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CYSTAMINE TREATMENT OF CHEMICALLY-INDUCED HEPATOTOXICITY

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CYSTAMINE TREATMENT OF CHEMICALLY-INDUCED HEPATOTOXICITY

By

John Robert MacDonald

A Dissertation Submitted to the Faculty of the

COMMITTEE ON PHARMACOLOGY AND TOXICOLOGY (GRADUATE)

In partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1984

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by John Robert MacDonald

entitled Cystamine Treatment of Chemically-Induced Hepatotoxicity

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

	Sept 11, 1984 Date
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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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SIGNED: John R. MacDonald

To my Parents,

for their life-long support

of my academic ambitions

and to my wife, Sherri

for unselfish support and hard work

which have helped make this possible

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to Dr. A. Jay Gandolfi and Dr. I. Glenn Sipes whose guidance, patience and friendship were essential in bringing these efforts to fruition. I would like to thank the members of my committee, Drs. Ryan Huxtable, Tom Lindell, Tim Bowden, and Klaus Brendel for their contributions of time and expertise.

I would also like to thank the graduate students, technicians, post docs, and secretaries whose friendship, criticism, and assistance have all had positive influences on my graduate career. In particular I would like to acknowledge the major influences and companionship of Matthew Miller, Mark Lafranconi, Carl Potter, Richard Lind, Anthony DiRenzo, Steve Weldon, Steve Waters, Tom Petry, Rick Schnellmann, Dave Wiersma, Eric Stine, and Mrs. Pamela Murray.

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ABSTRACT

There are few examples of therapeutic treatments in chemically-induced toxicity compared to pretreatments that protect against chemical injury. Cystamine treatment 12 hours after carbon tetrachloride (CCl_4) was reported to have therapeutic effects on CCl_4-induced hepatic necrosis via an unknown mechanism. The objectives of this project were to develop quantitative animal models to characterize cystamine treatment of chemically-induced hepatotoxicity and to use the models to investigate possible mechanisms of the therapeutic effect.

Cystamine produced dose related therapeutic effects against both CCl_4 and galactosamine-induced hepatic necrosis in male Sprague-Dawley rats. The therapeutic effect on galactosamine-induced damage demonstrated that cystamine has therapeutic effects that are unrelated to inhibition of early biochemical events initiating damage. This was an important finding since cystamine pretreatment will prevent CCl_4-induced hepatic damage by inhibiting the bioactivation of CCl_4.

Cystamine-induced hypothermia did not cause a delay in the appearance of maximal hepatic damage. Cystamine also did not stimulate hepatic protein synthesis in intoxicated

rats. Although cystamine was reduced to cysteamine in the livers of galactosamine treated rats the hepatic sulfhydryl content was only transiently affected by cystamine.

Cystamine did not reduce toxicant-induced hepatic calcium accumulation, despite the fact that the influx of extracellular calcium into toxicant damaged cells is considered by many to be an irreversible event causing cell death. Cystamine also did not alter subcellular calcium distribution in toxicant treated rats or enhance recovery of microsomal calcium sequestration in CCl_4 treated rats.

Since cystamine is metabolized to cysteamine **in vivo** and cysteamine can chelate calcium the effect of chelating agents and cysteamine analogs on galactosamine-induced hepatic damage was tested. Therapeutic effects were observed for the calcium chelators EDTA and EGTA, agents with a chelating structure similar to cysteamine (ie. a free amine and a free sulfhydryl on adjacent carbons), or agents which may be metabolized to such structures.

The results suggest that calcium chelation may be a mechanism of therapeutic action in chemically-induced hepatotoxicity. A reduction of free calcium concentration via chelation would explain reduced cytotoxic consequences of toxicant-induced hepatic calcium accumulation.

INTRODUCTION

Exposure to potentially noxious agents has come to be an acknowledged fact of the human condition. Chemicals with the potential to produce toxic consequences may be found in the food we eat, the air we breath, the water we drink, and the drugs we consume.

Along with the realization of the ubiquitous nature of toxic agents in our lives a great deal has been learned about the how toxic agents adversely affect biological processes. The understanding of how chemicals produce toxic reactions in biological systems has made it easier to treat chemical intoxications, to identify hazardous chemicals in our environment, and to make rational decisions about regulating and controlling our exposure to toxicants.

Although much has been learned about how different chemicals cause toxicity, man may still be exposed to noxious agents accidentally, unknowingly or intentionally. Hence, it is important that rational treatments for chemical intoxications be developed. Toward this end it is desirable to know the biochemical mechanisms that initiate toxic damage to cellular or organ function. In be too late to prevent the biochemical events initiating

the toxicity. Therefore, for treating chemical intoxications it is probably more important to understand how the initial biochemical lesion progresses to a life threatening condition or to permanent damage of an organ.

Many compounds or treatments that prevent the effects of known toxicants from being expressed have been described. These have been very useful tools in understanding how chemicals initiate toxic injury in animals. Elucidating the biochemical and/or physiologic alterations induced by the preventive agents or treatments has yielded valuable knowledge about the mechanisms via which toxicants themselves cause damage to cells, organs, or organisms.

While studying means by which toxicity may be prevented is a very useful means of discerning mechanisms by which toxic reactions are initiated. However, the real world offers few instances in which dangerous toxic exposures may be anticipated and preventive or protective agents administered prior to an exposure. Generally, direct exposure to acute or potent toxicants is avoided. When exposure to acute toxicants does occur it is usually unintentional, accidental, or the result of an intentional poisoning. Since treatment would normally follow exposure, agents which strictly prevent or protect against specific toxicities may be of little or no use in treating

cases of human intoxication. Therefore, agents which have therapeutic effects when administered following an exposure to a toxicant are inherently of greater use and interest to the clinician than agents which can only prevent toxicant-induced damage when administered concurrently or prior to toxicant exposure.

There are many agents that protect against the toxic effects of specific toxicants. However, there are few therapeutic treatments that are effective when administered after toxicant exposure. Therapeutic intervention in chemical intoxications often consists of attempts to remove the toxicant from the body by emesis, dialysis, or perfusion techniques and supportive treatments for toxicant-induced alterations of biochemical or physiological homeostasis.

Even when a specific therapeutic treatment is widely accepted the mechanism of therapeutic action is not always clear. For instance, N-acetylcysteine has been used to treat the hepatotoxic effects of acetaminophen overdose (Prescott, et al, 1977). Acetaminophen is metabolized by the liver to produce electrophilic reactive intermediates that react with glutathione and other tissue nucleophiles resulting in the depletion of hepatic glutathione stores, covalent binding to tissue macromolecules, and coagulative centrilobular necrosis of the liver (Mitchell, et al, 1973

a and b, Jollow, et al, 1973 and Potter, et al, 1973). N-Acetylcysteine was simply thought to provide additional nucleophilic sulfhydryl groups to react with electrophilic reactive intermediates of acetaminophen metabolism, reducing the depletion of glutathione, covalent binding, and necrosis (Buckpitt, et al, 1979). However, recent reports suggest that N-acetylcysteine may also inhibit gastric emptying, delaying the absorption of ingested acetaminophen (Whitehouse, et al, 1981). In addition, it has recently been shown that N-acetylcysteine does not react directly with reactive intermediates of acetaminophen **in vivo**, but must be deacetylated within liver parenchymal cells to cysteine which in turn, is incorporated into glutathione before a reaction with metabolites of acetaminophen occurs (Lauterburg, et al, 1983).

By achieving a better understanding of the mechanisms of therapeutic actions of various agents in chemical intoxications it is hoped that more effective therapeutic agents and treatment regimens for chemical and drug intoxications may be developed. The development of animal models for assessing the **in vivo** efficacy of putative therapies for chemical intoxications are therefore important both in the identification of new therapeutic agents and as a tool for investigating the mechanisms of

therapeutic action.

A Potential Model Therapeutic Agent in Chemically-Induced Hepatotoxicity

A potential example of an agent having therapeutic effects against chemically-induced hepatotoxicity was reported by DeFerreyra, et al, (1977). Cystamine appeared to reduce the degree of carbon tetrachloride (CCl_4)-induced hepatotoxicity attained when cystamine was administered 12 hours after the toxicant. This was an intriguing finding for several reasons. Prior to this report no other compound had been found to affect CCl_4-induced hepatotoxicity when given so long after the toxicant. In addition to an apparent therapeutic effect cystamine may also protect against CCl_4-induced hepatotoxicity when given prior to CCl_4 (Castro, et al, 1972 and 1973). This protective effect appeared to be the result of the binding of cystamine to hepatic cytochrome P-450, thus inhibiting the bioactivation of CCl_4 to toxic reactive intermediates (Castro, et al, 1973). However, the therapeutic effect of cystamine on CCl_4-induced hepatic injury when administered 12 hours after CCl_4 was believed to occur by a different mechanism, since hepatic biotransformation of CCl_4, covalent binding of reactive intermediates to cellular macromolecules, and CCl_4-induced lipid peroxidation all reach maximal levels within the

liver much earlier than 12 hours after CCl_4 administration (Uehleke and Werner, 1975, Rao and Recknagel, 1968 and Reynolds and Ree, 1971). Cystamine also appears to have therapeutic effects against other hepatotoxicants that must undergo bioactivation to produce hepatic damage. Hepatic injury due to either dimethylnitrosamine, thioacetamide, or bromobenzene were reduced when cystamine were administered within 3 hours after dimethylnitrosamine or 12 hours after bromobenzene or thioacetamide (DeFerreyra, et al, 1979). It was speculated that cystamine must alter the response of hepatic parenchymal cells to the toxicants, but the authors did not suggest any mechanism by which this could occur. Achieving a better understanding of the mechanism of this therapeutic effect could lead to the rational development of therapeutic agents effective against a variety of hepatotoxicants. Since no organ or biologic process is immune from some type of toxic damage, an understanding of the mechanism of action of cystamine in chemically-induced hepatotoxicity may have wider implications and applications.

Study Rationale

The mechanistic study of therapeutic effects in chemically-induced hepatotoxicity offers an advantage over the study of therapeutic effects against toxicity produced in other organs. The liver is very homogeneous in comparison to other organs in terms of diversity of cell types and functions. This relative homogeneity can be used to advantage to discern biochemical effects of putative therapeutic agents, since toxic effects in other organs may be the result of damage to highly specialized sub-populations of cells comprising only a small fraction of the total cell population of the organ.

The reported therapeutic effect of cystamine treatment after CCl_4 on CCl_4-induced hepatotoxicity (DeFerreyra, et al, 1977) suggested a possible model for studying a unique or novel mechanism of therapeutic action in intoxicated animals. In addition, an adequately developed animal model based on this observation could provide valuable information about critical biological processes altered between the initial insult to the liver and toxic cell death. An understanding of these processes could be utilized in the development of therapeutic agents for use in treatment of human intoxications.

In order to investigate possible mechanisms of a therapeutic effect of cystamine in chemically-induced

hepatotoxicity it was necessary to establish quantitative animal models of cystamine treatment of chemically-induced hepatotoxicity. Previous reports did not clearly quantify nor adequately describe the effect of cystamine treatment on measures of hepatotoxicity. Histological examinations were only semi-quantitative; only a single time point was assessed as the end point for toxicity; and therapeutic effects and the effect of varying cystamine doses was not examined. For this study three major assessments of hepatocellular injury were used in establishing animal models. The extent of hepatocellular necrosis was determined quantitatively using the method of Chalkley (1949) as described by MacDonald, et al, (1982). Increases in the activity of hepatic enzymes in plasma and increases in total hepatic calcium content were also assessed as quantitative measures of chemically-induced hepatic damage. By utilization of these measures of hepatic damage, the therapeutic effect of cystamine can be defined and characterized quantitatively in an animal model for dose dependency of therapeutic effect, time dependency of cystamine administration, and time dependency of hepatotoxic assessment. A quantitatively described model may then be employed to investigate possible mechanisms of therapeutic effects.

Properties of Cystamine

Figure 1. Structure of Cystamine.

Cystamine (Fig. 1) is the disulfide form of cysteamine (B-mercaptoethylamine). Both of these compounds have been studied extensively in connection with their radioprotective properties. Unlike the reported therapeutic effect of cystamine on CCl_4-induced hepatic injury however, cystamine or cysteamine must be administered prior to radiation challenge to be effective (Bacq, 1965). Radioprotective doses of cystamine are reduced to cysteamine **in vivo** (Sorbo, 1962 and Heiffer, et al, 1962) suggesting that cysteamine is the active radioprotective species. Several mechanisms have been proposed to explain the observed radioprotective effects of cystamine/ cysteamine. Cystamine/cysteamine induced tissue hypoxia, formation of mixed disulfides with target proteins, and general metabolic alterations have all been convincingly discounted (Bacq, 1965). Detoxification of radiation-induced free radicals appears to be the most tenable mechanism of the radioprotective effects of these agents (Bacq, 1965). This would not appear to be the

mechanism of the therapeutic effect of cystamine on CCl_4-induced hepatic damage when administered 12 hours after CCl_4, since free radicals produced by the bioactivation of CCl_4 and secondary lipid peroxides are formed rapidly from the metabolism of CCl_4 (Albano, et al, 1982 and Reynolds and Ree, 1971). Furthermore, cystamine/ cysteamine must be present at the time of radiation-induced free radical generation in order to have radioprotective effects (Bacq, 1965).

Possible Mechanisms of Cystamine's Therapeutic Action

Inhibition of CCl_4 Bioactivation

Bioactivation via cytochrome P-450 mediated metabolism to reactive intermediates is the critical initiating event in CCl_4-induced hepatotoxicity (Recknagel, 1967). Cystamine pretreatment prevents CCl_4-induced hepatic damage by inhibiting the bioactivation of CCl_4 to reactive intermediates (Castro, et al, 1973). Since CCl_4 is almost completely cleared from the liver by 12 hours after an intraperitoneal administration (Castro, et al, 1972), cystamine treatment 12 hours after CCl_4 is not expected to alter bioactivation of CCl_4 in any significant manner. However, one cannot discount the possibility that some small degree of bioactivation of

CCl_4 at later time points may cause some further critical injury to toxicant damaged cells from which they cannot recover. Cystamine could inhibit residual CCl_4 activation at this critical juncture and produce a therapeutic effect via the same basic mechanism as its protective effect against CCl_4. One way to test this hypothesis is to determine whether cystamine had therapeutic effects against hepatotoxicants that do not require cytochrome P-450 bioactivation or do not involve reactive metabolic intermediates in the production of hepatic damage.

D(+)-Galactosamine is classified as an indirect hepatotoxicant (Zimmerman, 1978) which is not metabolized to electrophilic metabolic intermediates. The mechanism of hepatotoxicity of galactosamine is linked to the depletion of hepatic uridine stores by the formation of uridine-5′-diphosphate (UDP)-hexosamines (Decker and Keppler, 1972) as shown in figure 2. Depletion of uridine stores was confirmed as the biochemical event initiating galactosamine-induced hepatic injury by the ability of exogenous uridine to prevent the expression of toxicity when administered within 2-3 hours after galactosamine (Farber, et al, 1973). A therapeutic effect of cystamine on galactosamine-induced hepatic injury would demonstrate that cystamine has therapeutic effects in chemically-induced hepatic injury other than inhibition of the

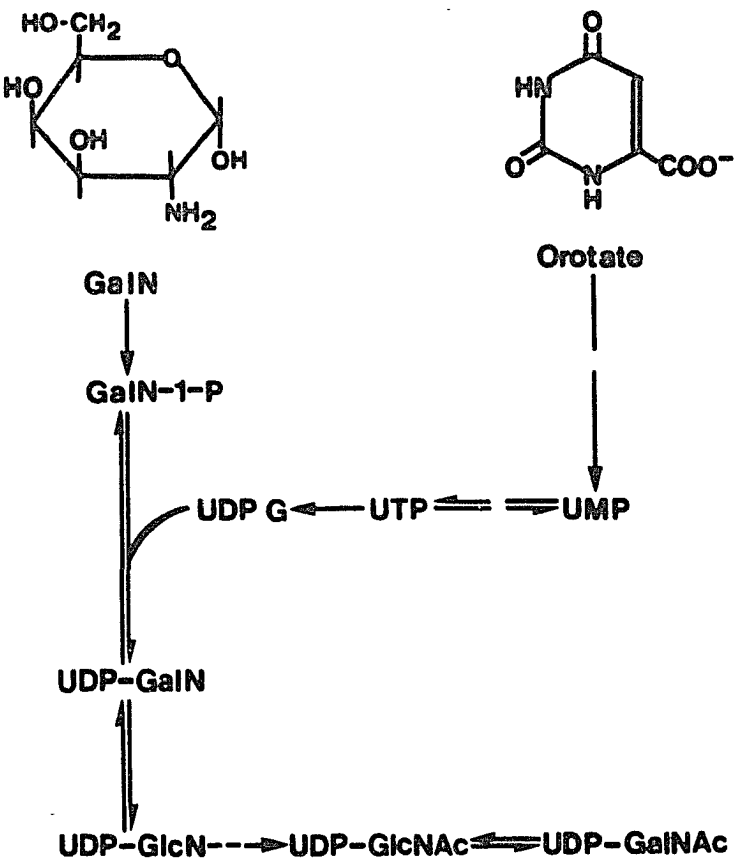


Figure 2. Metabolism of galactosamine. From Decker and Keppler (1972).

bioactivation of CCl_4.

Cystamine-Induced Hypothermia

Cystamine administration produces a hypothermic response in rats (Castro, et al, 1973). Hypothermia has been shown to be responsible for the apparent protective effect of spinal cord transection on CCl_4-induced hepatic liver injury when assessed 24 hours after the toxicant (Larson and Plaa, 1963 and 1965). When hepatic damage was assessed 48 and 72 hours after CCl_4, however, no protection was observed. Furthermore, when animals with transected spinal cords were artificially warmed to normal physiologic temperatures hepatic damage developed over the same time course as CCl_4 intoxicated animals with intact spinal cords. Therefore, the apparent protective effect observed by Calvert and Brody (1960) was simply a prolonging of the appearance of CCl_4-induced hepatic damage. It is important, therefore, to determine whether therapeutic administration of cystamine after CCl_4 or other hepatotoxicants results in a temperature-dependent shift in the time course for the appearance of hepatic damage.

Alterations in Hepatic Sulfhydryl Levels

$$NH_2CH_2CH_2S\text{-}SCH_2CH_2NH_2 \xrightarrow[\text{equivalents}]{\text{reducing}} 2\ NH_2CH_2CH_2SH$$

Figure 3. Reduction of cystamine to cysteamine.

Cystamine is rapidly reduced in vivo to cysteamine (Sorbo, 1962) as shown in figure 3. This may cause a transient increase in total free sulfhydryls within the liver (Fillipovich, et al, 1970). This increase represented approximately a 10% elevation above normal liver free sulfhydryl levels and lasted for 1 or 2 hours.

Alterations of free sulfhydryl content of the liver should be considered as a possible mechanism for the therapeutic effect of cystamine, since nucleophilic sulfhydryl compounds such as glutathione are known to protect against damage produced by a variety of hepatotoxicants (Mitchell, et al, 1974, Brown, et al, 1974, and MacDonald, et al, 1982). Glutathione is the most abundant free sulfhydryl compound found within cells (Kosower, 1976). This compound can react directly with some reactive intermediates produced by bioactivation of a number of hepatotoxicants (Mitchell, et al, 1976). Glutathione may also act as a substrate of glutathione transferases for the detoxification of toxicants (Jakoby,

1978) or as a free radical scavenger acting to detoxify peroxides and terminate peroxidation reactions (Chance, et al, 1978).

Since cystamine may alter the free sulfhydryl content of liver it was therefore important to determine the effect of cystamine on hepatic sulfhydryl content to evaluate the possible contribution of increased tissue sulfhydryl to the observed therapeutic effects.

Effects on Repair Processes

Another means by which cystamine could produce an apparent therapeutic effect on CCl^4-induced hepatic injury would be by stimulating repair processes in toxicant damaged cells. Many of the early biochemical events of CCl^4-induced hepatic damage have already occurred when cystamine is administered to rats 12 hours after CCl^4. Many structural and functional aspects of liver cells will have been altered by the effects of covalent binding of CCl^4-derived reactive intermediates to cellular constituents and by lipid peroxidation occurring prior to cystamine administration. If a cell is to survive toxicant-induced perturbations of structure and function, the proteins and lipids altered by the toxicant eventually must be repaired or replaced. If cystamine were capable of stimulating a more rapid or efficient repair within

toxicant damaged cells the net result could be fewer damaged cells progressing from being a toxicant damaged cell to a necrotic cell. This would then appear as a therapeutic effect of cystamine administration.

Effect on Calcium Homeostasis and Distribution

The therapeutic effect of cystamine when administered long after the early cytotoxic biochemical effects associated with CCl^4-induced hepatotoxicity, suggests that cystamine may affect a key cytotoxic event occurring late in the progression from initial toxic cell injury to cell death. Such an event may be the loss of the cells ability to regulate its intracellular calcium concentration which is believed to be an irreversible lethal event (Farber and El-Mofty, 1975 and Farber, 1979).

