to cellular macromolecules resulting in cellular injury (McCalla, Reuvers and Kaiser, 1971). Hydroxylamines have also been proposed as the reactive species in other aromatic 5-nitro derivatives including ronidazole (Miwa, West, Walsh et al., 1982).

In order to potentiate the hepatotoxicity of dantrolene, mice were pretreated with  $T_3$  for 5 days prior to receiving a single dose of dantrolene (50-400 mg/kg). A sporadic but significant increase in GPT

values were seen in the dantrolene (50 mg/kg)/T<sub>3</sub> group versus the dantrolene alone and T<sub>3</sub> alone groups. There apparently is some altered hepatocellular membrane permeability, but it is very sporadic and there is not histological evidence of cellular injury.

Mammalian tissues contain a variety of N-acetylating enzymes that catalyze the transfer of the acetyl group from acetyl coenzyme A to drugs (Weber, Miceli, Hearse et al., 1976). These hepatic and extra-hepatic N-acetyl-transferases differ from each other in substrate specificity and acetylating capacity (Hearse and Weber, 1973; DuSouich and Courteau, 1981; Friebova and Elis, 1976). In contrast to the microsomal MFO system, little is known about the possible regulating factors of the acetylation polymorphism (Olsen, 1982).

Acetylation of a compound is usually a detoxification mechanism (Lower and Bryan, 1973). In the case of dantrolene, amino-dantrolene is rapidly acetylated decreasing the chances of the formation of a hydroxylamine. By inhibiting the acetylation of dantrolene, there should be an increase in the amount of amino-dantrolene metabolite. This metabolite could then undergo N-hydroxylation as previously explained. Not knowing whether dantrolene is a preferred substrate for hepatic or extrahepatic N-acetyltransferases, both hepatic and extrahepatic substrates were used as competitors to the acetylation of dantrolene. Sulfamethazine (SMZ) was chosen as the hepatic substrate, with p-chloroaniline (PCA) as the extrahepatic substrate.

SMZ and PCA were administered to mice prior to dosing with dantrolene in order to decrease the levels of acetyl CoA available to dantrolene. No significant difference in GPT values of liver:body weight ratios were observed in the SMZ/dantrolene mice, however, there were sporadic increases as shown by the higher GPT value in the dantrolene/SMZ (150 mg/kg) group. No hepatic damage was seen in the histological slides.

Two dosing regimens with PCA were employed, but neither produced any significant increases in GPT values or liver:body weight ratios. Two mice which received the single PCA dose did exhibit increased GPT values over the control mice; histological assessment did not substantiate this increase. Methemoglobinemia was observed in the mice receiving PCA-water.

The biologic functions of glutathione are not fully understood. It has been proposed that glutathione peroxidase may protect cells from de novo generated hydrogen peroxide (Mills, 1960). Glutathione is also used in the biosynthesis of glutathione conjugates of foreign compounds that are then excreted as mercapturic acids (Boylard and Chasseaud, 1969). More recently, it was proposed that perhaps the most important role of glutathione in the body is to protect vital nucleophilic sites in hepatocytes from electrophilic attack by alkylating metabolites of drugs (Mitchell, Jollo, Potter et al., 1973).

Studies with ronidazole and nitrofurantoin lend credence to the latter theory of glutathione acting as a scavenger of electrophiles.

Binding studies with  $^{14}\text{C}$ -ronidazole using rat hepatic microsomes produced dramatic inhibition of protein alkylation in the presence of glutathione or cysteine (79% and 85%, respectively) in an anaerobic atmosphere. Inhibition of binding was not seen with methionine, indicating the requirement of free sulfhydryl group for inhibition. Since cysteine does not appear to alter the metabolism of ronidazole, it can be assumed that cysteine and glutathione inhibited binding by acting as competitors with ronidazole for the protein molecules. Work carried out with nitrofurantoin in hepatic microsomes from rats produced results similar to those seen with ronidazole, i.e. a decrease in binding in the presence of glutathione (Boyd, Stiko and Sasame, 1979). No change in the rate of nitroreduction was observed, therefore glutathione probably acted as a scavenger to the reactive species.

The in vivo and in vitro binding of  $^{14}\text{C}$ -dantrolene to hepatic proteins was studied by Roy, Francis, Born et al. (1980). They reported that glutathione depletion (produced by diethylmaleate pretreatment) resulted in an increased in vivo binding of dantrolene to hepatic proteins. This led to the observation that dantrolene or one of its metabolites may be acting as an electrophilic agent to complex hepatic proteins.