Calcium accumulates in necrotic tissues (Thiers, et al, 1960 and Reynolds, et al, 1962) and mitochondrial calcium accumulation occurs soon after toxic cell injury (Reynolds, et al, 1962). More recently, calcium influx into damaged liver cells has been postulated to be the key event occurring late in toxic cell injury that leads irreversibly to hepatic cell death caused by CCl^4 (Farber, 1979), D(+)-galactosamine (Farber and El-Mofty, 1975), anoxia (Okuno, et al, 1983), and a variety of other agents, including the calcium ionophore A23187 (Schanne,

et al, 1979). The negative influence of calcium accumulation on cell survival is clearly demonstrated by the rapid cell death observed in the presence of the calcium ionophore, A23187, and physiologic extracellular calcium concentrations and the lack of cell death in the presence of the ionophore and low extracellular calcium in both primary hepatocyte monolayer cultures (Schanne, et al, 1979) and cultured fibroblasts (Shier and DuBourdieu, 1982).

It is not surprising that the maintenance of cellular calcium homeostasis is so important for cell survival, given the many biological effects of calcium that may be considered detrimental to normal cellular function (Figure 4). Toxicant-induced alteration of cell membrane permeability to calcium or other perturbations in calcium homeostasis leading to increased intracellular free calcium concentrations may produce depolymerization of cytoskeletal proteins (Sauk, 1976) and activation of phospholipases (Chien, et al, 1980), both of which may exacerbate the challenge to the cell by further increasing cell membrane permeability. Toxicant-induced increases in free intracellular calcium concentrations will also lead to increased mitochondrial accumulation of calcium (Reynolds, 1964 and Cohn, et al, 1968). This can lead to an inhibition of normal mitochondrial function and a

decrease in ATP production (Reynolds, et al, 1962 and Reynolds, 1964). ATP is required for the function of calcium pumps in the endoplasmic reticulum (Moore, et al, 1975), mitochondria (Lehninger, et al, 1978), and the plasma membrane (Izutsu and Smuckler, 1978) which help to control intracellular calcium concentrations by active sequestration of calcium within membranes or extrusion from the cell.

Calcium homeostasis is clearly an important cellular function which is susceptible to perturbations by toxicants with potentionally lethal consequences for the cell. In fact, inhibition of energy dependent sequestration of calcium by the hepatic endoplasmic reticulum occurs within minutes of CCl^4 exposure (Moore, et al, 1976). This is the earliest occurring loss of a normal cellular function following the administration of CCl^4 with subsequent influxes of extracellular calcium presumed to represent the point of irreversibility of the toxicant-induced damage. It was therefore of great interest to determine whether cystamine had any effect on toxicant-induced hepatic calcium accumulation or calcium homeostasis, since cystamine-induced alterations could account for cystamine's therapeutic effects in CCl^4-induced hepatotoxicity.

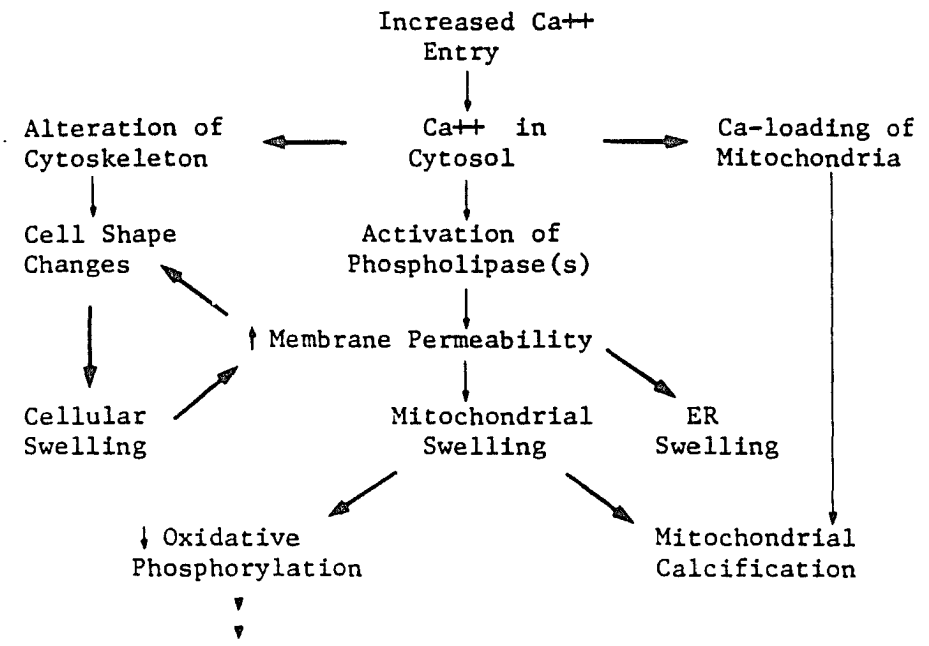


Figure 4. Consequences of cellular calcium accumulation. From Trump, et al, (1980).

Statement of the Problem

Cystamine has been reported to have a therapeutic effect on CCl^4-induced hepatic toxicity in rats, when administered orally up to 12 hours after the toxicant. The mechanism of the therapeutic effect is unknown, but has been postulated to be attributable to late effect on toxicant damaged cells which allowed them to survive the initial biochemical perturbations caused by the toxic insult. The purpose of this research endeavor was to establish quantitative animals for assessing the therapeutic effect of cystamine and other agents in chemically-induced hepatotoxicity and to use the animal models to investigate possible mechanisms of the observed therapeutic effect.

MATERIALS AND METHODS

Experimental Animals

Male Sprague-Dawley rats weighing at least 200 g were used in all experiments. The rats were obtained from the breeding colony maintained by the Division of Animal Resources of the Arizona Health Sciences Center and housed in an isolated room with an independent air source in stainless steel wire cages. The animals were maintained on a 12 hour light/dark cycle and allowed standard laboratory chow (Wayne Lab Blox) and water ad libitum unless otherwise noted.

Chemicals

Hepatotoxicants

Spectrophotometric grade carbon tetrachloride was purchased from Mallinckrodt Chemical Works (St. Louis, MO). D(+)-Galactosamine was obtained as the hydrochloride salt from Sigma Chemical Company (St. Louis, MO).

Hepatoprotective Agents

Cystamine dihydrochloride, cysteamine hydrochloride, D(-) penicillamine, taurine, 2-aminoethylisothiouronium bromide (2-AET), N-acetylcysteine, thioproline (L-thiazolidine-4-carboxylic acid), uridine, ethylenediamine-

ethyl ether) N,N′-tetraacetic acid (EGTA) were all purchased from Sigma Chemical Company (St. Louis, MO). N-Acetylcysteamine was purchased from Chemical Dynamics Corporation (South Plainfield, NJ) and S-methylcysteamine from Fluka Chemical Corporation (Hauppauge, NY). Dimercaptopropane-sulfonic acid (DMPS) was obtained from Johnson and Johnson Co. and dimercaptosuccinic acid (DMSA) from Heyl and Company (Berlin).

Radiochemicals

Tritiated thymidine (specific activity, 20 Ci/mmole)for the assessment of hepatic DNA synthesis and ^{14}C labeled leucine (specific activity, 339 mCi/mmole) for the assessment of hepatic protein synthesis were obtained from New England Nuclear Corporation (Boston, MA). Radioactive $^{45}CaCl_2$ (specific activity, 14.40 mCi/mg) for the assessment of calcium sequestration by isolated microsomal vesicles was also obtained from New England Nuclear.

Reagents

All other chemicals used in these studies were of reagent grade or better.

Animal Treatments

Carbon tetrachloride (CCl_4) was administered at a dose of 2.5 ml/kg as a 50% solution in corn oil by oral intubation (po) or 0.25 ml/kg as a 25% solution in corn oil by intraperitoneal injection (ip). Animals receiving CCl_4 orally were fasted for 14 hours prior to administration to prevent possible interference with the absorption of the toxicant. Chow was then allowed ad libitum 6 hours after administration of CCl_4. Animals receiving CCl_4 by ip injection were allowed free access to food and water at all times.

D(+)-Galactosamine hydrochloride was administered Intraperitoneally at a dose of 400 mg/kg as a 200 mg/ml aqueous solution neutralized with NaOH. Animals receiving galactosamine were allowed free access to food and water at all times.

Cystamine dihydrochloride in aqueous solution was neutralized with NaOH and doses up to 900 mg/kg were administered po at various times before or after hepatotoxicant administration. All other agents administered as putative protective or therapeutic agents were prepared in pH neutral aqueous solution and administered by ip injection. Cysteamine hydrochloride, N-acetylcysteamine, S-methylcysteamine, D(-)penicillamine, taurine, thioproline, dimercaptopropanesulfonic acid

(DMPS), and dimercaptosuccinic acid (DMSA) were all administered at a dose of 1.5 mmol/kg. EDTA and EGTA were administered at a dose of 0.75 mmol/kg. 2-Aminoethylisothiouronium bromide (2-AET) was administered at doses of 1.5 and 0.5 mmol/kg, ip. Uridine was administered to galactosamine treated rats at a dose of 1200 mg/kg.

Animals were killed by cervical dislocation at different times following hepatotoxicant administration. Heparinized blood was drawn from the inferior vena cava for quantification of plasma enzyme markers of hepatic damage. Hepatic sections from the left lobe of the liver (1 mm thick) were fixed in phosphate buffered formalin for histologic processing. The remaining hepatic tissue was then frozen and stored at -20 ^{o}C for subsequent analysis of tissue calcium levels.

Plasma Enzyme Markers of Hepatic Damage

Glutamate-pyruvate transaminase (GPT) activity was determined in heparinized plasma samples by the method of Wroblewski and LaDue (1956) as described in Sigma Technical Bulletin No. 155-UV (1977). The assay depends on a coupled reaction which is rate limited by GPT dependent conversion of alanine and a-ketoglutarate to glutamate and pyruvate. In the presence of NADH and lactate dehydrogenase, pyruvate is rapidly reduced to lactate and

NADH oxidized to NAD. The assay determines the rate of consumption of NADH by measuring the decline in absorbance by NADH at 340 nm over time using a Beckman ACTA CIII spectrophotometer. In this assay the rate of NADH consumption is a direct function of the amount of GPT activity in a given plasma sample. The results are expressed as Wroblewski-LaDue units/ml of plasma.

Isocitrate dehydrogenase (ICDH) activity was determined in some heparinized plasma samples according to the method described in Sigma Technical Bulletin No. 153-UV (1977). This enzyme in the presence of manganese catalyzes the formation of NADPH, CO_2, and a-ketoglutarate from L-isocitrate and NADP. The activity of the enzyme present in plasma is directly proportional to the rate of increase of absorbance measured at 340 nm due to the formation of NADPH. The results are expressed as Sigma units/ml.

Plasma activity of ornithine carbamyl transferase (OCT), a mitochondrial enzyme, was also determined by a spectrophotometric method (Snodgrass and Parry, 1969) as an indicator of hepatic damage. This method employs OCT catalyzed conversion of carbamyl phosphate and ornithine to citrulline. Citrulline is then quantitated by reaction with diacetyl monoxime and phenazone which forms a yellow complex with an absorbance maximum at 464 nm and a molar

absorptivity of 37,800. Sample absorbance was determined using a Gilford Instruments Stasar II spectrophotometer at 464 nm and the results were expressed as milli-international units/ml of plasma.

Histologic Examination

The extent of toxicant-induced hepatic necrosis was evaluated by light microscopic examination of hepatic sections. Thin sections (1 mm) from the left lobe of the liver were taken immediately after removal of the whole liver. The tissue was then fixed in phosphate buffered formalin. The tissue was processed using standard techniques of histological preparation (Luna, 1968) by the Division of Animal Resources of the Arizona Health Sciences Center. Sections (5 micron) were cut from the paraffin embedded tissue, slide mounted, and stained with eosin and counterstained with hematoxylin (H&E stain) for assessment of hepatocellular necrosis.

The extent of necrosis was quantified in individual sections using the method of Chalkley (1949) as modified and described previously (MacDonald, et al, 1982). Nine reference points on an ocular reticle grid in 20 randomly selected 400X fields were scored as corresponding to a necrotic hepatocyte, a non-necrotic hepatocyte, or non-hepatocyte. The results are then expressed as the

percentage of total hepatocytes scored as necrotic for each section.

A mitotic index was calculated from histologic examination as a quantitative measure of hepatic regeneration following toxic insult. The mitotic index is the mean number of mitotic figures observed per 400X field in 20 random fields in H & E stained hepatic sections.

Tissue and Subcellular Ca^{2+} Analysis

The total calcium content of hepatic tissues and hepatic subcellular fractions was determined by flame emission spectrophotometry (MacDonald, et al, 1984). Tissue and subcellular fractions were prepared for analysis by nitric acid digestion (Chvapil, et al, 1974). The calcium content of whole liver homogenates was expressed as micrograms of calcium/g of tissue (dry weight) or parts per million (dry weight).

Subcellular fractions were prepared for calcium analysis in the following manner. Freshly excised livers were homogenized in 4 volumes of an ice cold buffer consisting of 24 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 75 mM sucrose, 225 mM mannitol, and 1 mM EDTA at a pH of 7.2. This buffer was selected for its ability to limit calcium redistribution during preparation of subcellular fractions (Cohn, et al, 1968).

Mitochondrial, microsomal, and cytosolic fractions were then isolated by differential centrifugation (Cohn, et al, 1968) and analyzed for calcium as above. The calcium content of subcellular fractions was expressed as micrograms of calcium/g of tissue (wet weight) or parts per million (wet weight).

Body Temperature Measurements

Body temperature of the rats following CCl_4 and cystamine treatments was monitored using a Series 400 small animal rectal probe (Yellow Springs Instrument Company) attached to a YSI Model 46 TVC Telethermometer.

Hepatic DNA Synthesis

Hepatic DNA synthesis following CCl_4 and cystamine treatments was assessed by quantitating the incorporation of ^{3}H-thymidine into hepatic DNA at different times after CCl_4. ^{3}H-Thymidine (50 µCi/animal) in sterile physiologic saline (200 µl total volume) was administered by lateral tail vein injection 2 hours prior to end points of 24, 36, and 48 hours after CCl_4 administration. Freshly excised hepatic tissue was homogenized in 50 mM Tris buffer (pH 7.4) and hepatic DNA isolated by the sequential acid hydrolysis technique of Ruddick and Runner (1974). Extracted DNA was quantitated by the diphenylamine assay

(Burton, 1956) and incorporation of ^{3}H-thymidine was determined by liquid scintillation spectrophotometry. The results were expressed as DPM's/μg DNA.

Calcium Sequestration by Hepatic Endoplasmic Reticulum

The ability of isolated endoplasmic reticulum (microsomes) to actively sequester ionic calcium was assessed as described by Moore, et al, (1975) following CCl_4 and cystamine treatments. At different times after CCl_4 rats were killed by cervical dislocation, the livers rapidly excised and homogenized in ice-cold 0.25 M sucrose. The homogenate was then centrifuged at 1500 X g for 10 minutes and then at 12,500 X g for 20 minutes in a Sorvall RC-2B refrigerated centrifuge. Microsomal vesicles in the supernatant were then sedimented by centrifuging at 50,000 rpm for 40 minutes in a Beckman 50.2 TI rotor on a Beckman L8-55 preparative ultracentrifuge. Microsomal vesicles were resuspended in incubation buffer and the protein content was assayed (Lowry, et al, 1951).

The basic incubation buffer consisted of 100 mM KCl, 30 mM histidine and 30 mM imidazole adjusted to pH 6.8. Additions to the buffer prior to incubation were made to yield the following concentrations: between 0.075 and 0.15 mg microsomal protein/ml, 5 mM sodium azide (to

inhibit calcium uptake by any mitochondria contaminating the preparation), 5 mM $MgCl_2$, 5 mM ATP (pH adjusted to 6.8 with imidazole prior to addition), 20 μM $CaCl_2$ and 0.1 μCi/ml $^{45}CaCl_2$ in a total volume of 3 ml/incubation.

Incubations were conducted at 37 °C and initiated by the addition of microsomes. At various times 1 ml aliquots of the incubation mixture were removed and filtered through Whatman GF/F glass fiber filters with the aid of a vacuum manifold. The filters were then washed with two 10 ml aliquots of the imidazole-histidine KCl buffer, removed and placed in mini-scintillation vials containing 4.5 ml of Cytoscint counting fluor (West Coast Scientific Co.) added. The samples were then counted to determine the ^{45}Ca radioactivity trapped on the filter. The results were then expressed as nanomoles of calcium taken up per mg of microsomal protein/30 minutes of incubation. Complete incubation systems lacking only in ATP served as background controls for non-energy dependent non specific binding of calcium to microsomal membranes.

Hepatic Non-Protein Sulfhydryl and Total Sulfhydryl Determinations

Hepatic levels of non-protein sulfhydryls and protein sulfhydryls were determined spectrophotometrically by the method of Sedlak and Lindsay (1968). This assay depends

on the reaction of Ellman's reagent (5,5'-dithiobis-nitrobenzoic acid) with free sulfhydryls at pH 8 to 9 to form a mixed disulfide and a chromophore with an absorbance maximum at 412 nm. Reduced glutathione served as the sulfhydryl standard. Determination of hepatic non-protein sulfhydryl levels involved homogenization of hepatic tissue, precipitation of proteins with 5% sulfosalicylic acid, centrifugation of the samples, and spectrophotometric determination of the free sulfhydryl content of the resulting supernatant by the addition of Ellman's reagent. Total tissue sulfhydryls were determined by reacting Ellman's reagent with whole liver homogenate, followed by sulfosalicylic acid precipitation of the proteins, centrifugation, and spectrophotometric quantification of the chromophore in the resulting supernatant. Protein sulfhydryl content was calculated as the difference between total tissue sulfhydryl levels and non-protein sulfhydryl levels.

Tissue non-protein sulfhydryl levels were also determined for hepatic tissue following a 30 minute incubation of tissue homogenate with glutathione reductase (Sigma Chemical Company, St. Louis, MO) and an NADPH generating system (Ilett, et al, 1973) in order to assess the level of acid soluble disulfide, including cystamine, present in the liver of treated rats.

Specific Cysteamine Assay

In order to determine the fate of cystamine/cysteamine in the liver of toxicant treated rats a specific assay was developed. The method employed was a an adaptation of the HPLC assay for separating low molecular weight tissue sulfhydryl compounds developed by Reeve, et al, (1980).

Hepatic tissue was homogenized in 4 volumes of ice-cold 10 mM phosphate buffer, pH 6.0. The protein in 1 ml aliquots of the homogenates were immediately precipitated by the addition of 2 ml of ice-cold 100% ethanol. Samples were vortexed and centrifuged for 20 minutes at 27,000 X g in a Sorvall SM-24 rotor in a Sorvall RC-2B refrigerated centrifuge at 4 ^{o}C. Aliquots (2 ml) of the supernatant were evaporated to dryness under nitrogen and the residue redissolved in 1 ml of 50 mM phosphate buffer, pH 8.5. Free sulfhydryls were derivatized by the addition of 10 µl of Ellman's reagent (100 mM in methanol).

Cysteamine analysis was performed reversed-phase high performance liquid chromatography using a Beckman model 322 MP liquid chromatograph with a 210 sample injector and an Altex 280 nm fixed wavelength detector. Separations were performed on an Alltech C-18 analytical column (10 micron, 250 X 4.6 mm). Sample injections were 20 µl.

Samples were eluted with ammonium formate (0.023 M, pH 5) - methanol at a flow rate of 2 ml/min. The cysteamine adduct was eluted using a 90:10 mixture of ammonium formate : methanol. Following elution of the cysteamine derivative the methanol concentration was increased to 50% to facilitate the elution of glutathione, excess unreacted Ellman's reagent, and other unidentified slowly eluting substances with absorbance properties at 280 nm. Peak areas were integrated using a Hewlett-Packard 3380A integrator. Tissue cysteamine levels were quantitated by comparison of peak areas to tissue cysteamine standards prepared by spiking homogenates from livers of untreated control rats.

Statistical Analysis

Data were analyzed by one-way analysis of variance. Group means were compared with a Student Newman-Keuls test. Differences were considered significant if $p < 0.05$.

RESULTS

The Carbon Tetrachloride Model

Time Course of CCl_4-induced Damage

Figure 5 demonstrates the time course over 24 hours of both an early and a late occurring marker of CCl_4-induced hepatic damage. Within 4 hours of administration CCl_4 caused a 90% reduction in the ability of hepatic endoplasmic reticulum to actively sequester calcium. There was a slight trend toward recovery with calcium sequestering ability reaching approximately 20% of normal values by 24 hours after CCl_4. Cytochrome P-450 content determined by a CO difference spectra was also decreased by 75% by 4 hours after CCl_4 administration (data not shown). In contrast, the plasma enzyme marker of hepatic damage, glutamate-pyruvate transaminase (GPT) activity, did not reach maximal levels until 18-24 hours after toxicant administration (Figure 5). In assessing the apparent therapeutic effects of cystamine administration after CCl_4 on CCl_4-induced hepatic damage, late occurring markers of damage such as plasma GPT levels and histologically apparent hepatocellular necrosis were routinely employed.

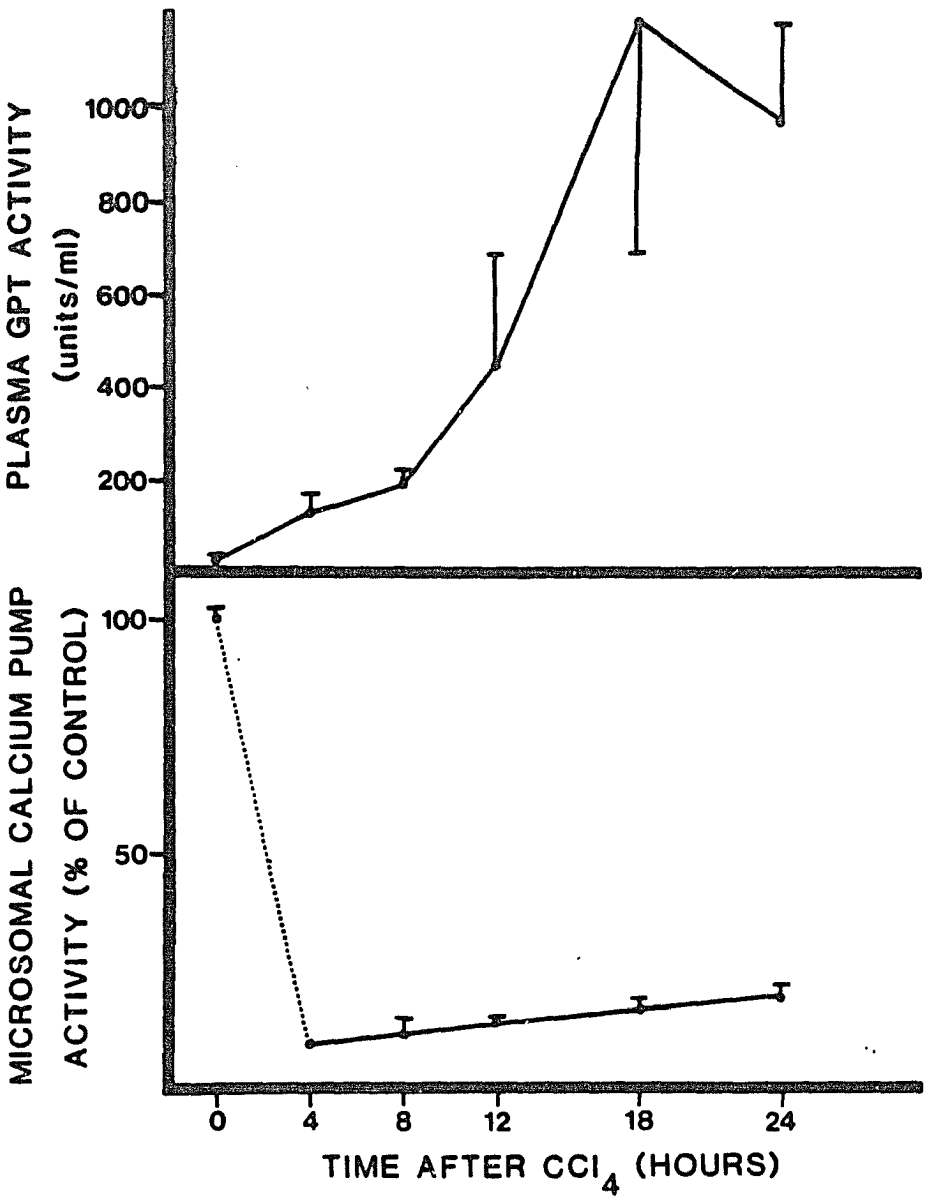


Figure 5. Time course of CCl_4-induced elevation of plasma GPT activity and inhibition of microsomal calcium pump activity. GPT activity in control animals = 24 $\pm$ 2. Microsomal calcium uptake in control animals was 33 $\pm$ 4 nanomoles/mg protein/30 minutes.

Cystamine Treatment Following Oral Administration of CCl_4

Castro, et al, (1972 and 1973) reported the protective effect on CCl_4-induced hepatotoxicity of a 600 mg/kg oral dose of cystamine administered 30 minutes or 3 hours prior to a 2.5 ml/kg dose of CCl_4. In contrast, when the same dose of cystamine was administered 6 hours after the same oral dose of CCl_4 (Table 1) only 1 of 9 rats survived 24 hours past CCl_4 administration. The mechanism of this effect is unknown, but was related to the dose of cystamine administered (Table 1). Cystamine alone, however, at an oral dose of 600 mg/kg caused no animal deaths or hepatotoxicity (data not shown). An oral dose of 300 mg/kg cystamine given 6 hours after CCl_4 resulted in significant reduction of CCl_4-induced necrosis for the 14 of 17 animals surviving 24 hours after CCl_4 (Table 1). Cystamine treatment did not prevent CCl_4-induced elevation of plasma GPT, despite reducing necrosis.

Cystamine Treatment of Intraperitoneally Administered CCl_4

Since oral cystamine administration may delay absorption of orally administered CCl_4 (Castro, et al, 1972) and since significant lethality was observed when both CCl_4 and cystamine were administered orally (Table 1), the effect of oral cystamine administration on CCl_4-

Table 1. Effect of oral cystamine administration 6 hr after orally administered CCl_4 on CCl_4-induced hepatotoxicity.[a]

cystamine dose[b] (mg/kg)	GPT[c]	24 hr survival[d]	% necrosis[e]
0	2574 ± 672	18/19	49.4 ± 2.2
300	2234 ± 537	14/17	32.0 ± 3.1[f]
600	630	1/9	---
900	---	0/4	---

a hepatotoxicity assessed 24 hr after oral administration of 2.5 ml/kg CCl_4

b administered by gastric intubation 6 hr after CCl_4

c glutamate-pyruvate transaminase (Wroblewski-LaDue units/ml plasma ± SE)

d animals surviving 24 hr / animals dosed

e % of hepatocytes scored as necrotic ± SE

f $p < 0.05$ compared to CCl_4 alone

induced hepatic damage was examined following intraperitoneal administration of CCl_4.

Within 24 hours an ip dose of 0.25 ml/kg CCl_4 produced a substantial and reproducible degree of hepatic injury with approximately 40% necrosis of the liver parenchyma and 50-100 fold elevations of plasma GPT activity evident by 24 hours after CCl_4 administration (Table 2). Orally administered cystamine reduced CCl_4-induced hepatic necrosis in a dose dependent manner when administered 6 hours after CCl_4 (Table 2). Cystamine did not reduce the extent of CCl_4-induced elevation of plasma GPT activity. As was observed with orally administered CCl_4 (Table 1), oral cystamine treatment produced some animal deaths at doses of 600 and 900 mg/kg in rats receiving CCl_4 by ip injection (Table 2). As a consequence of this finding an oral dose of 300 mg/kg of cystamine diHCl was used in further experiments.

Effect of Time of Cystamine Administration After CCl_4

Cystamine was equally effective in reducing CCl_4-induced hepatic necrosis whether administered 2, 6, or 12 hours after CCl_4 (Table 3). CCl_4-induced elevation of plasma GPT activity was not alleviated by cystamine administration at any time after CCl_4. Cystamine was administered 12 hours after CCl_4 in subsequent studies

Table 2. Dose-response of cystamine treatment 6 hr after intraperitoneally administered CCl_4 on CCl_4-induced hepatotoxicity.[a]

cystamine dose[b] (mg/kg)	GPT	24 hr survival[d]	%necrosis
0	645 ± 98	13/13	42.3 ± 3.1
150	899 ± 140	11/11	44.9 ± 5.0
300	1183 ± 309	7/7	24.2 ± 3.1[f]
600	598 ± 296	7/8	18.5 ± 7.2[f]
900	114	1/3	16.9

a assessed 24 hr after 0.25 ml/kg CCl_4

b administered by gastric intubation 6 hr after CCl_4

c Glutamate-pyruvate transaminase (Wroblewski-LaDue units/ml plasma ± SE)

d animals surviving 24 hr / animals dosed

e % of hepatocytes scored as necrotic ± SE

f $p < 0.05$ compared to CCl_4 alone

Table 3. Effect of time of cystamine administration after CCl_4 on treatment of CCl_4-induced hepatotoxicity.[a]

Time of cystamine treatment (hr after CCl_4)[b]	(n)	GPT[c]	% necrosis[d]
CCl_4 alone	(18)	739 ± 102	36.7 ± 2.2
2	(4)	476 ± 46	23.2 ± 3.6[e]
6	(7)	1183 ± 309	24.2 ± 3.1[e]
12	(12)	1296 ± 238	26.5 ± 4.1[e]

a assessed 24 hr after 0.25 ml/kg CCl_4, ip

b 300 mg/kg cystamine diHCl, po

c Glutamate-pyruvate transaminase (Wroblewski-LaDue units/ml plasma ± SE)

d % of hepatocytes scored as necrotic ± SE

e $p < 0.05$ compared to CCl_4 alone

since that time of administration most clearly focuses on late therapeutic effects of cystamine, rather than protection from early events in CCl_4-induced hepatic injury.

Histological Damage Produced By Carbon Tetrachloride

The histological damage seen 24 hours after CCl_4 (0.25 ml/kg, ip) is shown in figure 6. Characteristic "balloon" cells circumscribe a coagulative necrosis of the centrilobular region in the figure. Pyknotic nuclei, loss of normal cellular architecture and the loss of cellular boundaries may be observed. In animals receiving oral cystamine (300 mg/kg) 12 hours after CCl_4 histological damage also exhibits a centrilobular orientation bounded by "balloon" cells (figure 7). In contrast to animals receiving CCl_4 alone, however, there is a much greater preservation of both cellular and anatomical architecture of the centrilobular region.

Effect of Cystamine Treatment on Plasma Enzyme Markers of CCl_4-induced Hepatic Damage

DeFerreyra, et al, (1977) reported that cystamine treatment reduces the extent of CCl_4-induced elevation of plasma isocitrate dehydrogenase (ICDH) activity. Since plasma GPT elevation was not affected by cystamine

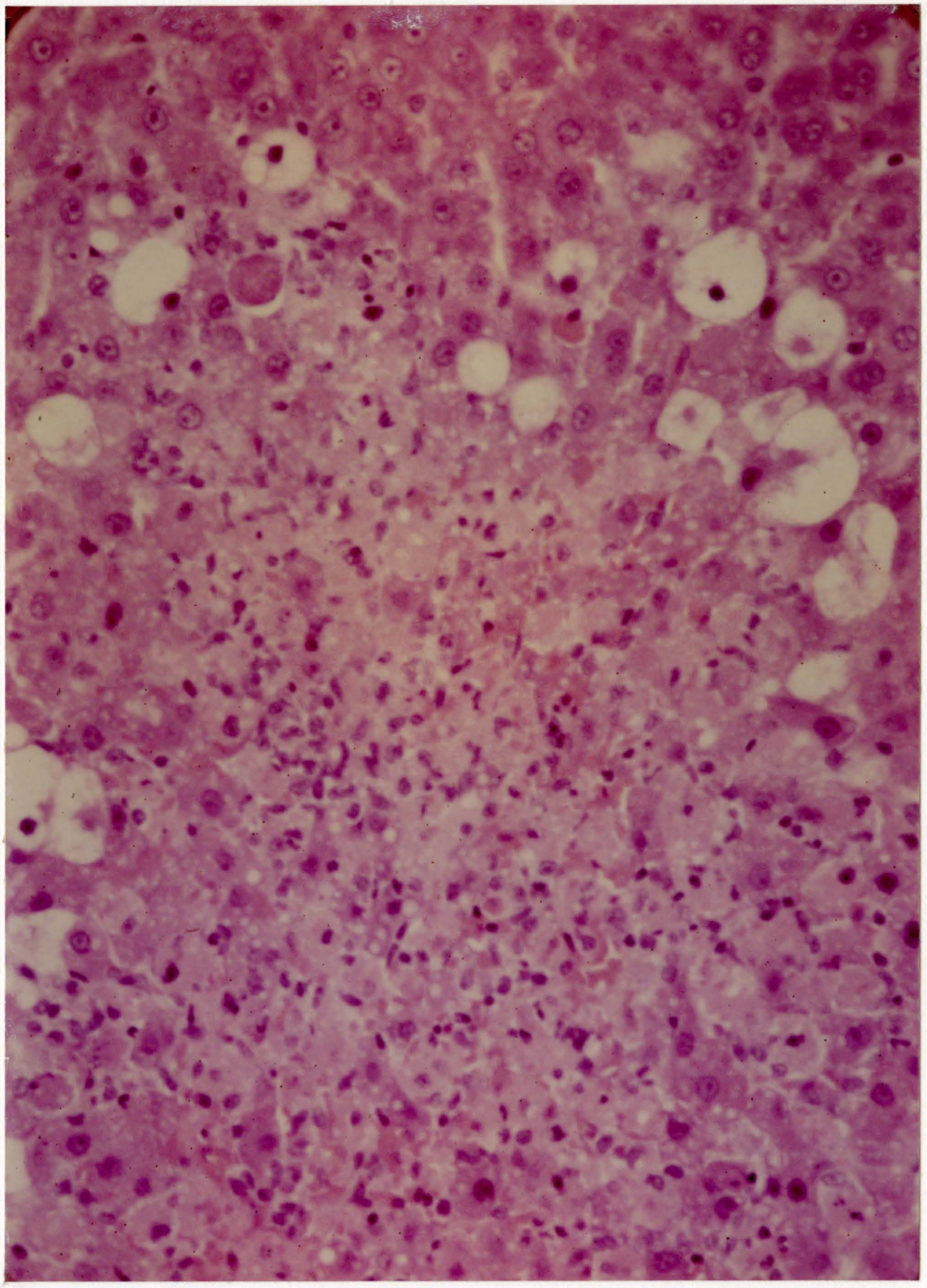


Figure 6. Hepatic histology 24 hr after CCl_4 (0.25 ml/kg, ip) (H&E x 100).

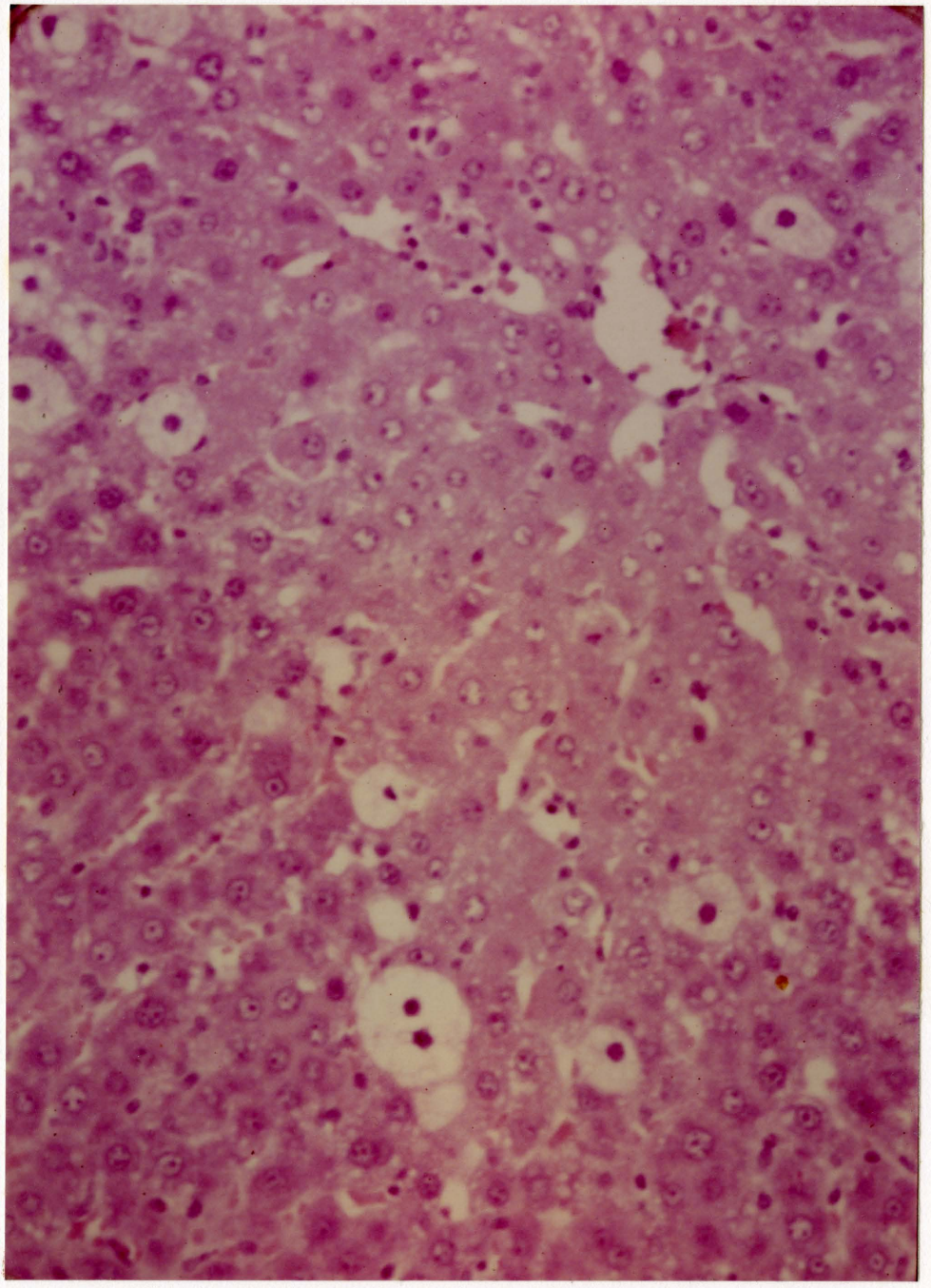


Figure 7. Hepatic histology 24 hr after CCl_4 (0.25 ml/kg, ip) and cystamine diHCl treatment (300 mg/kg, po) 12 hr later (H&E x 100).

treatment after CCl_4 other plasma enzyme markers of hepatic damage were examined as possible indicators of a therapeutic effect of cystamine. ICDH, ornithine carbamyl transferase (OCT) and GPT were assessed 24 hours after CCl_4 administration in animals receiving CCl_4 alone or CCl_4 and 300 mg/kg of cystamine po, 12 hours after CCl_4 (Table 4). While all three enzymes were markedly elevated 24 hours after CCl_4 compared to control rats, there was no apparent therapeutic effect of cystamine administration (Table 4). No elevation of GPT, ICDH, or OCT occurred in cystamine treated animals when assessed 12 hours after cystamine administration.

Effect of Cysteamine Administration on CCl_4-induced Hepatic Damage

Since cystamine is reduced to cysteamine **in vivo** (Sorbo, 1962) it was of interest to see if cysteamine would also have therapeutic effects when administered 12 hours after CCl_4. Because cysteamine is a potent ulcerogen (Lichtenberger, et al, 1977) it was administered by ip injection. As was seen following oral cystamine administration to CCl_4 intoxicated rats, cysteamine markedly decreased the extent of CCl_4-induced necrosis observed 24 hours after administration of the toxicant without reducing plasma GPT activity (Table 5).

Table 4. Plasma enzyme markers for hepatic damage in cystamine treatment of CCl_4-induced damage.[a]

	control[b]	cystamine alone	CCl_4 alone[a]	CCl_4 + cystamine[a,d]
GPT[e]	20 ± 4	26 ± 4	935 ± 238	1178 ± 210
ICDH[f]	237 ± 81	177 ± 36	2414 ± 1322	5332 ± 1485
OCT[g]	17 ± 1	16 ± 1	320 ± 129	441 ± 277

a assessed 24 hr after 0.25 ml/kg CCl_4, ip

b untreated control rats

c assessed 12 hr after 300 mg/kg cystamine diHCl, po

d 300 mg/kg cystamine diHCl, po 12 hr after CCl_4

e glutamate-pyruvate transaminase (Wroblewski-LaDue units/ml plasma ± SE)

f isocitrate-dehydrogenase (Sigma units/ml ± SE)

g ornithine carbamyl transferase (mIU/ml ± SE)

Table 5. Effect of cysteamine administration 12 hr after CCl_4 on CCl_4-induced hepatotoxicity.[a,b]

	(n)	GPT[c]	% necrosis[d]
CCl_4	(4)	630 ± 208	49.3 ± 2.4
CCl_4 + cysteamine	(4)	829 ± 248	22.8 ± 4.9[e]

a 100 mg/kg cysteamine HCl, ip

b assessed 24 hr after 0.25 ml/kg CCl_4, ip

c glutamate-pyruvate transaminase (Wroblewski-LaDue units/ml plasma ± SE)

d % of hepatocytes scored as necrotic ± SE

e $p < 0.05$ compared to CCl_4 alone

Effect of Cystamine on CCl_4-induced Alterations in Hepatic Calcium Levels

Since the influx of extracellular calcium into CCl_4 damaged liver cells has been postulated to be the key cytotoxic event leading irreversibly to hepatic cell death (Farber, 1979). It was of interest to determine whether cystamine treatment would alter CCl_4-induced hepatic calcium accumulation. Cystamine was found not to alter the extent of hepatic calcium accumulation following CCl_4 despite decreasing the extent of CCl_4-induced necrosis (Table 6).

Since cystamine administration prior to CCl_4 is presumed to protect against CCl_4-induced hepatic damage via a different mechanism than cystamine administration after CCl_4 (DeFerreyra, et al, 1977) CCl_4-induced hepatic calcium accumulation was also determined in cystamine pretreated rats. Cystamine pretreatment was found to effectively prevent CCl_4-induced hepatic calcium accumulation and elevations in plasma GPT, while almost completely preventing hepatic necrosis (Table 6). Cystamine alone had no effect on plasma GPT activity or hepatic calcium content.