From the above results it seemed plausible that by depleting glutathione levels, dantrolene's ability to produce hepatic injury would be potentiated. Previous work showed that pretreatment with diethylmaleate, although lowering glutathione levels, did not produce

any adverse effects. Therefore, methyl iodide, a more potent glutathione depleter was employed in this study.

Serum GPT levels and liver:body weight ratios were significantly increased in both the dantrolene/methyl iodide (0.70 mmole/kg) group and methyl iodide (0.70 mmole/kg) control group. As both these groups exhibit apparent toxicity, it is difficult to assess this toxicity as to the formation of a dantrolene reactive metabolite or PCA itself. Sporadic increases were seen in the GPT values for the dantrolene/methyl iodide (0.25 mmole/kg) group, however, they were not significant. Histological assessment shows no hepatic damage.

Previous work studying the effects of dantrolene on hepatic function employed rats as the test animal. Therefore, a single study using rats was performed. The rats were subjected to a combination of pretreatments. Sulfamethazine was administered as an acetylation competitor to dantrolene, with buthionine sulfoximine given to decrease glutathione levels. Buthionine sulfoximine is a potent inhibitor of gamma-glutamylcysteine synthetase, an enzyme in the synthesis of glutathione (Griffith and Meister, 1979). Phenobarbital was used to induce the hepatic mixed function oxidase system of the rats prior to dosing with sulfamethazine and buthionine sulfoximine. After induction, and pretreatment with sulfamethazine and buthionine sulfoximine, the rats were given an oral dose of dantrolene. Half the rats were then placed in a hypoxic chamber for 6 hr. The ability of previously

employed methods to alter biotransformation was not enhanced by phenobarbital pretreatment. No increase in GPT or liver to body weight ratios were exhibited; no hepatic damage was seen in the liver sections.

The biotransformation of dantrolene was manipulated in numerous ways in an attempt to produce a reactive metabolite capable of reacting with cellular constituents, thus producing hepatotoxicity. As measured by serum GPT levels and histological assessment of liver sections, no overt hepatotoxicity was produced.

Although it was demonstrated that an oral dose of dantrolene was absorbed, perhaps not enough was reaching the liver in amounts high enough to alter the liver's metabolizing ability. Studies by Utili, Boitnott and Zimmerman (1977) showed dantrolene-associated hepatic injury to occur more often after having taken the drug for at least 8 weeks, and at a dosage level of at least 300 mg daily. Other commonly administered drugs, including nitrofurantoin and diphenylhydantoin have also been associated with time-dependent hepatic injury, i.e., long-term exposure. In an effort to mimic this time-related injury over a short time period, mice were given multiple doses of dantrolene in an effort to overload the liver, thus producing hepatic damage. However, no increase in GPT levels or liver:body weight ratios were seen.

It is difficult to determine why the attempts at manipulating dantrolene biotransformation did not produce any hepatotoxicity. A major reason could be due to insolubility of dantrolene. The drug is very insoluble even in some of the more common organic solvents

including chloroform and acetone. The drug was administered to the animals in corn oil, in which dantrolene is also not very soluble (20 mg/ml). Because of this, a suspension of dantrolene was administered, probably affecting the rate of absorption and the amount reaching the liver at one time. The possibility also exists that a majority of the dantrolene was excreted before absorption occurred.

No measurement was ever made of dantrolene levels in the liver, or of metabolites during the various metabolic manipulations. This raises questions as to whether the manipulations really altered the metabolism of dantrolene. However, these same methods for biotransformation have produced adverse hepatic effects when used with other drugs. Therefore, one can probably assume the manipulation did work, but the toxicity of dantrolene may require the manipulation of other components not examined during this study.

Francis and Hamrick (1979) found that dantrolene produces a dose-related inhibition of the hepatic mixed function oxidase system. Therefore investigations using  $^{14}\text{C}$ -dantrolene were performed to further study the biotransformation of dantrolene and its effect on the production of hepatotoxicity. The in vivo and in vitro results lead to the conclusion that it is dantrolene rather than its metabolites which is acting as the binding agent. This idea is also substantiated by the findings that phenobarbital pretreatment decreased the inhibition of the hepatic mixed function oxidase system activity by dantrolene (Roy, Francis, Born et al., 1980).