Table 6. Effect of cystamine pretreatment and post-treatment on CCl_4-induced hepatic damage.[a,b]

	cystamine treatment	GPT[c]	% necrosis	hepatic Ca^{2+}
control[f]	-	20 ± 4	0	99 ± 6
cystamine alone[g]	+	26 ± 4	0	104 ± 4
CCl_4 (0.25 ml/kg) ip	-	739 ± 102	37 ± 2	280 ± 58
	30 min prior	82 ± 28[h,i]	<1%[h,i]	138 ± 19[h,i]
	12 hr after	1296 ± 238	20 ± 4	276 ± 32

a 300 mg/kg cystamine diHCl, po, 30 min prior to or 12 hr after 0.25 ml/kg CCl_4, ip

b toxicity assessed 24 hr after CCl_4

c glutamate-pyruvate transaminase (Wroblewski-LaDue units/ml plasma ± SE)

d % of hepatocytes scored as necrotic ± SE

e µg Ca^{2+}/g liver (dry weight) ± SE

f untreated control rats

g assessed 12 hr after 300 mg/kg cystamine diHCl, po

h $p < 0.05$ compared to CCl_4 alone

i $p < 0.05$ compared to CCl_4 + cystamine 12 hr later

Effect of Cystamine on Subcellular Calcium Distribution

Subcellular calcium levels were assessed after CCl_4 and cystamine treatments to determine whether cystamine altered the subcellular distribution of calcium in CCl_4 intoxicated rats. Treatment with CCl_4 caused a 10 fold increase in total hepatic calcium (ug Ca^{2+}/g liver wet weight) by 18 hours after intraperitoneal administration compared to the livers of untreated control rats. This increase was also reflected in increased subcellular concentrations of calcium (Table 7). Mitochondrial and cytosolic calcium were elevated 10 fold over control levels, while microsomal calcium content was increased by 15 fold. Cystamine treatment 12 hours after CCl_4, however, did not affect CCl_4-induced increases in any of the subcellular fractions.

Cystamine treatment 12 hours after CCl_4 also did not affect the recovery of the ability of hepatic endoplasmic reticulum to actively sequester calcium (Figure 8).

Cystamine-induced Hypothermia and Extended Time Course of Cystamine Effects on CCl_4-induced Hepatic Damage

Cystamine will produce hypothermia in rats (Castro, et al, 1973) and hypothermia produces an apparent protective effect against CCl_4-induced hepatic damage by delaying the appearance of maximal injury (Larson and

Table 7. Effect of cystamine treatment on hepatic subcellular calcium distribution in CCl_4 treated rats.[a]

	control[b]	CCl_4	CCl_4 + cystamine[c,d]
homogenate	33 ± 4	373 ± 40[e]	366 ± 20[e]
mitochondria	5.6 ± 0.2	68 ± 5[e]	65 ± 5[e]
microsomes	3.2 ± 0.2	44 ± 6[e]	43 ± 3[e]
cytosol	8.5 ± 0.6	85 ± 7[e]	72 ± 5[e]

a μg Ca^{2+} / g liver (wet weight) ± SE

b untreated control rats

c 18 hr after 0.25 ml/kg CCl_4, ip

d 300 mg/kg cystamine diHCl, po, 12 hr after CCl_4

e $p < 0.05$ compared to control

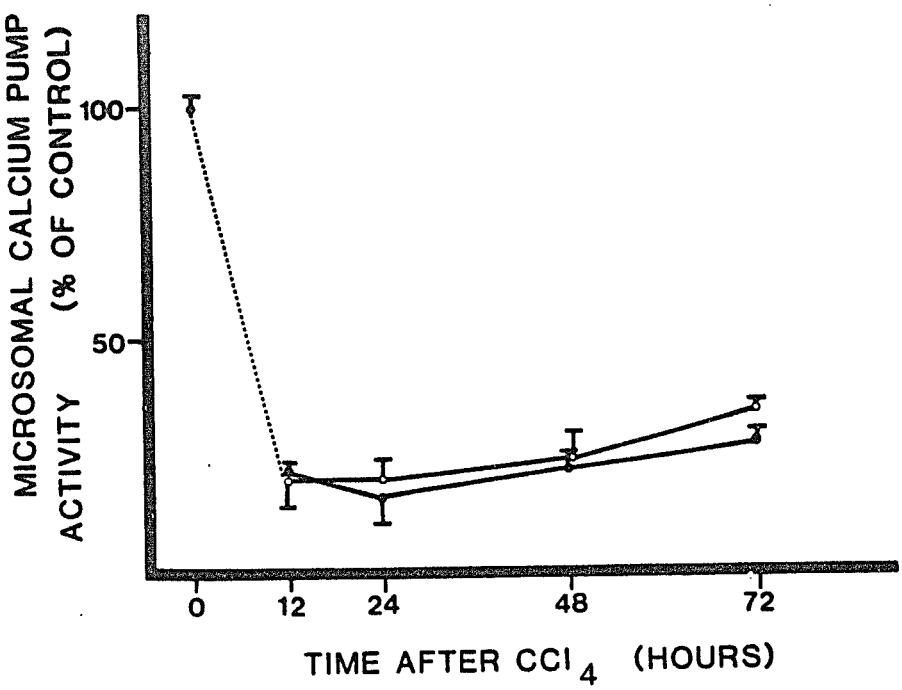


Figure 8. Effect of oral cystamine administration (300 mg/kg) on recovery of microsomal calcium pump activity following CCl_4 (2.5 ml/kg, po). CCl_4 alone (●). CCl_4 + cystamine 6 hours later (○). Control activity was 38 ± 4 nanomoles Ca^{2+}/mg protein/30 minutes.

Plaa, 1963 and 1965). To test whether cystamine-induced hypothermia contributed to the therapeutic effect on CCl_4-induced hepatic necrosis, the degree of hypothermia produced by an oral dose of 300 mg/kg was quantified and the extent of CCl_4-induced damage was assessed 24, 48, and 72 hours after CCl_4 administration in cystamine-treated rats. Cystamine produced a rapid and sustained decrease of approximately 1.5 ^{o}C in mean body temperature (Figure 9). A dose of 0.25 ml/kg of CCl_4, ip, produced no hypothermic response, nor did the combination of CCl_4 plus cystamine 12 hours later produce a greater degree of hypothermia than cystamine alone. The mild hypothermia induced by cystamine did not cause a delay in the appearance of CCl_4-induced hepatic damage. Hepatic necrosis was greatest in all rats 24 hours after CCl_4 administration with cystamine-treated rats exhibiting less hepatic necrosis 48 and 72 as well as 24 hours after CCl_4 than rats receiving CCl_4 alone. Cystamine treatment did not alter CCl_4-induced elevations of hepatic calcium levels or plasma GPT activities at any of the time points (Table 8).

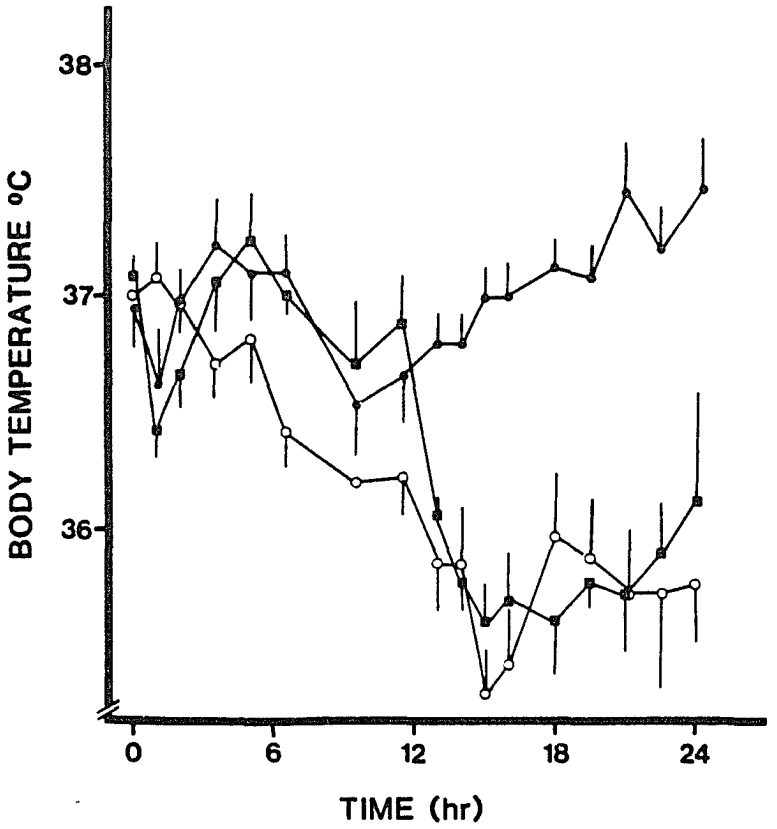


Figure 9. Cystamine-induced hypothermia in control and CCl_4-treated rats. Cystamine diHCl was administered orally (300 mg/kg) to control and CCl_4 treated rats 12 hr after CCl_4 administration (0.25 ml/kg, ip). CCl_4 alone (●), CCl_4 + cystamine (○); cystamine alone (■).

Table 8. Effect of cystamine treatment 12 hr after CCl_4 administration on CCl_4-induced hepatotoxicity 24, 48, and 72 hr after CCl_4.

Hr after CCl_4 (+/-) cystamine	(n)	GPT[c]	% necrosis[d]	hepatic Ca^{2+}[e]
24 (-)	(18)	739 ± 102	36.7 ± 2.2	280 ± 58
(+)	(12)	1296 ± 238	26.5 ± 4.1	276 ± 32
48 (-)	(7)	1232 ± 398	25.5 ± 2.3	570 ± 122
(+)	(8)	944 ± 157	19.6 ± 1.7	444 ± 32
72 (-)	(4)	138 ± 20	10.4 ± 2.3	67 ± 3
(+)	(4)	185 ± 40	4.9 ± 0.8	78 ± 12

a 0.25 ml/kg CCl_4, ip

b 300 mg/kg cystamine diHCl po, 12 hr after CCl_4

c glutamate-pyruvate transaminase (Wroblewski-LaDue units/ml plasma ± SE)

d % of hepatocytes scored as necrotic ± SE

e µg Ca^{2+}/g liver (dry weight) ± SE

f $p < 0.05$ compared to CCl_4 alone

Effects of Cystamine on Hepatic Regeneration Following CCl_4

Cystamine treatment appeared to facilitate a slightly earlier regeneration of necrotic liver tissue. Livers from animals receiving cystamine 12 hours after CCl_4 exhibited a higher mitotic index than those from livers of rats receiving CCl_4 alone (Table 9). In addition, animals receiving cystamine exhibited a higher level of ^3H-thymidine incorporation into hepatic DNA between 34 and 36 hours after CCl_4 than animals receiving CCl_4 alone (Figure 10).

Table 9. Effect of cystamine treatment 12 hr after CCl_4 administration on mitotic index of liver 24, 48, and 72 hr after CCl_4.[a]

Time after CCl_4 (hr)	cystamine treatment[b]	n	mitotic index[c]
24	-	18	0
	+	12	0
48	-	7	0.81 ± 0.22
	+	8	1.89 ± 0.62[d]
72	-	4	1.44 ± 0.22
	+	4	1.78 ± 0.36

a 0.25 ml/kg CCl_4, ip

b 300 mg/kg cystamine diHCl, po, 12 hr after CCl_4

c mitotic figures/400x microscopic field in H&E stained hepatic sections ± SE

d $p < 0.05$ compared to CCl_4 alone

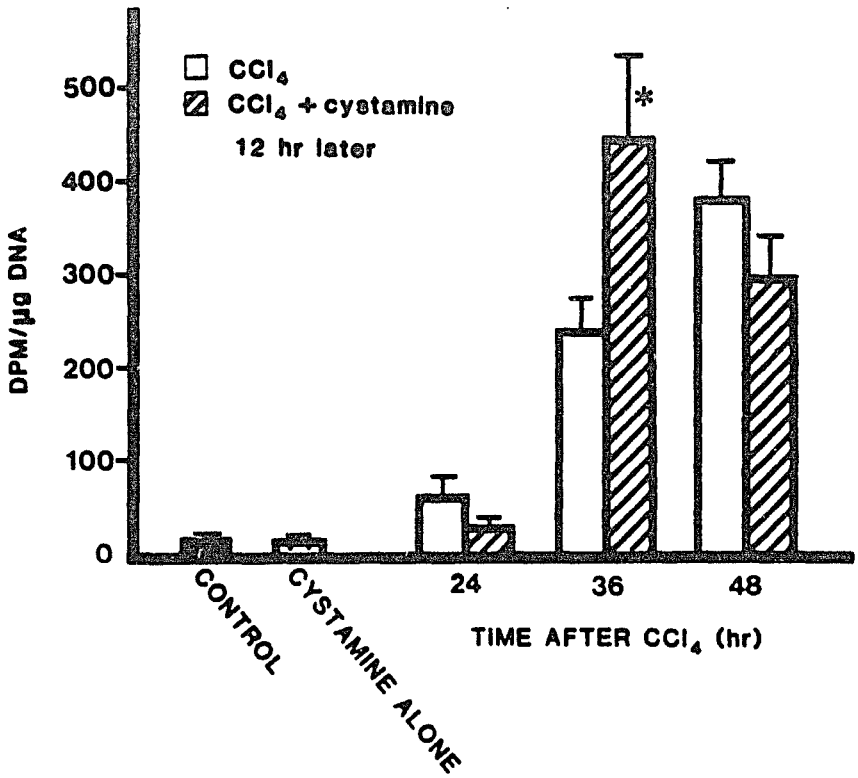


Figure 10. ^{3}H-Thymidine incorporation into hepatic DNA following cystamine treatment in CCl_4-treated rats. Cystamine diHCl (300 mg/kg, po) was administered 12 hr after CCl_4 (0.25 ml/kg, ip). * $p < 0.05$ compared to CCl_4 alone.

The Galactosamine Model

To test whether cystamine had therapeutic effects on chemically-induced hepatotoxicity that were unrelated to inhibition of the bioactivation of CCl_4 to toxic reactive intermediates, the effect of cystamine treatment on D(+)-galactosamine-induced hepatic necrosis was assessed.

Time Course for Galactosamine-induced Hepatic Damage

The time course for the development of galactosamine-induced hepatic damage showed all indices of damage to be maximal 24 hours after galactosamine administration (Figure 11). GPT which is normally found in both cytosol and mitochondria was elevated earlier than OCT, a mitochondrial enzyme. Consistent with the plasma membrane being an early site of galactosamine-induced damage, hepatic calcium levels were elevated as early as 4 hours after galactosamine administration (Figure 11). Necrosis paralleled the increases in GPT and OCT with some necrotic cells apparent by 4 hours after galactosamine and maximal necrosis observed by 24 hours. By 48 hours after administration of galactosamine regeneration was evident as indicated by the appearance of mitotic figures and less necrosis than was seen after 24 hours.

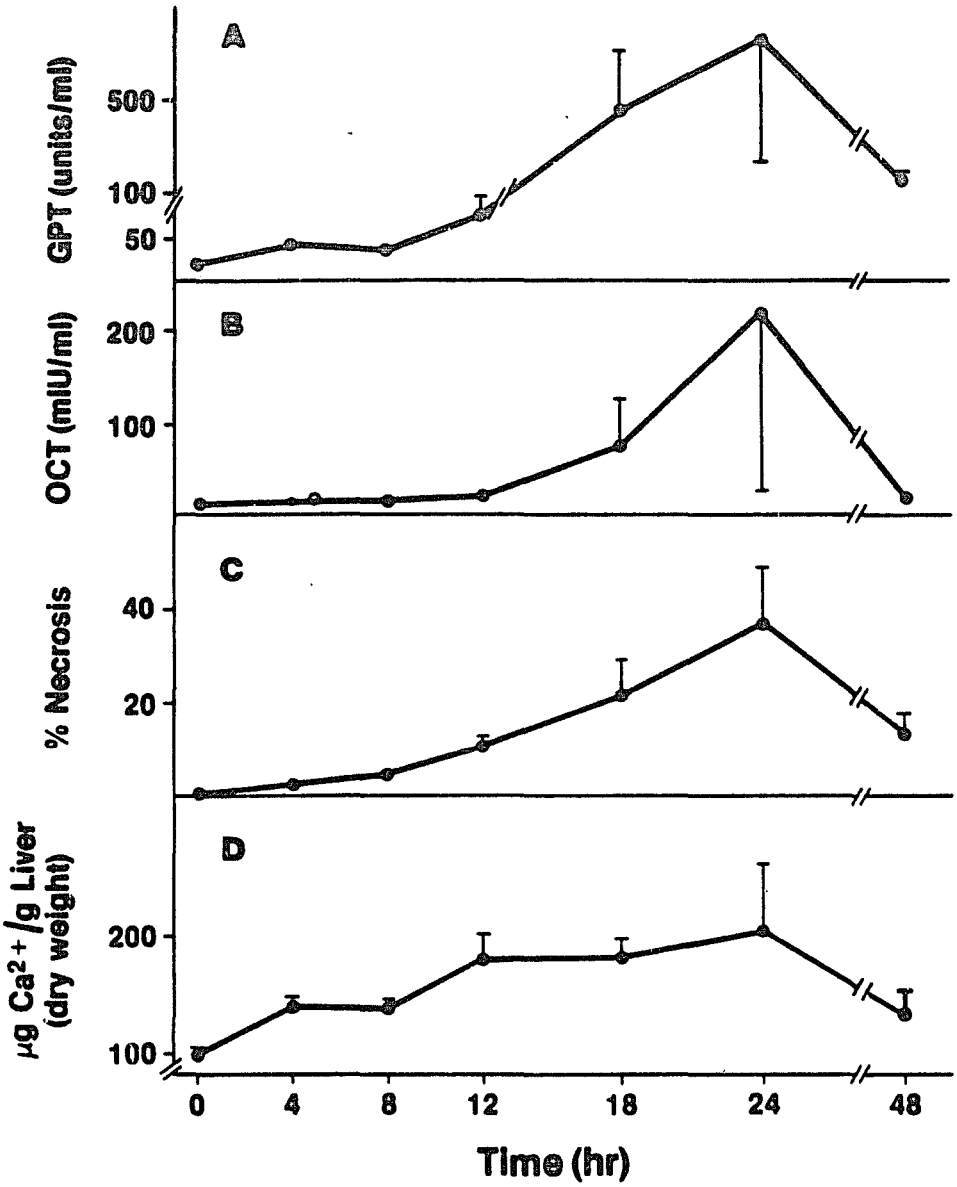


Figure 11. Indices of hepatic damage following D(+)-galactosamine HCl (400 mg/kg, ip). (A) Plasma glutamate-pyruvate transaminase (GPT) activity (B) Plasma ornithine carbamyl transferase (OCT) activity. (C) Hepatocellular necrosis, (percent of total hepatocytes). (D) Total hepatic calcium content.

Effect of Time of Cystamine Treatment on Galactosamine-induced Hepatic Damage

Cystamine was administered at various times after galactosamine in order to assess its therapeutic effects on galactosamine-induced liver injury (Table 10). Plasma GPT and OCT activities, hepatic levels, and cellular necrosis were determined 24 hours after the administration of galactosamine. An oral dose of 300 mg/kg of cystamine diHCl reduced the degree of galactosamine-induced hepatic necrosis when administered at times ranging from 30 minutes prior to galactosamine to 12 hours after the toxicant. Cystamine administered as a therapeutic post-treatment was even more effective in limiting galactosamine-induced necrosis than against CCl_4-induced cell death. Unlike treatment of CCl_4-induced hepatic damage cystamine was no more effective as a pretreatment than as a post-treatment in reducing the extent of galactosamine-induced hepatic injury. Also in contrast to treatment of CCl_4-induced damage cystamine treatment of galactosamine challenged rats resulted in significantly lower plasma GPT and OCT levels than were seen in animals receiving the toxicant alone.

Unlike plasma GPT and OCT activities and necrosis, total hepatic calcium levels did not uniformly reflect a protective effect of cystamine. In fact, as was seen in

Table 10. Effect of time of administration of oral cystamine diHCl administration (300 mg/kg) on indices of hepatic injury 24 hr after intraperitoneal administration of D(+) galactosamine HCl (400 mg/kg).[a]

Time of Cystamine Treatment[b]	GPT[c]	OCT[d]	%necrosis[e]	Hepatic Calcium[f]
---	2710 ± 1668	537 ± 148	49 ± 14	518 ± 149
0.5 hr (prior)	161 ± 18[g]	32 ± 4[g]	19 ± 2[g]	271 ± 32
2 hr (after)	212 ± 170[g]	78 ± 46[g]	23 ± 6[g]	292 ± 50
4 hr (after)	207 ± 66[g]	37 ± 8[g]	11 ± 3[g]	236 ± 29
6 hr (after)	176 ± 84[g]	31 ± 7[g]	14 ± 4[g]	237 ± 34
8 hr (after)	120 ± 86[g]	28 ± 8[g]	13 ± 4[g]	262 ± 54
12 hr (after)	191 ± 64[g]	93 ± 67[g]	8 ± 2[g]	512 ± 172
untreated control	24 ± 2	16 ± 1	0	99 ± 6
cystamine treated control[h]	26 ± 2	---	0	108 ± 4

a all values are mean ± SE (N=4 animals/group)
b prior to or after galactosamine
c glutamate-pyruvate transaminase (Wroblewski-LaDue units/ml plasma ± SE)
d ornithine carbamyl transferase (mIU/ml ± SE)
e % of hepatocytes scored as necrotic ± SE
f μg Ca^{2+}/g liver (dry weight) ± SE
g $p < 0.05$ compared to galactosamine alone
h 12 hr after cystamine administration

CCl_4-intoxicated rats, administration of cystamine 12 hours after galactosamine did not alter hepatic calcium levels assessed 24 hours after the toxicant compared to animals receiving the toxicant alone. This was observed despite an 80% reduction in hepatocellular necrosis (Table 10).

Extended Time Course of Cystamine Effects on Galactosamine-induced Hepatic Damage

As was seen with CCl_4-induced hepatic damage, the effect of cystamine was not simply a delay in the appearance of maximal hepatic injury, since the therapeutic effect was sustained over the period of time in which the galactosamine-induced damage was resolved (Table 11). When cystamine was administered 12 hours after galactosamine, plasma GPT activities and hepatic necrosis in cystamine treated animals never reached the levels observed in animals receiving galactosamine alone (Table 11), but hepatic calcium levels once again did not reflect the therapeutic effect of cystamine.

Dose Dependency of the Therapeutic Effect of Cystamine on Galactosamine-Induced Hepatic Damage

A dose-response relationship for the therapeutic effect of cystamine administration on galactosamine-induced hepatic injury was also evident (Table 12).

Table 11. Time course of effects of cystamine treatment on galactosamine-induced liver injury.[a,b]

Hr after galactosamine	cystamine	GPT[c]	% necrosis[d]	hepatic calcium[e]
control animals	-	24 ± 2	0	99 ± 6
	+	26 ± 2	0	104 ± 4
24	-	1728 ± 856	51 ± 4	227 ± 48
	+	322 ± 203[f]	8 ± 3[f]	198 ± 10
48	-	802 ± 374	50 ± 11	160 ± 28
	+	108 ± 58[f]	8 ± 4[f]	175 ± 47
72	-	33 ± 6	8 ± 2	142 ± 14
	+	69 ± 33	5 ± 2	127 ± 16

a cystamine diHCl (300 mg/kg, po) 12 hr after D(+)-galactosamine HCl (400 mg/kg, ip)

b All values mean ± SE ($n \geq 6$ animals/group)

c glutamate-pyruvate transaminase (Wroblewski-LaDue units/ml plasma)

d % of hepatocytes scored as necrotic

e µg Ca^{2+}/g liver (dry weight)

f $p < 0.05$ compared to galactosamine alone

Table 12. Dose-response for cystamine treatment of galactosamine-induced hepatic injury assessed 24 hr after galactosamine.[a,b]

Cystamine Dose (mg/kg)	GPT[c]	% necrosis[d]
0	3520 + 1680	45 + 13
150	2338 + 1386	25 + 9
300	2143 + 1612	18 + 8[e]
600	1267 + 432	11 + 2[e]

a cystamine diHCl, po, 12 hr after 400 mg/kg D(+)-galactosamine HCl, ip

b All values mean $\pm$ SE ($n \geq 4$ animals/group)

c glutamate-pyruvate transaminase (Wroblewski-LaDue units/ml plasma)

d % of hepatocytes scored as necrotic

e $p < 0.05$ compared to galactosamine alone

Hepatic necrosis 24 hours after galactosamine was reduced in a dose related fashion by doses of 150, 300 and 600 mg/kg of cystamine administered by gastric intubation 12 hours after galactosamine administration.

Histology

The histological damage seen 24 hours after galactosamine (400 mg/kg, ip) is shown in Figure 12. Focal areas of necrotic hepatocytes and single necrotic cells with pyknotic nuclei are apparent. Infiltration of inflammatory cells with a general loss of normal hepatic architecture can also be observed. In animals receiving oral cystamine (300 mg/kg) 12 hours after galactosamine a characteristic focal necrosis was observed with infiltration of inflammatory cells, but with significantly fewer necrotic cells and a greater preservation of hepatic architecture (Figure 13).

Effect of Uridine Treatment on Galactosamine-induced Hepatic Damage

To establish that the therapeutic effect of cystamine was not related to prevention of early biochemical events in the toxicity of galactosamine, the time course for effective treatment and the protection afforded by uridine treatment was determined. Uridine treatment (1200 mg/kg,

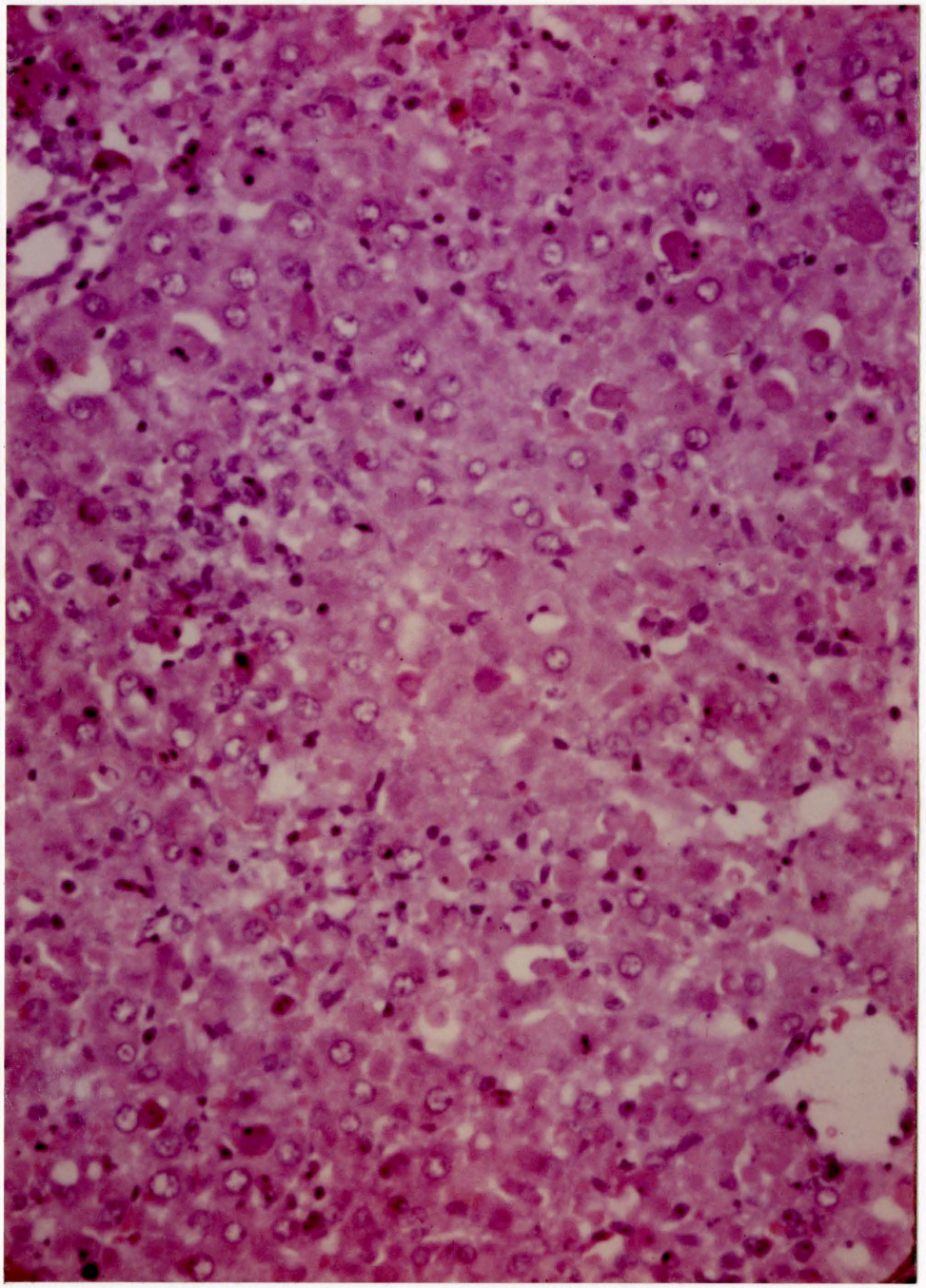


Figure 12. Hepatic histology 24 hr after galactosamine HCl (400 mg/kg, ip) (H&E x 100).

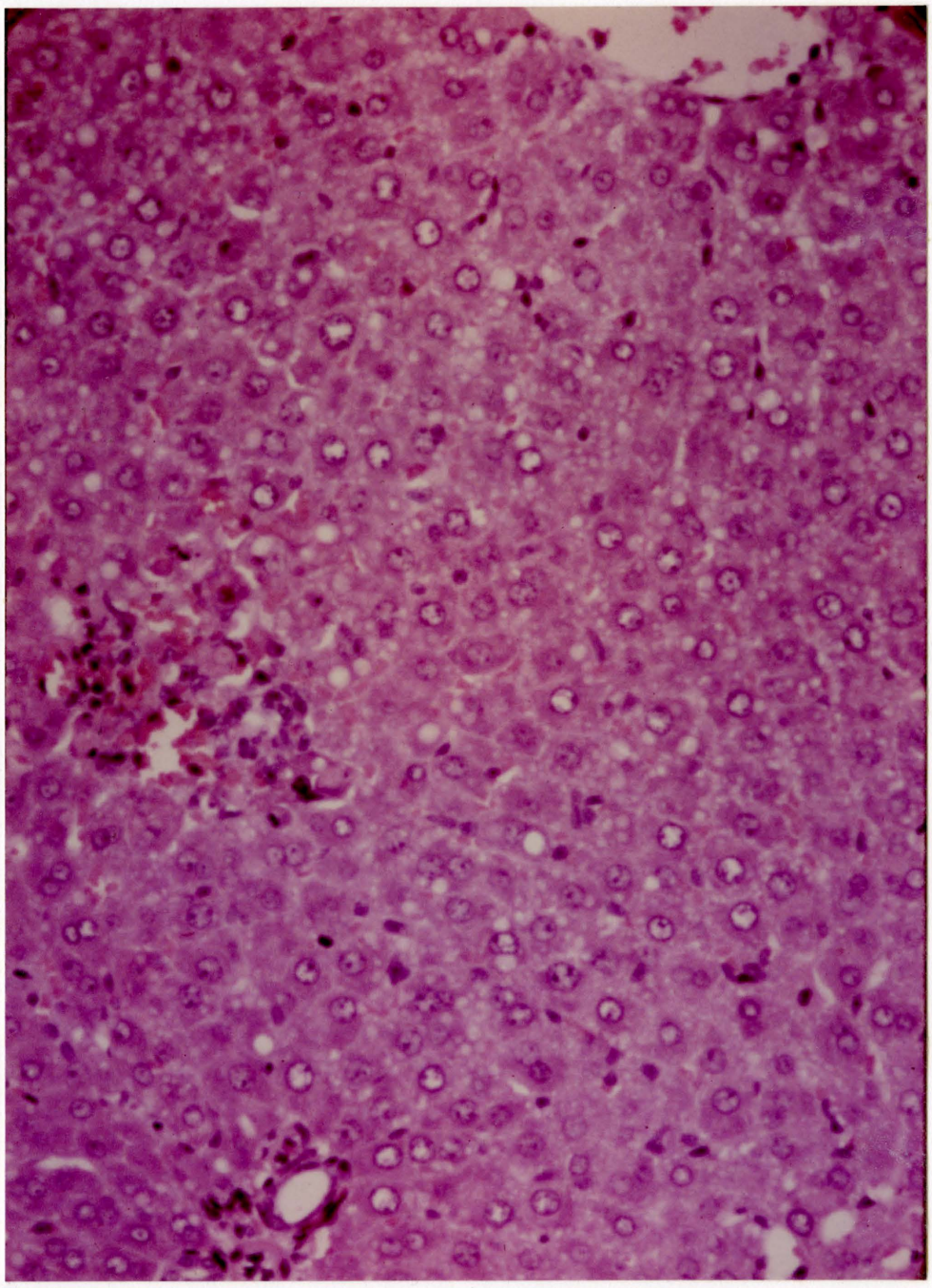


Figure 13. Hepatic histology 24 hr after galactosamine HCl (400 mg/kg, ip) and cystamine diHCl treatment (300 mg/kg, po)]2 hr later (H&E x 100).

ip) was found to prevent galactosamine-induced necrosis when administered 2 hours after galactosamine (Table 13). This treatment prevented galactosamine-induced increases in plasma GPT activities, hepatic calcium content, and hepatic necrosis assessed 24 hours after galactosamine. However, when uridine (1200 mg/kg, ip) was administered 6 hours after galactosamine there was no protective effect on any measure of hepatic damage (Table 13). Cystamine, in contrast, was able to limit the extent of galactosamine-induced hepatic damage whether given as a pretreatment or 12 hours after galactosamine (Table 10). In further contrast to protection by uridine, cystamine did not completely prevent galactosamine-induced elevations of plasma GPT activities, hepatic calcium accumulation or necrosis regardless of the time of administration in relation to the toxicant.

Correlation of Hepatic Calcium Levels with Necrosis

Hepatic calcium levels 24 hours after toxicant administration correlated well with the degree of necrosis for individual animals receiving galactosamine alone ($r = 0.73$; $n = 20$). However, in animals receiving 300 mg/kg cystamine 12 hours after galactosamine, necrosis did not correlate well with hepatic calcium levels 24 hours after toxicant administration ($r = 0.15$; $n = 28$)

Table 13. Effect of uridine treatment on galactosamine-induced hepatic damage.[a,b]

	Galactosamine alone	Time of uridine administration: hr after galactosamine 2	6
GPT[c]	1846 ± 706	21 ± 5[f]	878 ± 459
% necrosis[d]	34 ± 6	<1%[f]	23 ± 7
hepatic[e] calcium	334 ± 136	98 ± 6[f]	200 ± 28

a 1200 mg/kg uridine, ip following 400 mg/kg D(+)-galactosamine HCl, ip

b All values are mean ± SE ($n \geq 8$ animals/group) assessed 24 hr after galactosamine

c glutamate-pyruvate transaminase (Wroblewski-LaDue units/ml plasma)

d % of hepatocytes scored as necrotic

e μg Ca^{2+}/g liver (dry weight)

f $p < 0.05$ compared to galactosamine alone

Time Course for the Appearance of the Therapeutic Effects of Cystamine on Galactosamine-induced Hepatic Damage

A study of the time course for the development of galactosamine-induced injury between 12 and 18 hours after administration was undertaken. The purpose of this study was to determine the time at which the therapeutic effects of cystamine administration 12 hours after galactosamine became apparent. The therapeutic effect was found to occur within 4-6 hours after cystamine administration when cystamine was given 12 hours after galactosamine (Figure 14). The therapeutic effect is evidenced by gradually increasing plasma GPT activity and hepatic necrosis in animals not receiving cystamine, while those parameters remain at approximately the same levels present 12 hours after galactosamine administration in the cystamine treated animals. Elevation of hepatic calcium levels were found to be already maximal 12 hours after galactosamine administration.

Effect of Cystamine Treatment on Hepatic Protein Synthesis Following Galactosamine

Since the therapeutic effect of cystamine treatment 12 hours after galactosamine was already apparent by 18 hours after toxicant administration the effect of cystamine treatment on hepatic protein synthesis was

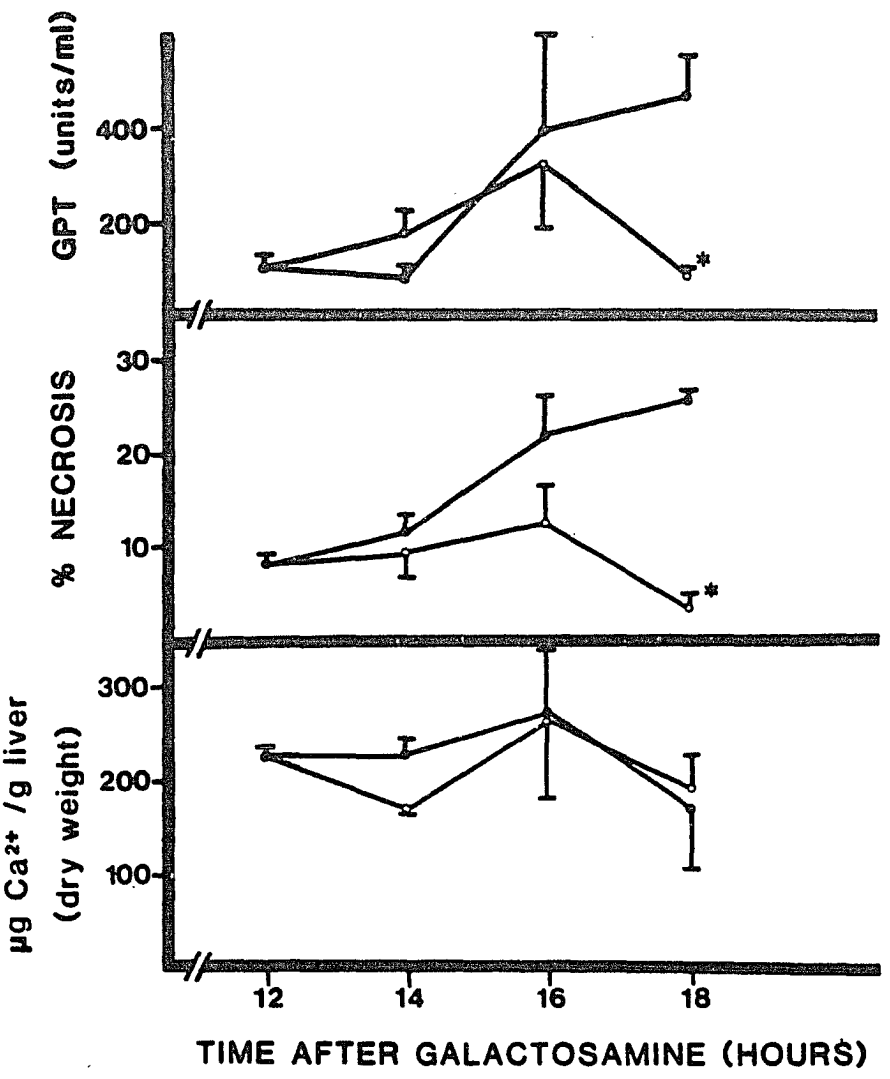


Figure 14. Indices of galactosamine-induced hepatic damage following cystamine diHCl (300 mg/kg, po) treatment 12 hr after D(+)-galactosamine HCl (400 mg/kg, ip).

assessed at that time. Cystamine treatment did not alter the incorporation of ^{14}C-leucine into hepatic proteins in galactosamine treated rats (Table 14).

Effect of Cystamine on Subcellular Calcium Levels

The subcellular distribution of calcium following galactosamine and cystamine treatments was also assessed 18 hours after galactosamine treatment. Calcium content of whole liver homogenate was elevated approximately 2 fold by 18 hours after galactosamine (Table 15). Cystamine treatment 12 hours after galactosamine did not appreciably alter this accumulation.

The mitochondrial fraction exhibited a 5 fold increase in calcium on a wet weight basis while microsomal calcium was elevated 2 fold and cytosolic calcium levels were not altered compared to control by 18 hours after galactosamine. This is in contrast to CCl_4-induced increases in hepatic calcium where all subcellular fractions exhibited greater than a 10 fold increase by 18 hours after CCl_4. As was the case with CCl_4, however, cystamine did not appear to alter subcellular increases in calcium levels or the subcellular distribution.

Table 14. Effect of cystamine treatment on hepatic protein synthesis in galactosamine intoxicated rats.[a,b]

	^{14}C-leucine in hepatic protein[c] DPM / mg protein
galactosamine	388 ± 59
galactosamine + cystamine	413 ± 32

a 400 mg/kg D(+)-galactosamine HCl, ip

b 300 mg/kg cystamine diHCl, po, 12 hr after galactosamine

c 50 µCi ^{14}C-leucine / animal, iv, 17.5 hr after galactosamine; animals killed 30 min later

Table 15. Effect of cystamine treatment on hepatic subcellular calcium distribution in galactosamine treated rats.[a]

	control[b]	galactosamine[c]	galactosamine +cystamine[c,d]
homogenate	34 ± 4	71 ± 6[e]	53 ± 4[e]
mitochondria	4.2 ± 0.2	26 ± 2[e]	21 ± 3[e]
microsomes	1.7 ± 0.1	2.7 ± 0.3[e]	3.0 ± 0.4[e]
cytosol	3.3 ± 0.7	2.6 ± 0.6	4.3 ± 2.0

a results in μg Ca^{2+}/g liver (wet weight)

b untreated control rats

c 18 hr after 400 mg/kg D(+)-galactosamine HCl, ip

d 300 mg/kg cystamine diHCl, po, 12 hr after galactosamine

e $p < 0.05$ compared to control

Effect of Cystamine/Cysteamine Treatment on Hepatic Sulfhydryl and Cysteamine Levels in Galactosamine-treated Rats

The effect of cystamine/cysteamine on tissue thiol and disulfide levels was assessed. When cystamine (300 mg/kg, po) was administered to control rats at 8:00 A. M. there was no measurable alteration of non-protein, protein, or total hepatic sulfhydryl content (Table 16). Cystamine was found to be reduced by glutathione reductase and an NADPH generating system **in vitro** to free sulfhydryls (data not shown). Incubation of liver homogenates from cystamine dosed rats with glutathione reductase and an NADPH generating system, however, did not indicate a higher level of reducible disulfides than in livers from control rats. This indicated that cystamine administration to rats did not cause an increase in acid soluble non-protein disulfides (Table 16). Hepatic non-protein sulfhydryl levels were found to decrease considerably between 12 and 24 hours after galactosamine administration (Table 17). However, neither cystamine nor cysteamine administration 12 hours after galactosamine was found to prevent this decrease (Table 17).