The findings from in vitro  $^{14}\text{C}$ -dantrolene binding to hepatic microsomal protein in this study, confirm some of the findings of Roy, Francis, Born et al (1980).  $^{14}\text{C}$ -dantrolene was found to bind to hepatic microsomal proteins in the presence or absence of a NADPH system, and was actually enhanced in the absence of a generating system. Major differences, however, were seen in the amount of binding. This study reports picomoles bound/mg protein, while Roy, Francis, Born et al., (1980) reported nmoles bound/mg protein. This raises the question of whether the binding measured by Roy, Francis, Born et al., (1980) was truly covalent binding of  $^{14}\text{C}$ -dantrolene, and not just an extremely high affinity for, but not covalent binding to, hepatic proteins. In addition to in vitro binding studies in which exhaustive washing was used to eliminate all unbound  $^{14}\text{C}$ -dantrolene, dialysis with SDS buffer was also employed (Sun and Dent, 1980). Buffer changes were done every 8 hours, and it still took more than 2 days to reach a count equal to background. This correlates well with the fact that numerous extra washings with 100% ethanol are required in order to remove all unbound radioactivity. If the protein pellet was not thoroughly washed, binding results would appear to be higher than they really were.

The insolubility of dantrolene is also a major factor in  $^{14}\text{C}$ -radioactivity studies.  $^{14}\text{C}$ -dantrolene was added to the incubation tubes in a methanol carrier, which was then dried off under  $\text{N}_2$  gas before continuing with the experiment. Unfortunately, after evaporation the dantrolene was left as particulate at the bottom of the tube,

which did not completely dissolve in the Tris-KCL buffer. Problems were then encountered after incubation, when the protein was precipitated. The unbound <sup>14</sup>C-dantrolene was also precipitated and trapped in the ensuing protein pellet.

Unfortunately, this study did not produce an animal model capable of reproducing the hepatotoxicity seen in humans. That is not to say none of the manipulations performed might actually be the route by which dantrolene produces its toxicity, but as sporadic and unpredictable as this expression of toxicity is in humans, it is not surprising that it could not be routinely reproduced in this study.

Faults in experimental design could also be a cause of no toxicity. One major factor is the species of animals used. All previous studies on the hepatotoxicity of dantrolene used rats as the experimental animals (Francis and Hamrick, 1979; Roy, Francis, Born et al., 1980; Abernathy, Utili and Zimmerman et al., 1978). Although not overt, some hepatotoxicity was observed. Studies recently published, indicate rats to be less sensitive than mice to dantrolene (Arnold, Epps, Cook et al., 1983). Hamsters appear to be the most responsive of the three animals. All the studies in this project utilized mice except for one, in which rats were used. Hamsters were not used due to cost limitations.

The manipulations of dantrolene's biotransformation should have been examined to ensure the alteration of metabolism did occur. The best way to do this through the measurement of metabolites in the blood

and liver over the time course of the experiment. Although the HPLC assay used in this study to quantitate serum levels of dantrolene can be adapted to detect the 5-hydroxy- and aceto-metabolites as well as the parent, it is not simple. The mobile phase is not an isocratic one, but a gradient, and one run requires approximately 30 min. However, just recently, a new HPLC assay capable of determining dantrolene and the 5-hydroxy and aceto-metabolite with an isocratic mobile phase was published (Wuis, Grutters, Vree et al., 1982). This assay allows the detection of dantrolene and its metabolites in plasma and urine, and a run takes just over 10 min. If future studies are performed with dantrolene, the measuring of metabolite formation is a definite must, and this new assay might be of great help.

Other parameters that should be followed include the periodic measurement of GSH levels in the animals treated with methyl iodide or any other glutathione depletor utilized. Although preliminary work with methyl iodide and its ability to deplete glutathione indicated a 30-40% decrease from control levels within 30 min to 1 hr after an ip injection (appendix A), no follow-up studies were done when given in conjunction with dantrolene. It is possible a second dose may be required a few hours later in order to keep the glutathione levels depressed. This is an easy assay and the procedure by Sedlak and Lindsay (1958) is recommended.

Probably a major deletion from this study, was a chronic study involving dantrolene as the parent drug without any outside biotransformation alterations. Reports on human hepatic injury due to

dantrolene state that this drug-associated injury occurs most often after a therapy of at least 8 weeks (Utili, Boitnott and Zimmerman, 1977). This time-related phenomenon of hepatotoxicity is not unique in dantrolene. Nitrofurantoin, to which dantrolene is structurally similar, and diphenylhydantoin have also be implicated in this type of hepatic toxicity. Therefore, a study of at least 8 weeks in length should be performed. Liver:body weight ratios, GPT levels or any other method to follow liver function, as well as gross and microscopic changes in the liver should be followed. A study of this magnitude was not done due to expense and time committment.

This study was based on the premise that through the biotransformation of dantrolene, reactive metabolites are formed which are then capable of binding to cellular macromolecules thus producing toxicity. In hindsight, this was not an appropriate assumption, as the toxicity of the parent compound is still in question. However, using the premise that the biotransformation of dantrolene is the route to hepatotoxicity, the question arises as to whether it would not have been more appropriate to bypass completely the parent compound and dose the animal with the amino-metabolite. This could be done through an i.v. injection, thereby eliminating absorption and allowing a small dose to be administered. More amino-dantrolene would be presented to the liver increasing the chances for the formation of a N-hydroxylamine. This study was not performed due to the low quantity of amino-dantrolene available to this researcher.

More information on the biotransformation and subsequent binding of dantrolene and metabolites to cellular macromolecules would be available if phenobarbital and/or diethylmaleate pretreatment had been given prior to making hepatic subcellular fractions.  $^{14}\text{C}$ -radioactivity binding could then be compared between the control microsomes and the pretreated ones. Binding studies with the soluble fraction should also have been followed. Results from this would give a better idea of what specific enzyme(s) may metabolize dantrolene. An alternate species, i.e., hamsters, should also be examined.

Using the indices of serum GPT levels and histological assessment of liver sections to identify any hepatic damage may not have been sensitive enough for this specific study. A more logical approach might have been through the measurement of hepatic function such as alterations in the secretion and excretion of sulfobromophthalein, as in the studies done by Abernathy, Utili, Zimmerman et al. (1978). Changes in metabolism enzymes are another possibility. These could include assessment of cytochrome P-450 concentration, cytochrome c reductase or aminopyrine N-demethylase activities as well as many others.

An alteration of calcium metabolism has been shown to be an early consequence of intoxication with some hepatotoxins. This disruption has been implicated in the early events of cellular necrosis produced by carbon tetrachloride as well as other hepatotoxins. Inhibition of the calcium-ATPase pump can be determined by uptake of  $^{45}\text{Ca}$  by microsomal fractions. This assay is a very sensitive measurement

of early cellular disruption and therefore could be a good assay to incorporate into the dantrolene studies. Unfortunately, calcium pump alteration is usually seen only when the end point of cellular injury is necrosis. With this in mind, it may not be feasible to use it until more information has been gathered on the hepatotoxicity of dantrolene (Moore, Davenport and Landon, 1976).

The possibility still exists that the toxicity produced by dantrolene is an idiosyncratic, hypersensitivity reaction, the mediation of which is not dependent upon the metabolism of dantrolene. If this is the case, many more studies are needed, possibly including studies with endotoxin and its effect upon the cells in the presence of dantrolene.

The liver stands as an effective barrier to the passage of bacteria and their products from the gastrointestinal tract to the systemic circulation. Failure to normally perform the function of detoxifying endotoxin, a lipopolysaccharide unique to gram-negative bacteria, might initiate or perturbate liver injury (Nolan, 1975). Evidence is present that a common pathway for hepatic injury by a variety of agents may result from impairment of the liver's ability to detoxify bacterial endotoxins, as seen with sub-lethal doses of  $CCl_4$  in guinea pigs (Farrar and Magnani, 1964).

Studies by Lind (1982) indicate that the hepatocytes only need to be compromised in order to be more sensitive to endotoxins. Dantrolene has been shown to alter the ability of the liver to excrete sulfobromophthalein (Abernathy, Utili, Zimmerman et al., 1978)

and to decrease hepatic mixed function oxidase activity (Francis and Hamrick, 1979). Thus, exposure to endotoxin after receiving dantrolene may be useful to enhance compromised liver function due to dantrolene.

Studies with nitrofurantoin and other aromatic 5-nitro derivatives have revealed that their metabolism is not hepatic cytochrome P-450 mediated. Instead, it is felt xanthine oxidase and/or cytochrome c reductase are responsible (Wang, Behrens, Ichiwaka et al., 1974; West, Wislocki, Wolf et al., 1982). Just recently, it has been reported that under anaerobic conditions and the presence of a NADPH generating system, nitrofurantoin caused the metabolic activation of oxygen, resulting in a nitro anion free radical (Peterson, Combs, Holtzman, et al., 1982; Youngman, Osswald and Elster, 1982). Due to the similarities of dantrolene with nitrofurantoin, it is feasible to assume that dantrolene may undergo the same chemical reaction. Studies on this might lead to a further understanding of the toxicity produced by dantrolene.