When hepatic non-protein sulfhydryl levels were assessed along with hepatic cysteamine levels between 15 minutes and 4 hours after cystamine or cysteamine

Table 16. Effect of cystamine treatment on hepatic sulfhydryl levels in control rats.[a,b]

	Time after cystamine			
	2 hr		6 hr	
	control	cystamine	control	cystamine
NPSH[c]	8.3 ± 0.4	6.5 ± 1.0	6.6 ± 0.7	5.3 ± 0.7
NPSH + reductase[d]	8.8 ± 0.3	7.0 ± 0.7	7.8 ± 0.8	6.3 ± 0.7
S-S[e]	0.3 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
TSH[f]	23.7 ± 1.3	22.3 ± 1.3	20.9 ± 0.8	19.4 ± 1.8
PSH[g]	15.4 ± 1.3	15.9 ± 0.9	14.3 ± 0.4	14.1 ± 1.2

a 300 mg/kg cystamine diHCl, po

b all values nanomoles sulfhydryl/mg tissue ± SE (n = 4 animals/group)

c non-protein sulfhydryl

d non-protein sulfhydryl after 30 min incubation with glutathione reductase

e non-protein acid soluble disulfides (NPSH + reductase minus NPSH)

f total tissue sulfhydryls

g protein sulfhydryls (TSH minus NPSH)

Table 17. Hepatic non-protein sulfhydryl levels 24 hr after galactosamine following cystamine and cysteamine treatments.[a,b]

treatment	Time after galactosamine (hours) 12	24
---	7.6 ± 0.4	3.0 ± 0.1
cystamine[c]	---	2.2 ± 0.7
cysteamine[d]	---	2.4 ± 0.3

a 400 mg/kg D(+)-galactosamine HCl, ip

b nanomoles sulfhydryl/mg tissue (n = 4 animals/group)

c 300 mg/kg cystamine diHCl, po, 12 hr after galactosamine

d 170 mg/kg cysteamine HCl, ip, 12 hr after galactosamine

administration some effects were discernible. Cystamine appeared to delay the decrease in hepatic non-protein sulfhydryl levels observed between 8:00 A.M. and 2:00 P.M. in control rats (Figure 16), in contrast to the results presented in table 16. Cysteamine administration did not show this effect (Figure 17). In galactcsamine treated rats, cystamine administration 12 hours after the toxicant appeared to maintain hepatic non-protein sulfhydryls at the level present at the time of cystamine administration (Figure 18). However, cystamine did not prevent the fall of hepatic non-protein sulfhydryls to below 3 nanomoles/mg tissue by 24 hours after galactosamine (Table 17). Cysteamine administration 12 hours after galactosamine caused a rapid increase in hepatic non-protein sulfhydryls in toxicant treated rats (Figure 19) which decreased back to pre-cysteamine levels within an hour. Cysteamine also did not prevent decreases of non-protein sulfhydryls to below 3 nanomoles/mg tissue by 24 hours after galactosamine (Table 17).

Cysteamine was also measured directly in hepatic tissue by HPLC analysis following cysteamine and cystamine administration. A typical chromatogram from a sample from a cysteamine dosed animal is shown in figure 15. An ip dose of 1.5 mmole/kg (170 mg/kg) cysteamine HCl produced a hepatic level of 1-2 nanomoles of cysteamine/mg tissue by

15 minutes after administration in both control and galactosamine treated rats (Figures 17 and 19). Similar results were obtained following an oral dose of 1.33 mmole/kg (300 mg/kg) cystamine diHCl, although peak cystamine derived cysteamine levels were observed between 30 and 60 minutes after cystamine administration (Figures 16 and 18). With the exception of cysteamine administration to control animals hepatic cysteamine levels were found to parallel effects of cysteamine/ cystamine administration on hepatic non-protein sulfhydryl levels (Figures 16 - 19).

Cystamine was found to be rapidly and quantitatively reduced to cysteamine by incubation in hepatic homogenates (data not shown). Incubation of liver homogenates from cysteamine or cystamine dosed rats did not measurably increase the amount of detectable cysteamine. It was therefore concluded that cysteamine predominated as the molecular species present in the liver following either cysteamine or cystamine administration in both control and toxicant treated rats.

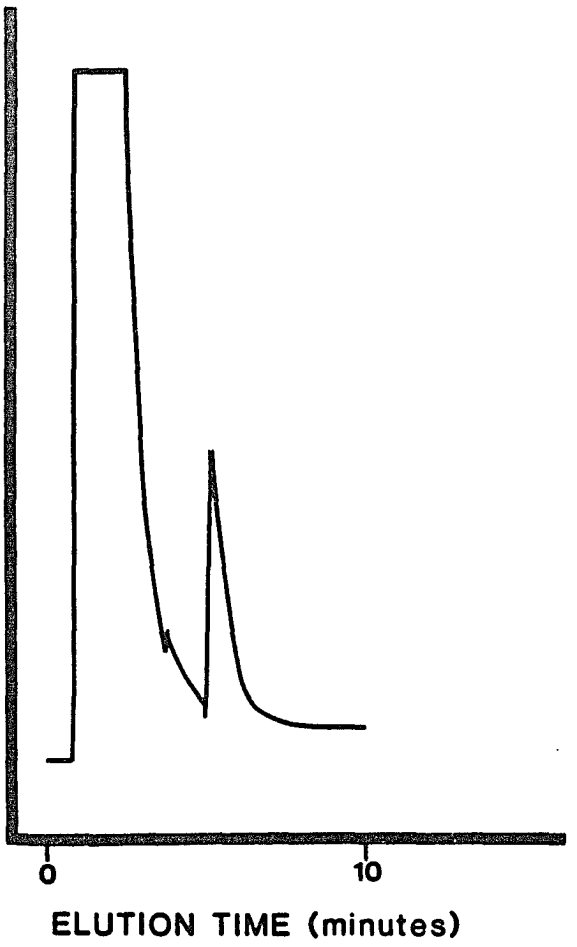


Figure 15. Typical HPLC chromatogram of cysteamine extracted from the liver of a cysteamine treated rat.

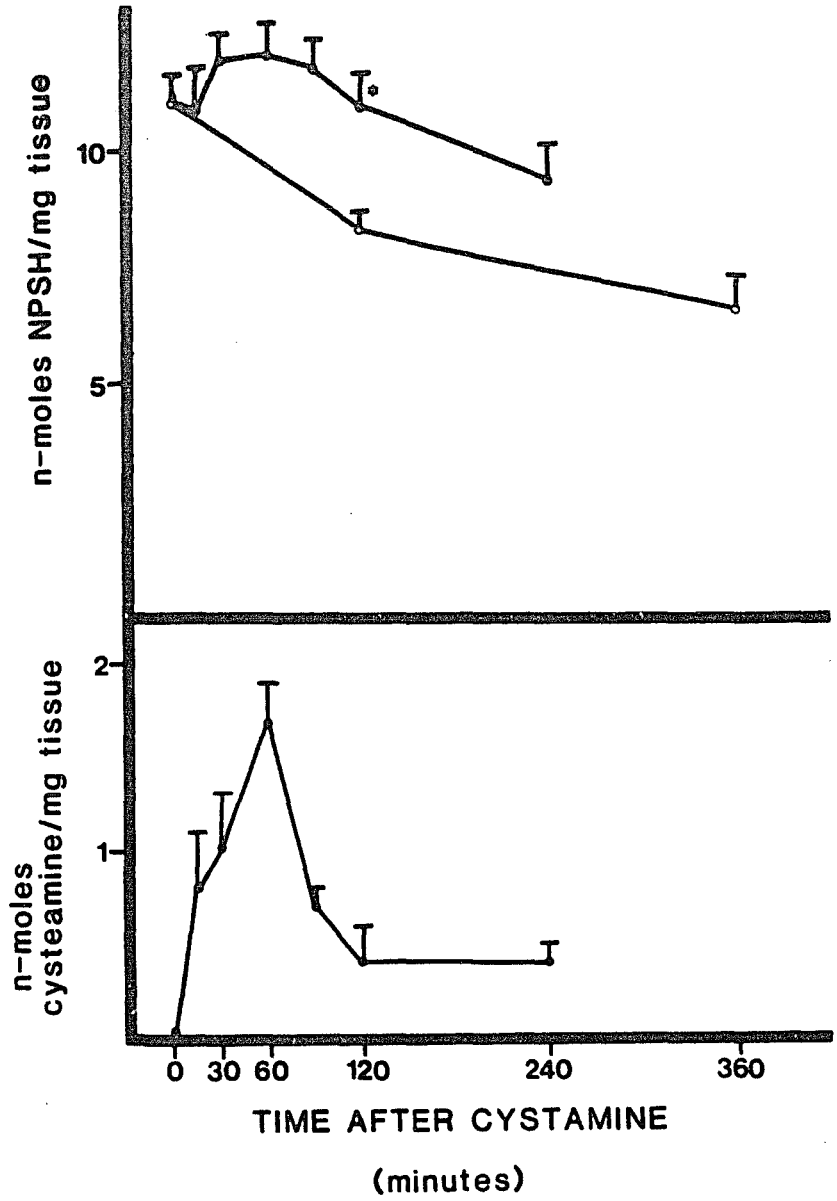


Figure 16. Effect of oral cystamine diHCl treatment (300 mg/kg) on hepatic non-protein sulfhydryl and cysteamine levels in control rats. Cystamine treated (●), untreated controls (○). Cysteamine levels in untreated controls were undetectable. * $p < 0.05$ compared to untreated control values.

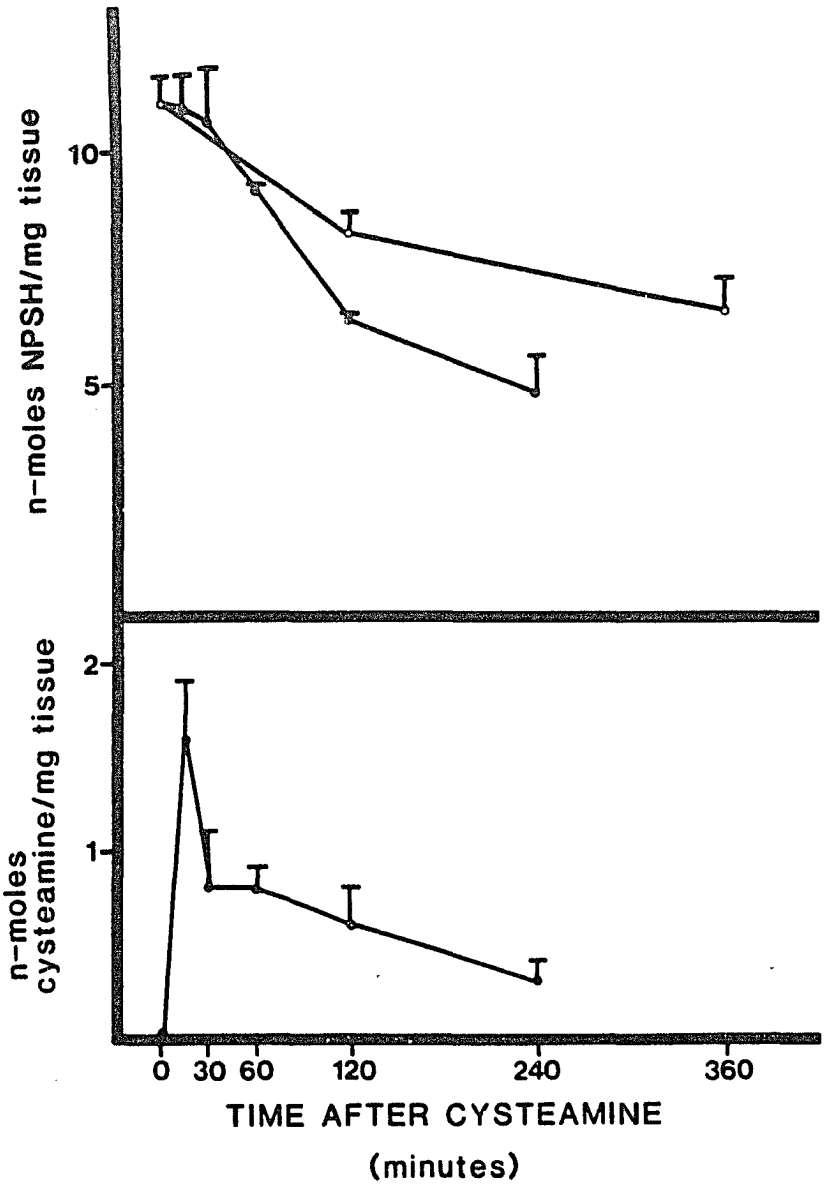


Figure 17. Effect of cysteamine HCl (170 mg/kg, ip) on hepatic non-protein sulfhydryl and cysteamine levels in control rats. Cysteamine treated (●), untreated control (○). Cysteamine levels in untreated controls were undetectable.

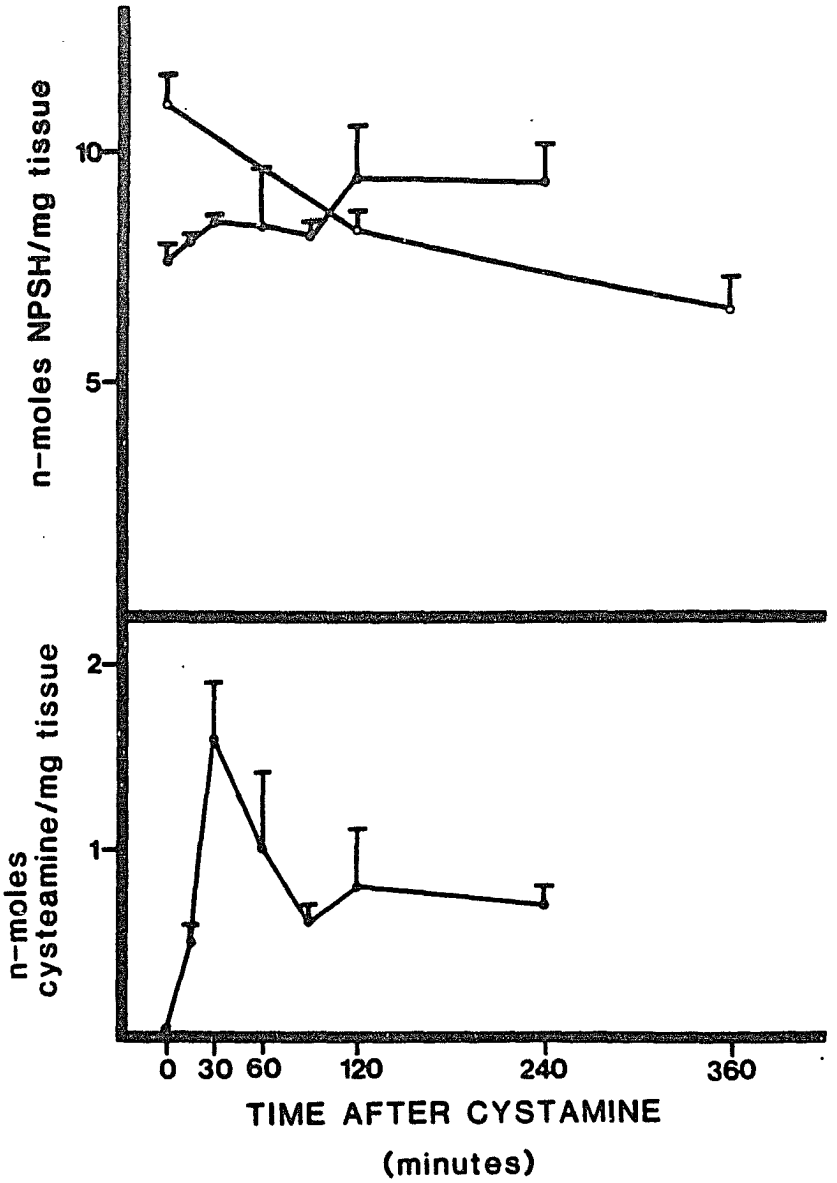


Figure 18. Effect of oral cystamine diHCl (300 mg/kg) administered 12 hr after D(+)-galactosamine HCl (400 mg/kg, ip) on hepatic non-protein sulfhydryl and cysteamine levels. Cystamine treated (●), untreated controls (○). Cysteamine levels in untreated controls were undetectable.

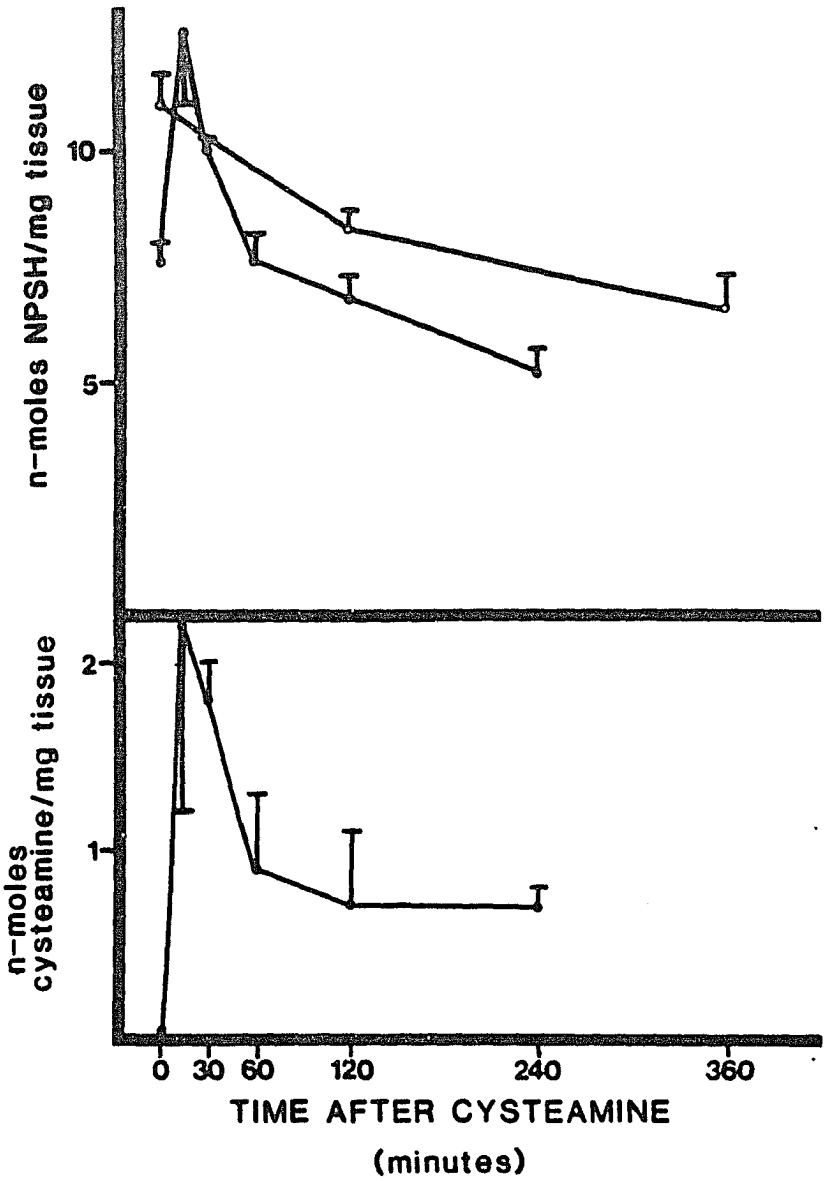


Figure 19. Effect of cysteamine HCl (170 mg/kg, ip) administered 12 hr after D(+)-galactosamine HCl (400 mg/kg, ip) on hepatic non-protein sulfhydryl and cysteamine levels. Cysteamine treated (●), untreated controls (○). Cysteamine levels in untreated controls were undetectable.

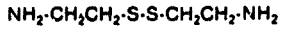
Structure-Activity Relationships for Agents with Therapeutic Effects Against Galactosamine-Induced Hepatic Necrosis

Compounds tested in these studies (Table 18) were administered by intraperitoneal injection to help reduce variations due to differences in absorption. Intraperitoneal administration of cystamine was also attempted. However, when cystamine was administered by ip injection at doses of 0.75 and 0.5 mmoles/kg (170 and 113 mg/kg) 12 hours after galactosamine administration to rats, most of the animals did not survive to the 24 hour end point for the experiments (Table 19), although animals receiving cystamine alone at doses of 0.5 and 0.75 mmoles/kg exhibited no mortality within 12 hours of administration (data not shown).

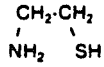
Effect of Chelating Agents on Galactosamine-induced Hepatic Damage

Cysteamine and its structural analog penicillamine (Table 18) reduced the extent of galactosamine-induced hepatic necrosis when administered 12 hours after the toxicant (Table 20). The potent calcium chelators EDTA and EGTA, which bear no structural similarity to cysteamine (Table 18), were found to have the same

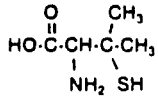
Table 18. Structures of agents tested for therapeutic effects on galactosamine-induced hepatic damage.