The fact that dantrolene binding to hepatic proteins is not dependent upon a NADPH generating system is, to say the least, uncommon since many mechanisms of hepatotoxicity are related to the binding of electrophiles after biotransformation of the compound. No studies were performed which altered the hydroxylation of the hydantoin ring in the dantrolene structure. Therefore, it is very possible that the reactive intermediate actually is formed by action on the hydantoin ring. Cytosolic amidases could attack either of the 2 amide bonds on

the hydantoin ring thus opening up the ring, exposing it to other enzymes which could create a reactive site. It is postulated that the hepatic injury produced by furosemide results from metabolic activation of the furan ring, possibly by epoxidation (Mitchell, Nelson, Potter et al., 1976). Activation of the furan ring has been documented for other hepatotoxins including 2-(N-ethyl-carbamoylhydroxymethyl) furan and aflatoxins (Guengerich, 1977). Thus, hepatotoxicity of dantrolene could be postulated to be due to metabolic activation of the furan ring. The presence of an azomethane linkage (Schiff base) in the dantrolene structure allows this position to also be the site of activation by electron shift or breaking of the azomethane bond.

This study has examined only a small portion of the possibilities of how dantrolene actually results in hepatic toxicity. There are still many avenues both in in vivo and in vitro that should be examined in order to fully understand how this drug results in hepatic toxicity.

## APPENDIX A

Effect of Intraperitoneal (ip) and Oral (po) dosage of methyl iodide on mouse hepatic nonprotein sulfhydryl content

<u>Dose (mmole/kg)</u>	<u>Route of Administration</u>	<u>Time after dose (min)</u>	<u>Nonprotein-sulfhydryl content (% control)</u>
0.53	po	15	70
		30	60
		60	80
		90	95
0.70	po	15	90
		30	73
		60	37
		90	61
		120	81
		180	65
1.0	po	60	37
		120	46
		180	40
1.0	ip	60	40
		180	31
		240	21

## REFERENCES

- Abernathy, C. O., Utili, R., Zimmerman, H. J., Ezekiel, M. (1978): The effects of dantrolene sodium on excretory function in the isolated perfused rat liver. *Tox. Appl. Pharm.* 44:441.
- Arnold, T. H., Jr., Epps, J. M., III, Cook, H. R., Hamrick, M. E. (1983): Dantrolene sodium: Urinary metabolites and hepatotoxicity. *Res. Comm. Chem. Pathol. Pharmacol.* 39:381.
- Bowman, W. C. and Khan, H. H. (1977): Effects of dantrolene sodium on isolated skeletal, smooth, and cardiac muscle of the guinea pig. *J. Pharm. Pharmac.* 29:628.
- Boyd, M. R., Stiko, A. W., Sasame, H. A. (1979): Metabolic activation of nitrofurantoin - possible implications for carcinogenesis. *Biochem. Pharmacol.* 28:601.
- Boylard, E. and Chasseaud, L. F. (1969): The role of glutathione and glutathione S-transferases in mercapturic and biosynthesis. *Advan. Enzymol.* 32:173.
- Calvert, D. N. and Brody, T. M. (1961): The effects of thyroid function upon carbon tetrachloride hepatotoxicity. *J. Pharmacol. Exp. Ther.* 134:304.
- Chyatte, S. B. and Basmajian, J. V. (1973): Dantrolene sodium: Long-term effects in severe spasticity. *Arch. Phys. Med. Rehabil.* 54:311.
- Combes, B. and Schenker, S. (1969): Laboratory Tests. In *Diseases of the Liver*. L. Schiff (ed.), J. B. Lippincott, Philadelphia, PA, pp. 165-208.
- Cox, P. L., Heotis, J. P., Polin, D., Rose, G. M. (1969): Quantitative determination of dantrolene sodium and its metabolites by differential pulse polarography. *J. Pharm. Sci.* 58:987.
- Donegan, J. H., Donegan, W. L., Cohen, E. B. (1978): Massive hepatic necrosis associated with dantrolene therapy. *Digestive Diseases.* 23:48.
- DuSouich, P. and Correau, H. (1981): Induction of acetylating capacity with complete Freund's adjuvant and hydrocortisone in the rabbit. *Drug Metab. Disp.* 3:279.

- Dykes, M. (1975): Evaluation of a muscle relaxant: Dantrolene sodium (Dantrium). JAMA. 231:862.
- Ellis, K. O. and Carpenter, J. F. (1974): Mechanism of control of skeletal muscle contraction by dantrolene sodium. Arch. Phys. Med. Rehabil. 55:362.
- Ellis, K. O. and Wessels, F. L. (1978): Muscle relaxant properties of the identified metabolites of dantrolene. Naunyn-Schmiedeberg's Arch. Pharmacol. 301:237.
- Farrar, W. E., Jr. and Magnani, T. J. (1964): Endotoxin susceptibility following hepatic injury by carbon tetrachloride. Proc. Soc. Exp. Biol. Med. 115:596.
- Federal Drug Administration (1975): FDA Drug Bulletin. July-Aug.
- Francis, K. T. and Hamrick, M. E. (1979): Dantrolene inhibition of the hepatic mixed function oxidase system. Res. Comm. Chem. Pathol. Pharmacol. 23:69.
- Francis, K. T. and Hamrick, M. E. (1980): Effects of dantrolene on adrenal cortical function. Biochem. Pharmacol. 29:1669.
- Friebova, M. and Elis, J. (1976): Effect of splenectomy on N-acetyltransferase activity in mice. Chem. Biol. Interactions. 15:199.
- Gandolfi, A. J., MacDonald, J. R., Sipes, I. G. (1979): Comparative microsomal bioactivation of organohalogenes. Pharmacologist, 21:194.
- Gandolfi, A. J., White, R. D., Sipes, I. G., Pohl, L. R. (1980): Bioactivation and covalent binding of halothane in vitro. J. Pharmacol. Exp. Ther. 214:721.
- Gelenberg, A. J. and Poskanzer, D. C. (1973): The effect of dantrolene sodium on spasticity in multiple sclerosis. 23:1313.
- Goodman, C. R., Beckman, I., Carlson, R. (1977): Death associated with dantrolene sodium. N.Y. State J. Med. 77:1759.
- Gornall, A. G., Bardawill, C. S., David, M. D. (1949): Protein measurement with the Biuret reagent. J. Biol. Chem. 177:751.
- Graves, S., Dretchen, K. L., Kruger, G. O. (1978): Dantrolene sodium: Effects on smooth muscle. European J. Pharmacol. 47:29.
- Griffith, O. W. and Meister, A. (1979): Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). J. Bio. Chem. 254:7558.

- Guengerich, F. P. (1977): Studies on the activation of a model furan compound - toxicity and covalent binding of 2-(N-ethylcarbamoyl-hydroxymethyl)furan. *Biochem. Pharmacol.* 26:1909.
- Hackett, L. P. and Dusci, L. J. (1979): Determination of dantrolene sodium in human plasma using high-performance liquid chromatography. *J. Chromatography.* 179:222.
- Hainaut, K., Brunko, E., Desmedt, J. E. (1975): Effect of dantrolene sodium on skeletal muscle contraction. *Arch. Intern. Physiol. Biochem.* 83:162.
- Hearse, D. J. and Weber, W. W. (1973): Multiple N-acetyltransferases and drug metabolism. *Biochem. J.* 132:519.
- Herman, R., Mayer, N., Mecomber, S. A. (1972): Clinical pharmacophysiology of dantrolene sodium. *Am. J. Phys. Med.* 51:296.
- Hollifield, R. D. and Conklin, J. D. (1973): Determination of dantrolene in biological specimens containing drug-related metabolites. *J. Pharm. Sci.* 62:271.
- Johnson, M. K. (1965): The influence of some aliphatic compounds on rat liver glutathione levels. *Biochem. Pharmacol.* 14:1383.
- Johnson, M. K. (1966): Metabolism of iodomethane in the rat. *Biochem. J.* 98:38.
- Joynt, R. L. (1976): Dantrolene sodium: Long-term effects in patients with muscle spasticity. *Arch. Phys. Med. Rehabil.* 57:212.
- Lietman, P. S., Haslam, R. H. A., Walther, J. R. (1974): Pharmacology of dantrolene sodium in children. *Arch. Phys. Med. Rehabil.* 55:388.
- Lind, R. C. (1982): The role of endotoxins in halothane-associated liver injury. Masters Thesis, University of Arizona.
- Lower, G. M., Jr. and Bryan, G. T. (1973): Enzymatic N-acetylation of carcinogenic aromatic amines by liver cytosol of species displaying different organ susceptibilities. *Biochem. Pharmacol.* 22:1581.
- Lowry, O. H., Rosenbrough, N. J., Farr, A. L., Randall, R. J. (1951): Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265.