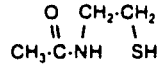
cystamine



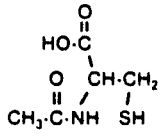
cysteamine



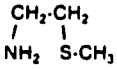
D(−) penicillamine



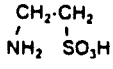
N-acetylcysteamine



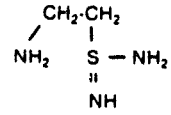
N-acetylcysteine



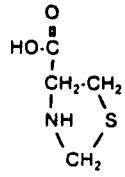
S-methylcysteamine



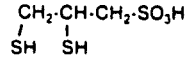
taurine



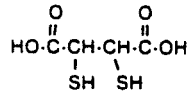
2-aminoethylisothiourea (2-AET)



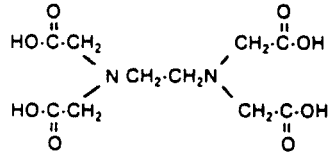
thioproline



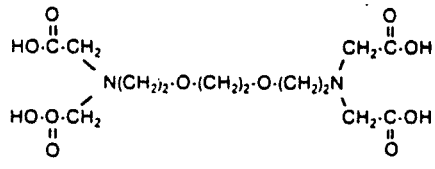
dimercaptopropane sulfonic acid (DMPS)



dimercaptosuccinic acid (DMSA)



ethylenediaminetetraacetic acid (EDTA)



ethyleneglycol-bis-(β-aminoethyl ether)
N, N'-tetraacetic acid (EGTA)

Table 19. Effect of route of administration on cystamine treatment of galactosamine-induced hepatic damage.[a,b]

	Cystamine Dose and Route of Administration (mmole/kg)			
	---	1.33 (po)	0.75 (ip)	0.5 (ip)
mortality[c]	---	---	9/11	3/5
(n)	(11)	(8)	(2)	(2)
GPT[d]	2247 ± 719	299 ± 117g	485	375
% necrosis[e]	41.3 ± 6.3	11.4 ± 2.0g	37.0	6.1
hepatic calcium[f]	317 ± 71	340 ± 103	344	230

a assessed 24 hr after 400 mg/kg D(+)-galactosamine HCl, ip

b cystamine diHCl administered 12 hr after galactosamine

c animals not surviving 24 hr after galactosamine / animals dosed

d glutamate-pyruvate transaminase (Wroblewski-Ladue units/ml plasma ± SE)

e % of hepatocytes scored as necrotic ± SE

f µg Ca^{2+}/g liver (dry weight) ± SE

g $p < 0.05$ compared to galactosamine alone

Table 20. Effect of divalent cation chelators on galactosamine-induced hepatic damage.[a]

Treatment (mmole/kg)[b]	(n)	GPT[c]	% necrosis[d]	hepatic calcium[e]
---	(17)	3769 ± 715	45.4 ± 3.3	635 ± 62
cysteamine (1.5)	(10)	1086 ± 786	12.3 ± 4.1[f]	324 ± 58[f]
penicillamine (1.5)	(7)	1668 ± 1079	22.2 ± 6.8[f]	347 ± 59[f]
EDTA (0.75)	(10)	1096 ± 490	24.9 ± 6.8[f]	539 ± 82
EGTA (0.75)	(7)	1404 ± 427	9.0 ± 1.6[f]	288 ± 53[f]
DMPS (1.5)	(5)	1002 ± 379[f]	37.1 ± 3.6	---
DMSA (1.5)	(5)	3202 ± 946	38.8 ± 4.7	---

a assessed 24 hr after 400 mg/kg D(+)-galactosamine HCl, ip

b 12 hr after galactosamine, ip

c glutamate-pyruvate transaminase (Wroblewski-LaDue units/ml plasma ± SE)

d % of hepatocytes scored as necrotic ± SE

e μg Ca^{2+}/g liver (dry weight) ± SE

f $p < 0.05$ compared to galactosamine alone

therapeutic effect as cysteamine (Table 20). In contrast, the heavy metal chelators dimercaptosuccinic acid (DMSA) and dimercaptopropanesulfonic acid (DMPS) which do bear some structural similarity to cysteamine (Table 18), but have two sulfhydryl groups on adjacent carbons instead of a free sulfhydryl and a free amine, had no therapeutic effect against galactosamine-induced necrosis. EDTA did not significantly reduce hepatic calcium accumulation compared to rats receiving galactosamine alone, despite significantly reducing the necrosis. Cysteamine, penicillamine, and EGTA treatments all appeared to reduce the extent of hepatic calcium accumulation by nearly half compared to levels found in animals receiving galactosamine alone. However, these levels still represent a 3 fold increase above those seen in untreated control animals. Only EDTA significantly reduced galactosamine-induced increases of plasma GPT activity.

Effect of N-Derivatives of Cysteamine on Galactosamine-induced Hepatic Damage

N-Acetylcysteamine and N-acetylcysteine have acetylated nitrogens on the carbon adjacent to a sulfhydryl carbon, while the sulfur and nitrogen atoms of thioproline are both contained within the thiazolidine ring (Table 18). N-Acetylcysteamine and N-acetylcysteine

both had a therapeutic effect on galactosamine-induced necrosis while thioproline actually appeared to enhance the toxicity of galactosamine (Table 21). N-Acetylcysteamine reduced galactosamine-induced necrosis by 75%, but reduced hepatic calcium accumulation by less than half. N-Acetylcysteine reduced hepatic necrosis by 50% without any significant reduction in hepatic calcium accumulation. Thioproline had no therapeutic effect on any of the indices of hepatic damage examined, while only N-acetylcysteamine significantly reduced the toxicant-induced increase of plasma GPT activity.

Effect of S-Derivatives of Cysteamine on Galactosamine-induced Hepatic Damage

S-methylcysteamine, taurine, and 2-aminoethyl-isothiouronium bromide (2-AET) do not contain free sulfhydryl groups (Table 18). However, 2-AET is known to rearrange to 2-mercaptoethylguanidine **in vivo** which does have a free sulfhydryl (Bacq, 1965). Unlike other compounds tested in the structure activity studies for therapeutic efficacy, 2-AET had to be administered at an ip dose of 0.5 mmoles/kg, since the animals did not survive until 24 hours after galactosamine when 1.5 mmoles/kg of 2-AET was administered 12 hours after galactosamine (data not shown). S-Methylcysteamine had no

therapeutic on any parameter of hepatic damage assessed. Taurine and 2-AET both reduced necrosis by nearly half without affecting galactosamine-induced elevation of plasma GPT activity or hepatic calcium accumulation (Table 22).

Table 21. Effect of treatment with N-substituted cysteamine derivatives on galactosamine-induced hepatic damage.[a]

Treatment (mmole/kg)[b]	(n)	GPT[c]	% necrosis[d]	hepatic calcium[e]
---	(12)	2056 ± 488	40.2 ± 5.5	501 ± 73
N-acetyl-cysteamine (1.5)	(6)	250 ± 174[f]	9.6 ± 6.0[f]	278 ± 91[f]
N-acetyl-cysteine (1.5)	(8)	2532 ± 1031	25.0 ± 7.5[f]	404 ± 85
thioproline (1.5)	(5)	2018 ± 200	68.1 ± 8.4[f]	670 ± 36

a assessed 24 hr after 400 mg/kg D(+)-galactosamine HCl, ip

b 12 hr after galactosamine, ip

c glutamate-pyruvate transaminase (Wroblewski-LaDue units/ml plasma ± SE)

d % of hepatocytes scored as necrotic ± SE

e μg Ca^{2+}/g liver (dry weight) ± SE

f $p < 0.05$ compared to galactosamine alone

Table 22. The effect of treatment with S-substituted cysteamine derivatives on galactosamine-induced hepatic damage.[a]

Treatment (mmole/kg)[b]	(n)	GPT[c]	% necrosis[d]	hepatic calcium[e]
---	(12)	3955 ± 997	47.5 ± 4.5	791 ± 77
S-methyl-cysteamine (1.5)	(5)	663 ± 301[f]	32.2 ± 9.3	397 ± 75
taurine (1.5)	(7)	2264 ± 1336	24.7 ± 8.5[f]	716 ± 211
2-aminoethyl-isothiouronium bromide	(6)	3393 ± 964	24.8 ± 5.8[f]	463 ± 165

a assessed 24 hr after 400 mg/kg D(+)-galactosamine HCl, ip

b 12 hr after galactosamine, ip

c glutamate-pyruvate transaminase (Wroblewski-LaDue units/ml plasma ± SE)

d % of hepatocytes scored as necrotic ± SE

e μg Ca^{2+}/g liver (dry weight) ± SE

f $p < 0.05$ compared to galactosamine alone

DISCUSSION

The research described was undertaken to meet two major objectives. The first objective was to establish quantitative animal models for the therapeutic action of cystamine against chemically-induced hepatic damage. The second was to use the animal models to investigate possible mechanisms of the observed therapeutic effect.

In describing the therapeutic effect of cystamine on CCl_4-induced hepatotoxicity, De Ferreyra, et al (1977) measured a single enzyme marker of hepatic damage 24 hours after toxicant administration and evaluated hepatic architecture histologically at this single time point in only a semi-quantitative manner. To begin to address questions concerning the mechanism of action of cystamine it was necessary to develop an animal model that was much more thoroughly characterized.

Since the ultimate objective was to investigate possible mechanisms of the therapeutic effects of cystamine on toxicant-induced damage it was important that the animal models incorporate a large degree of hepatic injury with a high degree of reproducibility in order to most clearly quantify therapeutic effects of treatment. In developing models of cystamine treatment of both CCl_4

and later D(+)-galactosamine-induced hepatic damage it was also important to establish optimal times for assessing hepatic damage after hepatotoxicant administration, optimal therapeutic doses of cystamine and optimal times of cystamine administration. Futhermore, it was important to more thoroughly assess and quantify end point measurements of hepatotoxicity. Toward this end hepatotoxicity was assessed using plasma enzyme markers of hepatic damage, quantitative histological assessment of hepatic necrosis and measurement of toxicant-induced hepatic calcium accumulation.

Male Sprague-Dawley rats were selected as the experimental animal since a therapeutic effect of cystamine on CCl_4-induced necrosis was first described in male rats (DeFerreyra, et al, 1977). Male rats are frequently used in investigations of CCl_4-induced hepatic damage in preference to females because of their increased susceptibility to damage due to increased ability to bioactivate CCl_4 to toxic reactive intermediates (Zimmerman, 1978).

CCl_4 was initially chosen as the model hepatotoxicant for investigating agents with therapeutic potential because it has been studied intensively and much is known about the biochemical events that initiate CCl_4-induced damage and the sequence of events that culminate in cell

death. However, the critical events leading irreversibly to cell death remain obscure and are a source of controversy. Studying a therapeutic effect of cystamine on CCl_4-induced hepatic damage is also complicated by the fact that cystamine has been shown to be an effective protective agent against CCl_4-induced hepatic damage when administered prior to CCl_4 (Castro, et al 1972, 1973).

Within the liver CCl_4 undergoes rapid cytochrome P-450 dependent bioactivation to reactive intermediates that covalently bind to cellular macromolecules (Reynolds, 1967 and Recknagel, 1967). Cytochrome P-450 activity itself is destroyed by the bioactivation of CCl_4 (Reynolds and Ree, 1971). Lipid peroxidation also begins within minutes of CCl_4 uptake into the liver of experimental animals (Rao and Recknagel, 1968 and Reynolds and Ree, 1971). A consequence of the early biochemical events of CCl_4-induced hepatic damage is the rapid loss of the endoplasmic reticulum's ability to actively sequester free calcium ions (Moore, et al 1976). Protein synthetic ability is also inhibited early in CCl_4-induced hepatocellular injury. Following these early events a number of alterations begin to occur and eventually the damage reaches a point of irreversibility from which the cell cannot recover and cell death ensues. Some of these events include but are not limited to alteration of

intracellular calcium homeostasis (Moore, et al, 1976), cellular calcium accumulation (Thiers, et al, 1960), decreased mitochondrial production of ATP (Reynolds, 1964), and decresed hepatic protein synthesis (Smuckler, et al, 1962).

As a protective pretreatment against CCl_4-induced damage Castro, et al, (1972) demonstrated that cystamine inhibited the covalent binding of reactive metabolites of CCl_4 to cellular macromolecules and prevented CCl_4-induced inhibition of cytochrome P-450 dependent metabolism of ethylmorphine. Subsequently it was demonstrated that both cystamine and its reduced free sulfhydryl form, cysteamine, bound to cytochrome P-450 and inhibited the reduction of cytochrome P-450 by cytochrome P-450 reductase (Castro et al 1973). Hence, it appears that the mechanism of cystamine protection against CCl_4-induced hepatic injury is by inhibition of the bioactivation of CCl_4 to a toxic reactive species.

Inhibition of the bioactivation of CCl_4 to a toxic reactive intermediate was not believed to account for the therapeutic effect of cystamine administration 12 hours after CCl_4 on CCl_4-induced hepatic damage (DeFerreyra, et al, 1977). While it was clear that cystamine pretreatment would inhibit the early biochemical events of CCl_4-induced damage (Castro et al 1972, 1973) administration of

cystamine 12 hours after CCl_4 is long after early biochemical alterations have occurred. For instance, by 12 hours after an intraperitoneal administration, CCl_4 is 90% cleared from the liver (Castro, et al, 1972), while covalent binding of CCl_4 metabolites to cellular macromolecules is maximal within an hour (Uehleke and Werner, 1975) as is CCl_4-induced peroxidation in the liver (Rao and Recknagel, 1968 and Reynolds and Ree, 1971). In addition, inhibition of calcium sequestering ability of the endoplasmic reticulum (Moore, et al, 1976 and Figure 5) and dissociation of polyribosomes (Smuckler, et al, 1962) reach maximal levels within an hour of CCl_4 administration. Release of intracellular enzymes and intracellular calcium accumulation (Figure 5 and Reynolds, 1964) also begin prior to 12 hours after CCl_4. These events must be kept in mind when considering possible mechanisms of therapeutic agents administered as late as 12 hours after CCl_4.

Cystamine (300 mg/kg, po) administered 6 hours after a 2.5 ml/kg oral dose of CCl_4 was found to reduce the extent of CCl_4-induced hepatic necrosis observed 24 hours after CCl_4 by 35% without reducing CCl_4-induced elevation of plasma GPT levels (Table 1). While this demonstrated a therapeutic effect of cystamine the model was not developed further using the same dosing parameters for

several reasons. First, orally administered CCl_4 may be only 50% cleared from the liver within 6 hours (Castro, et al, 1972) and therefore cystamine could be acting by inhibiting further bioactivation of CCl_4. In addition cystamine and cysteamine inhibit gastric emptying (Lichtenberger, et al, 1977) which could delay absorption of the toxicant and reduce toxicity to the liver. This mechanism in fact, appears to be at least partially responsible for the therapeutic effect of N-acetylcysteine against the hepatotoxic effects of oral overdoses of acetaminophen (Whitehouse, et al, 1981). Finally cystamine exhibited a dose-dependent systemic co-lethal effect with CCl_4 that was apparent even at the therapeutic dose of 300 mg/kg (Table 1).

The complications of assessing the effects of oral cystamine administration on hepatotoxicity produced by oral CCl_4 were avoided by assessing its effect against intraperitoneally administered CCl_4. In contrast to orally administered CCl_4, intraperitoneally administered CCl_4 is 90% cleared from the liver within 12 hours (Castro, et al, 1972). Orally administered cystamine would also not be able to affect absorption of CCl_4. An ip dose of 0.25 ml/kg of CCl_4 was found to cause necrosis of approximately 40% of all hepatocytes within 24 hours. This provided a high degree of damage which facilitated

assessment of the therapeutic effects of cystamine.

Dose-dependent reduction of CCl_4-induced hepatic necrosis was achieved by cystamine administration 6 hours after CCl_4 (Table 2). An oral cystamine dose of 300 mg/kg was found to substantially reduce necrosis without causing any lethality in CCl_4-treated rats. As was seen with orally administered CCl_4, doses of cystamine greater than 300 mg/kg caused death of some animals within 24 hours after ip administration of CCl_4. However, high doses cystamine alone caused no hepatotoxicity or animal deaths. Consequently, further studies of cystamine's effects on CCl_4-induced hepatic damage employed a dose of 300 mg/kg.

Since cystamine administration 2 or 6 hours after CCl_4 proved to be no more effective than when given 12 hours after CCl_4, subsequent studies employed the later time of administration since the effects seen when given 12 hours after the toxicant would most clearly focus on therapeutic rather than protective effects.

It was surprising that despite substantial therapeutic effects against CCl_4-induced hepatic necrosis, cystamine did not reduce the extent of CCl_4-induced elevation of plasma GPT activity, since DeFerreyra et al, (1977) reported an inhibition of CCl_4-induced elevation of plasma ICDH activity observed 24 hours after CCl_4 when cystamine was administered 12 hours after CCl_4. Due to

this discrepancy, plasma ICDH and OCT were also examined as markers of hepatic injury in CCl_4 and cystamine treated rats. As was the case for GPT, however, neither ICDH nor OCT activities reflected the therapeutic effect of cystamine against CCl_4-induced necrosis (Table 4). The reason these enzymes did not reflect the therapeutic effect in contrast to the report of DeFerreyra, et al (1977) is unclear. One difference between the studies is that DeFerreyra, et al, employed a four fold higher dose of CCl_4 which produced more than a 10 fold greater increase in plasma ICDH activity 24 hours after CCl_4 than the dose employed in these studies. The high values reported by this group included standard errors equal to their reported mean values indicating a very high variability in the animal response. This would at least suggest that plasma ICDH activity is not a definitive indicator of a therapeutic effect of cystamine treatment. Since neither ICDH nor OCT appeared to be better indicators of the therapeutic affect of cystamine, GPT was the only plasma enzyme marker of hepatic damage employed in subsequent studies.

The fact that the extent of hepatic calcium accumulation was not reduced by cystamine treatment was surprising (Tables 6 and 8). This finding was unexpected since the influx of extracellular calcium into toxicant

damaged cells is believed by many investigators to be the key irreversible event in cellular injury that leads to cell death (Farber, 1979, Schanne, et al, 1979, and Shier and DuBourdieu, 1982). Alternatively, others believe that calcium accumulation in tissue is simply a consequence of cell death (Smith, et al, 1981). In either case however, one would expect a lesser accumulation of hepatic calcium to accompany less necrosis (cell death). The role of calcium in cell death will be discussed in greater detail later, however it is clear that cystamine's protective effect was not due to prevention of hepatic calcium accumulation.

Assessment of hepatic calcium levels does help distinguish the protective effects of cystamine pretreatment from the therapeutic effects of cystamine post-treatment of CCl_4-induced hepatic damage. Cystamine pretreatment which apparently inhibits the bioactivation of CCl_4 to toxic reactive intermediates (Castro, et al, 1972 and 1973) almost completely inhibited CCl_4-induced hepatic necrosis, increase of plasma GPT activity and hepatic calcium accumulation. In contrast, cystamine treatment 12 hours after CCl_4 inhibited the development of hepatocellular necrosis but had no ameliorating effect on hepatic calcium accumulation and elevation of plasma GPT activity. As a protective agent preventing the

bioactivation of CCl_4 to reactive intermediates cystamine would prevent the early biochemical events that eventually lead to the perturbations that cause leakage of cellular enzymes, influx of extracellular calcium and cell death. As a post-treatment 12 hours after CCl_4, cystamine cannot inhibit these early biochemical events of CCl_4-induced damage and apparently some of the consequences of these events are not inhibited (ie. leakage of cellular enzymes and the influx of extracellular calcium). Despite the lack of an effect on these parameters hepatic necrosis is still inhibited. This would suggest that cystamine has an effect on some critical event in the progression from toxic cell injury to cell death that can be affected at a late time point in the progression.

Despite the difference in the cytoprotective effects of pre- versus post-treatment of CCl_4-induced hepatic damage with cystamine it was not possible to discount the possibility that some small degree of bioactivation of CCl_4 occurs at later time points. The production of small amounts of reactive intermediates in toxicant damaged cells could conceivably produce sufficient additional stress to cause death of the cell. Introduction of an agent with the potential to inhibit additional bioactivation could produce a therapeutic effect by this mechanism.

To test whether cystamine did indeed have a therapeutic effect that was unrelated to inhibition of bioactivation of CCl_4 the effect of cystamine administration on D(+)-galactosamine-induced hepatotoxicity was investigated. Galactosamine is classified as an indirect hepatotoxicant (Zimmerman, 1978) and does not undergo cytochrome P-450 dependent bioactivation to a reactive intermediate to cause hepatocellular damage. The mechanism of galactosamine-induced hepatic damage is dependent upon depletion of hepatic uridine stores via the irreversible formation of UDP-galactosamine (Decker and Keppler, 1972). The depletion of uridine stores or the incorporation of UDP-hexosamines into the plasma membrane leads to an alteration of the plasma membrane allowing the influx of extracellular calcium, which is believed to be the sequence of events leading irreversibly to hepatic cell death caused by galactosamine (Farber and El-Mofty, 1975). This metabolic uridine deficit can be overcome and galactosamine-induced hepatotoxicity prevented if exogenous uridine is administered within two hours of the hepatotoxicant (Farber, et al, 1973).

Cystamine was found to be a very effective therapeutic agent for galactosamine-induced hepatic necrosis. Necrosis was inhibited by up to 80% even when

cystamine (300 mg/kg po) was administered up to 12 hours after the toxicant. The effect was dose dependent. An oral dose of 300 mg/kg was chosen for additional studies since that dose was found to be able to limit galactosamine-induced hepatic necrosis to approximately the level already present by 12 hours after galactosamine administration (Figure 14). In addition the same dose was found to be effective against CCl_4-induced damage without producing any lethal cotoxicity with CCl_4.