- Lundin, S. R., Uden, D. L., Hanson, R. F. (1977): Dantrolene-associated hepatitis. *Drug Intelligence and Clinical Pharmacy*. 11:278.
- Mayer, N., Mecomber, A., Herman, R. (1973): Treatment of spasticity with dantrolene sodium. *Am. J. Phys. Med.* 52:18.
- McCalla, D. R., Reuvers, A., Kaiser, C. (1971): Activation of nitro-farantoin in animal tissues. *Biochem. Pharmacol.* 20:3532.
- McLain, G. E., Jr., Sipes, I. G., Brown, B. B., Jr. (1979): An animal model of halothane hepatotoxicity: Role of microsomal enzyme induction and hypoxia. *Anesthesiology*. 57:321.
- Meyler, W. J., Mols-Thurkow, I., Scaf, A. H. J., Sargo, S., Wesseling, H. (1979): The effect of dantrolene sodium on rat skeletal muscle in relation to the plasma concentration. *European J. Pharmacol.* 53:335.
- Meyler, W. J., Wesseling, H., Agoston, S. (1976): The effects of dantrolene sodium on cardiac and skeletal muscle in rats. *European J. Pharmacol.* 39:127.
- Mills, G. C. (1960): Glutathione peroxidase and the destruction of hydrogen peroxide in animal tissues. *Arch. Biochem. Biophys.* 86:1.
- Mitchell, J. R., Jollow, D. J., Potter, W. Z., Gillette, J. R., Brodie, B. B. (1973): Acetaminophen-induced hepatic necrosis IV. Protective role of glutathione. *J. Pharmacol. Exp. Ther.* 187:211.
- Mitchell, J. R., Nelson, W. L., Potter, W. Z., Sasame, H. A., Jollow, D. J. (1976): Metabolic activation of furosemide to a chemically reactive, hepatotoxic metabolite. *J. Pharmacol. Exp. Ther.* 199:41.
- Miwa, G. T., West, S. B., Walsh, J. S., Wolf, F. J., Lu, A. Y. H. (1982): Drug residue formation from - ronidazole, a 5-nitroimidazole .III. Studies on the mechanism of protein alkylation in vitro. *Chem. - Biol. Interactions*. 41:297.
- Monster, A. W., Herman, R., Meeks, S., McHenry, J. (1973): Cooperative study for assessing the effects of a pharmacological agent on spasticity. *Am J. Phys. Med.* 52:163.
- Moore, L. (1979): Inhibition of liver-microsome calcium pump by in vivo administration of  $CCl_4$ ,  $CHCl_3$ , and 1,1-dichloroethylene (vinylidene chloride). *J. Biol. Chem.* 257:951.

- Moore, L., Davenport, G. R., Landon, E. J. (1976): Calcium uptake of a rat liver microsomal subcellular fraction in response to in vivo administration of carbon tetrachloride. *J. Biol. Chem.* 251:1197.
- Morgan, K. G. and Bryant, S. H. (1977): The mechanism of action of danrolene sodium. *J. Pharmacol. Exp. Ther.* 201:138.
- Nolan, J. P. (1975): The role of endotoxins in liver injury. *Gastroenterology.* 69:1346.
- Ogburn, R. M., Myers, R. L., Burdick, G. E. (1976): Hepatitis associated with dantrolene sodium. *Am. Intern. Med.* 84:54.
- Olsen, H. (1982): Interaction between drug acetylation and ethanol, acetate, pyruvate, citrate and L (-) carnitine in isolated rat liver parenchymal cells. *Acta. Pharmacol. et Toxicol.* 50:67.
- Omura, T. and Sato, R. (1964): The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* 239:2370.
- Pandit, S. K., Kothary, S. P., Cohen, P. J. (1979): Orally administered dantrolene for prophylaxis of malignant hyperthermia. *Anesthesiology.* 50:156.
- Peterson, F. J., Combs, G. F., Jr., Holtzman, J. L., Mason, R. P. (1982): Metabolic activation of oxygen by nitrofurantoin in the young chick. *Tox. Appl. Pharmacol.* 65:162.
- Petusevsky, M. L., Faling, L. J., Rocklin, R. E., Snider, G. L., Merliss, A. D., Moses, J. M., Dorman, S. A. (1979): Pleuropericardial reaction to treatment with dantrolene. *JAMA.* 242:2772.
- Pinder, R. M., Brogden, R. N., Speight, T. M., Avery, G. S. (1977): Dantrolene sodium: A review of its pharmacological properties and therapeutic efficacy is spasticity. *Drugs,* 13:3.
- Putney, J. W. and Bianchi, C. P. (1974): Site of action of dantrolene in frog sartorius muscle. *J. Pharmacol. Exp. Ther.* 189:202.
- Roy, S., Francis, K. T., Born, C. K., Hamrick, K. (1980): Interaction of dantrolene with the hepatic mixed function oxidase. *Res. Comm. Chem. Pathol. Pharmacol.* 3:507.
- Schneider, R. and Mitchell, D. (1976): Dantrolene hepatitis. *JAMA.* 235:1590.
- Sedlak, J. and Lindsay, R. H. (1968): Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissues with Ellman's reagent. *Anal. Biochem.* 25:192.

- Shen, E. S., Garry, V. F., Anders, M. W. (1982): Effect of hypoxia on carbon tetrachloride hepatotoxicity. *Biochem. Pharmacol.* 31:3787.
- Silverman, H. and Hikida, R. S. (1977): Membrane systems of avian hepatocytes during chronic exposure to dantrolene sodium: A morphometric, ultrastructural and histochemical study. *Tissue and Cell*, 9:507.
- Snyder, H. R., Davis, C. S., Bickerton, R. K., Halliday, R. P. (1967): 1-[(5-arylfurfurylidene)amino]hydantoins. A new class of muscle relaxants. *J. Med. Chem.* 10:807.
- Statham, H. E. and Duncan, C. J. (1976): Dantrolene and the neuromuscular junction: Evidence for intracellular calcium stores. *European J. Pharmacol.* 39:143.
- Sun, J. D. and Dent, J. G. (1980): A new method for measuring covalent binding of chemicals to cellular macromolecules. *Chem.-Biol. Interactions*, 32:41.
- Utili, R., Boitnott, J. K., Zimmerman, H. J. (1977): Dantrolene-associated hepatic injury. *Gastroenterology*, 72:610.
- Van Winkle, W. B. (1976): Calcium release from skeletal muscle sarcoplasmic reticulum: Site of action of dantrolene sodium. *Science*, 193:1130.
- Wan, H. H. and Tucker, J. S. (1980): Dantrolene and lymphocytic lymphoma. *Postgrad. Med.* 56:261.
- Wang, C. Y., Behrens, B. C., Ichihawa, M., Bryan, G. T. (1974): Nitroreduction of 5-nitrofur derivatives by rat liver xanthine oxidase and reduced nicotinamide adenine dinucleotide phosphate - cytochrome c reductase. *Biochem. Pharmacol.* 23:3395.
- Weber, W. W., Miceli, J. N., Hearse, D. J., Drummond, G. S. (1976): N-acetylation of drugs. Pharmacogenetic studies in rabbits selected for their acetylase characteristics. *Drug Metab. Disp.* 4:94.
- Weisburger, J. H. and Weisburger, E. K. (1973): Biochemical formation and pharmacological, toxicological and pathological properties of hydroxylamines and hydroxamic acids. *Pharmacol. Rev.* 25:1.

- West, S. B., Wislocki, P. G., Fiorentini, K. M., Alvaro, R., Wolf, F. J., Lu, A. Y. H. (1982): Drug residue formation from ronidazole, a 5-nitroimidazole. I. Characterization of in vitro protein alkylation. *Chem. - Biol. Interactions*, 41:265.
- West, S. B., Wislocki, P. G., Wolf, F. G., Lu, A. Y. H. (1982): Drug residue formation from ronidazole, a 5-nitroimidazole. II. Involvement of microsomal NADPH-cytochrome P-450 reductase in protein alkylation in vitro. *Chem. - Biol. Interactions*, 41:281.
- Wilkinson, S. P., Portmann, B., Williams, R. (1979): Hepatitis from dantrolene sodium. *Gut*. 20:33.
- Wood, M., Berman, M. L., Harbison, R. D., Hoyle, P., Phythyon, J. M., Wood, A. J. J. (1980): Halothane-induced hepatic necrosis in tri-iodothyronine-pretreated animals. *Anesthesiology*, 52:470.
- Wroblewski, F. and LaDue, J. S. (1956): Serum glutamic-pyruvic transaminase in cardiac and hepatic disease. *Proc. Soc. Exp. Biol. Med.* 91:569.
- Wuis, E. W., Grutters, A. C. L. M., Vree, T. B., Van Der Kleyn, E. (1982): Simultaneous determination of dantrolene and its metabolites 5-hydroxydantrolene and nitro-reduced acetylated dantrolene (F 490), in plasma and urine of man and dog by high-performance liquid chromatography. *J. Chromatography*, 231:401.
- Youngman, R. J., Osswald, W. F., Elstner, E. F. (1982): Mechanisms of oxygen activation by nitrofurantoin and relevance to its toxicity. *Biochem. Pharmacol.* 31:3723.