The observed therapeutic effects of cystamine on galactosamine-induced hepatic damage were very similar to those seen in therapeutic post-treatment of CCl_4 intoxication. Hepatic necrosis was reduced while hepatic calcium accumulation was unaltered. Cystamine treatment did somewhat reduce galactosamine-induced elevation of plasma GPT activities. Unlike cystamine treatment of CCl_4-induced damage, cystamine pretreatment was no more effective than post-treatment in alleviating galactosamine-induced hepatic damage. In fact cystamine pretreatment may even be somewhat less effective than post-treatment of galactosamine treated animals. This was not an unexpected result since cystamine was not expected to affect early biochemical effects of galactosamine-induced injury. Uridine administration within 2 hours of galactosamine administration did, however, prevent the

development of galactosamine-induced hepatic damage as had been previously reported by Farber, et al, (1973). The effects of uridine treatment on prevention of galactosamine-induced hepatic damage mimicked the protective effects of cystamine pretreatment on CCl_4-induced damage. Elevations of plasma GPT activity, hepatic calcium accumulation, and hepatic necrosis were all almost entirely prevented. These results with galactosamine as the hepatotoxicant clearly demonstrate that cystamine has therapeutic effects against chemically-induced hepatotoxicity that are unrelated to the prevention of bioactivation or other early biochemical events associated with toxic cell injury. The two animal models using either CCl_4 or galactosamine as the hepatotoxicant were then utilized to address further questions about possible mechanisms of cystamine's therapeutic effects in chemically-induced hepatotoxicity.

Cystamine-induced hypothermia was one possible mechanism of an apparent therapeutic effect of cystamine in chemically-induced hepatotoxicity. In studying the protective effect of cystamine pretreatment against CCl_4-induced hepatic damage Castro, et al, (1973) found that doses of 1.0 ml/kg CCl_4, ip, and 600 mg/kg, po, cystamine each produced a hypothermic response of approximately 3 ^{o}C in rats. The effects were additive and resulted in a 6-7

^{o}C decrease in mean body temperature. This drop in body temperature is similar to that produced by spinal cord transection, a procedure known to delay the hepatotoxic effects of CCl_4 (Larson and Plaa, 1963 and 1965). In the present studies the dose of cystamine employed was found to produce only a mild hypothermic response of approximately 1.5 ^{o}C, while the dose of CCl_4 employed produced no measurable hypothermia and no potentiation of cystamine-induced hypothermia. The expected results of an apparent protective effect of cystamine-induced hypothermia would have been a delay in the appearance of maximal hepatotoxic damage. Cystamine-induced hypothermia therefore was clearly not the mechanism of an apparent therapeutic effect against either CCl_4 or galactosamine since no such delay was observed. Cystamine could also cause an apparent therapeutic effect by enhancing or facilitating cellular repair processes, a result that would decrease the number of necrotic cells. Although the field of chemical carcinogenesis has devoted much attention and study to mechanisms and control of DNA repair, somatic repair processes in toxicant damaged cells is a comparatively unexplored territory. Since both CCl_4 and galactosamine may produce many alterations of cellular function prior to a therapeutic administration of cystamine 12 hours later, an enhancement of cellular

repair would fit well with the observed effects of cystamine. If one considers hepatic calcium accumulation and elevated plasma GPT activity as indicators of cellular injury, enhanced cellular repair could easily account for reduced hepatic cell death despite equivalent evidence of cellular injury in cystamine treated rats. There was, however, no compelling evidence for an effect of cystamine treatment on regeneration repair processes in toxicant treated rats. Cystamine treatment had no effect of ^{14}C-leucine incorporation into protein in galactosamine treated rats. Cystamine treated rats did exhibit a slightly earlier regeneration of hepatic tissue lost to CCl_4-induced necrosis as evidenced by a higher mitotic index 48 hours after CCl_4 and greater incorporation of ^{3}H-thymidine into DNA between 34 and 36 hours after CCl_4. It is therefore possible that the apparent protective affect of cystamine administration observed 48 hours after CCl_4 may be partially due to a more rapid regeneration of necrotic tissue. Whether this effect is due to a direct effect of cystamine or a secondary effect due to less initial cell death is not clear. In any case, this effect does not account for the protective effect of cystamine administration 12 hours after CCl_4 on CCl_4-induced hepatic necrosis observed 24 hours, since no regeneration of damaged tissue occurs by 24 hours after CCl_4, as evidenced

by a lack of mitoses (Table 9) or significant ^{3}H-thymidine incorporation into hepatic DNA (Figure 10). In addition no mitotic figures are present by 24 hours after galactosamine. Furthermore, cystamine treated animals did not exhibit a higher mitotic index 48 or 72 hours after galactosamine administration (data not shown). An effect of cystamine on lipid synthesis was not examined in these studies. A recent report indicates that this may be an important avenue of further investigations. When untreated control rats were given a 600 mg/kg oral dose of cystamine a stimulation of ^{32}P incorporation into phospholipids was observed within 2.5 hours of cystamine administration (Fernandez, et al, 1984). Cystamine had no effect on protein or RNA synthesis or degradation in that study. Phospholipids are important structural and functional components of cellular membranes and alterations or disruption of cellular membranes is an important component of both CCl_4 and galactosamine-induced hepatic damage (James et al 1982 and Farber and El-Mofty, 1975). Therefore, the effect of cystamine on phosholipid synthesis or content should be examined in toxicant treated animals as a possible therapeutic effect of cystamine.

The role of hepatic sulfhydryl levels in hepatotoxic damage must be considered in the models of therapeutic

cystamine administration, since cystamine was largely reduced to the free sulfhydryl cysteamine in the liver (Figures 16 and 18). These findings are in agreement with previous reports (Sorbo, 1962 and Heiffer, et al, 1962). The results of the present studies demonstrate that hepatic non-protein sulfhydryl levels were not dramatically altered by cystamine administration to either galactosamine-treated or control rats (Tables 16 and 17 and Figures 16 - 19). Hepatic non-protein sulfhydryl content usually reaches a peak level around 8 A. M. in rats fed ad libitum and this level gradually decreases during the day while the rats are not feeding (Schnell, et al, 1983). Cystamine treatment did slightly delay this decline in non-protein sulfhydryl levels compared to animals receiving cysteamine in control animals (Figure 16). This was not observed in control animals receiving cysteamine (Figure 17). In galactosamine treated animals both cystamine and cysteamine treatments caused at least a transient increase in non-protein sulfhydryl levels (Figures 17 and 19). However, total non-protein sulfhydryl levels were not significantly elevated compared to galactosamine treated animals by 24 hours after galactosamine following either cystamine or cysteamine treatments (Table 17). The transient increase in non-protein sulfhydryls in galactosamine treated rats was

apparently due to hepatic cysteamine which occurred within 15 minutes after cysteamine and 30-60 minutes after cystamine (Figures 17 and 19). This transient elevation may be critical to some key intracellular process that enhances cell survival. However, the lack of a sustained effect raises doubts about whether sulfhydryl equivalents alone are responsible for the therapeutic effect. Depletion of the principal intracellular free sulfhydryl compound, glutathione, has been shown to be a key element in the hepatotoxicity caused by a number of agents including acetaminophen and bromobenzene (Mitchell, et al, 1976). In fact, N-acetylcysteine has been shown to be an effective therapeutic agent in acetaminophen-induced hepatic damage presumably because of its ability to enhance synthesis of glutathione, a potent nucleophile that detoxifies electrophilic reactive intermediates produced during the cytochrome P-450 catalyzed bioactivation of acetaminophen (Lauterburg, et al, 1983).

A similar role was not expected for the therapeutic action of cysteamine/cystamine in CCl_4 or galactosamine-induced hepatotoxicity, since neither toxicant has been reported to include depletion of hepatic non-protein sulfhydryl levels as a contributing factor in their hepatotoxic effects. By 24 hours after galactosamine hepatic non-protein sulfhydryl levels were greatly

depressed compared to control levels (Table 17), however this may be at least partially a function of the high degree of necrosis present by 24 hours after galactosamine, since hepatic non-protein sulfhydryl levels were not markedly depressed by 12 hours after galactosamine administration (Table 17). In addition, neither cystamine nor cysteamine administration 12 hours after galactosamine prevented this decrease (Table 17) despite the therapeutic effect on galactosamine-induced necrosis. Furthermore, a role for the simple addition of SH equivalents as the therapeutic mechanism of cysteamine/cystamine is contradicted by the finding that the disulfhydryl chelating agents DMSA and DMPS had no therapeutic effect on galactosamine-induced hepatic necrosis (Table 20).

An alternative mechanism of sulfhydryl protection against hepatotoxicity is the functioning of free sulfhydryls, notably glutathione, in the redox cycling-dependent detoxification of intracellular peroxidation products (Chance, et al, 1978). Peroxidation is not known to be a feature of galactosamine-induced hepatotoxicity, however CCl_4-induced hepatotoxicity could conceivably be affected by intracellular SH balance. The literature, however, would not support such a role for the therapeutic action of cysteamine/cystamine in CCl_4-induced

hepatotoxicity. First, as mentioned previously, CCl_4 is not known to cause a depletion of intracellular non-protein sulfhydryls and presumably cell death does not result from a deficiency of glutathione for glutathione peroxidase-dependent detoxification of CCl_4-induced lipid peroxides. Secondly, CCl_4-induced lipid peroxidation is maximal long before the administration of cystamine in this model (Rao and Recknagel, 1968 and Reynolds and Ree, 1971). In addition, cystamine treatment prior to large doses of CCl_4 has been shown to have little effect on the extent of CCl_4-induced lipid peroxidation, despite markedly reducing the extent of covalent binding of CCl_4 metabolites to cellular macromolecules and subsequent CCl_4-induced hepatic damage (Castro, et al, 1972).

Alterations of the non-protein sulfhydryl content of liver cells can also affect the sulfhydryl/disulfide balance in intracellular proteins as well (Kaplowitz, 1981). Cystamine administration did not appear to alter the free protein sulfhydryl levels in rat liver homogenate (Table 16) and was not considered further as a possible mechanism of cystamine's therapeutic effect in chemically-induced hepatotoxicity.

The reduction of cystamine to cysteamine in vivo is presumed to proceed by a disulfide exchange mechanism (Bacq, 1965). Glutathione reductase facilitates the

reduction of intracellular disulfides to free sulfhydryls at the expense of NAD(P)H reducing equivalents (Griffith and Meister, 1979). Alteration of the redox status of pyridine nucleotides has been shown to have a regulatory effect on some aspects of intracellular calcium ion homeostasis. Oxidation of mitochondrial NAD(P)H is associated with the release of calcium from calcium loaded mitochondria (Lehninger, et al, 1978). This contribution to altered intracellular calcium homeostasis is believed to contribute ultimately to toxic cell death as proposed by Orrenius and coworkers (Smith, et al, 1981 and Bellomo, et al, 1982 and 1984). In contrast, Beatrice, et al, (1982 and 1984) concluded from their studies that it was the glutathione/glutathione disulfide ratio that directly controlled calcium fluxes in isolated mitochondria by affecting membrane phospholipid lability. How these processes are actually controlled, how they relate to cytotoxic injury, and how they may be altered by therapeutic agents are yet to be clearly resolved. It was clear in the present studies that the cystamine doses employed did not measurably alter the overall hepatic sulfhydryl/disulfide balance. It therefore remains for further investigations to fully evaluate a possible effect of cysteamine/cystamine on cellular and subcellular redox states and thiol/disulfide balances that may relate to the

observed therapeutic effect of cysteamine/cystamine. These studies will require the development of models of therapeutic intervention in cell culture or even isolated mitochondria to effectively address these questions.

The most unique finding was that cystamine or other therapeutic agents could reduce the extent of toxicant-induced hepatic necrosis without reducing toxicant-induced hepatic accumulation of calcium. For the last several years the role of calcium in toxicant-induced cell death has been an area of intensive investigation and controversy. Carbon tetrachloride-induced alterations of hepatic calcium content and subcellular distribution associated with CCl_4-induced hepatic necrosis were being investigated as early as the late 1950's and early 1960's (Thiers, et al, 1960 and Reynolds, et al 1962). Following the early descriptive studies little attention was given to the role of calcium in toxicant-induced hepatic cell death and a great deal of research attention was focused on the role of bioactivation and lipid peroxidation in initiating CCl_4-induced hepatotoxicity (Recknagel, 1967).

In 1975 Farber and El-Mofty helped renew interest in the role of calcium in toxic cell death when they speculated that galactosamine-initiated damage to the plasma membrane allowed the influx of extracellular calcium and that this influx constituted the key

irreversible event in galactosamine-induced hepatic damage. This speculation was later supported by finding that chlorpromazine blocked the influx of extracellular calcium and prevented galactosamine-induced hepatic necrosis when administered within 2 hours after galactosamine (Farber, 1979). Similar results were obtained when CCl_4 was substituted as the toxicant (Farber, 1979). Recently a calcium deficient diet has also been shown to protect against acetaminophen-induced hepatotoxicity (DeVries, 1983).

Studies in cell culture on the role of calcium in toxicant-induced cell death culture have produced some interesting results and fueled an active controversy about the true involvement of calcium in cell death. Utilizing adult rat hepatocytes in primary monolayer culture Schanne, et al (1979) demonstrated an absolute dependence on the presence of extracellular calcium for ten different membrane active toxicants to produce cell death. Chenery, et al (1981) also employed primary monolayer cultures of adult rat hepatocytes to demonstrate that in the presence of physiologic concentrations of extracellular calcium carbon tetrachloride-induced cell damage was greater than in calcium free medium. In addition the calcium ionophore A23187 was found to enhance CCl_4-induced damage without affecting the bioactivation of CCl_4, while A23187 in the

presence of physiological extracellular calcium concentrations caused leakage of cellular enzymes into the medium and the characteristic "blebbing" of the plasma membrane observed after toxic damage to hepatocytes in culture. Shier and DuBourdieu (1982) were able to demonstrate using cultured fibroblasts that the cell death induced by A23187 in the presence of physiologic extracellular calcium concentrations could be antagonized by the presence of another divalent cation, manganese, which antagonizes intracellular effects of calcium.

The ability of calcium to cause cell death in these studies was not surprising in view of the many effects that elevated intracellular calcium may produce (Trump, et al, 1980). If normal cellular means of controlling the intracellular free calcium concentration are disrupted, as may be seen with CCl_4-induced damage to the endoplasmic reticulum's ability to sequester calcium (Moore, et al, 1976), or if normal control mechanisms are overwhelmed as may happen when toxicant-induced damage to the plasma membrane allows the influx of extracellular calcium, a sequence of calcium dependent events will occur (Figure 20). The first consequence of the alterations, is obviously an increase in the free cytosolic calcium concentration. Increased free cytosolic calcium can activate phospholipases within the cell (Chien, et al,

1980 and Shier and DuBourdieu 1982) which degrade membrane phospholipids. Breakdown of membrane phospholipids in turn may alter membrane structure and function (Chien, et al 1978). Altered membrane permeability may lead to further alteration of cellular calcium homeostasis (Figure 20). In addition, cytosolic calcium-activated proteinases have recently been discovered in the liver of rabbits (Pontremoli, et al, 1984). Activation of such enzymes could possibly have a role in calcium-induced liver cell injury.

Disruption of the cytoskeleton may also occur as a result of an increase of free cytosolic calcium (Sauk, 1976). In addition, the cell "blebbing" induced by A23187 and calcium mimics the "blebbing"" induced by vinblastine or colchicine which depolymerize microtubules (Trump, et al, 1981). This calcium-induced depolymerization of microtubules appears to be mediated by the calcium binding protein, calmodulin (Keith, et al, 1983). Changes in the cytoskeleton may in turn alter cell shape and affect membrane permeability (Figure 20). Mitochondrial accumulation of calcium is also known to occur in toxicant-induced liver injury and this accumulation precedes the disruption of normal mitochondrial function (Reynolds, et al, 1962 and Reynolds, 1964). Decreased ATP production can in turn inhibit the energy dependent

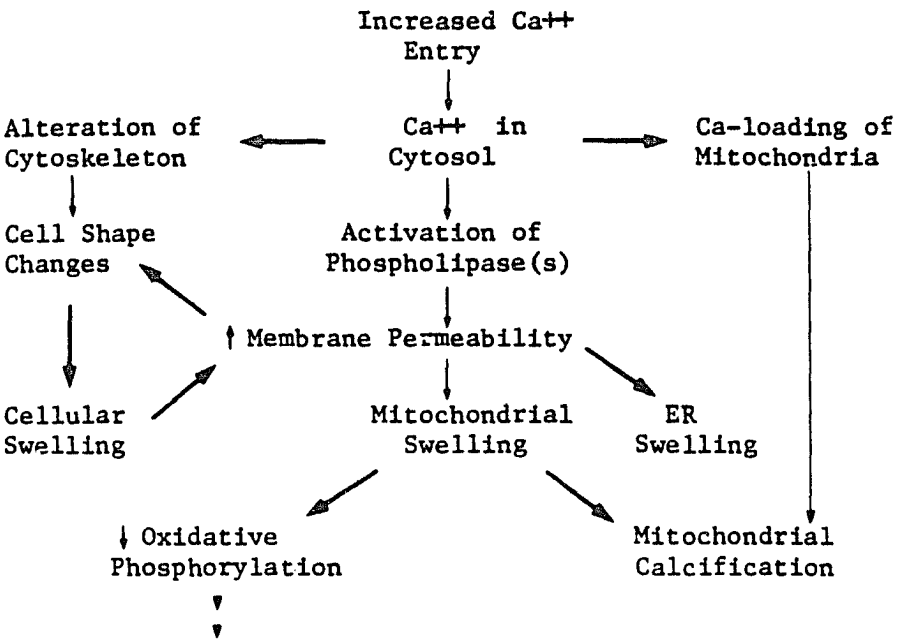


Figure 20. Consequences of cellular calcium accumulation. From Trump, et al, (1980).

calcium pump activities of the endoplasmic reticulum (Moore, et al 1975) and the plasma membrane (Izutsu and Smuckler, 1978) further decreasing the cells ability to maintain homeostasis with regard to free calcium concentration.

Despite the voluminous literature and convincing arguments for a critical role of calcium in mediating toxic cell death a number of contradictory reports exist. Smith, et al (1981) reported that CCl_4, bromobenzene, and ethylmethanesulfonate were more toxic to isolated hepatocytes in suspension culture in the absence of extracellular calcium than in its presence. Similar results were obtained by Acosta and Sorenson (1983) when the toxicity of $CdCl_2$ was assessed in hepatocytes in suspension culture. Reed and Fariss (1984) also found A23187 to cause a greater loss of cell viability in isolated hepatocytes in the absence of extracellular calcium than in the presence of 3.5 mM extracellular calcium. It is the generalized conclusion of these studies that the influx of extracellular calcium is not the key irreversible event leading to cell death, but that calcium accumulation occurs as a consequence of cell death and cell death itself is caused by a combination of altered intracellular calcium homeostasis and other biochemical events. These studies are subject to

criticism on several points. First, incubation in calcium free media represents a totally unphysiological situation and cellular responses to chemical challenges may not reflect those that would be observed in vivo. Secondly it should be considered that preparation of single cell suspensions of isolated hepatocytes are prepared by perfusing livers with a calcium free EDTA buffer to remove extracellular calcium from the liver to disrupt calcium dependent cell to cell contacts (Seglen, 1975). Cells isolated by this procedure may already be somewhat calcium deficient and very susceptible to effects of further depletion by incubation in a calcium free medium.

Whether one subscribes to the theory that the influx of extracellular calcium into toxicant-damaged hepatocytes constitutes an irreversible cytotoxic event leading to cell death or that calcium accumulation is a consequence of cell death the results of the present studies are not easily explained. Equivalent hepatic calcium increases should correlate with equivalent hepatocellular necrosis whether calcium accumulation is a cause or effect of cell death. With the exception of one published abstract (Schuman and Evans, 1982), the results of the present studies are unique in separating hepatic calcium accumulation from hepatic necrosis.

The therapeutic effect of cystamine on CCl_4 or

galactosamine-induced hepatic necrosis may be considered to approximate the protective effect of manganese against the cell death produced in cultured fibroblasts by physiological extracellular calcium concentrations and the ionophore A23187 (Shier and DuBourdieu, 1982). Manganese does not prevent the influx of calcium into the cells but prevents the cytotoxic effects of calcium influx by antagonizing the biological effects of calcium, such as phospholipase activation. Cystamine or its predominant **in vivo** form, cysteamine, are not considered to be calcium antagonists in the manner of manganese, yet their therapeutic effects must somehow account for decreased cytotoxic consequences of calcium accumulation in toxicant damaged cells.

Chelation or binding of calcium, reducing the free cytosolic concentration of calcium, was one plausible mechanism meeting the criteria of equivalent accumulation and decreased cytotoxic effects. This seemed a plausible explanation of cystamine's therapeutic effect since therapeutic doses of cystamine are reduced to cysteamine **in vivo** (Sorbo, 1962 and Figures 16 and 18). Intraperitoneally administered cysteamine also had a therapeutic effect on CCl_4-induced damage (Table 5) and cysteamine has a limited ability to chelate calcium (Tsuchitani, et al 1963). In addition, the potent calcium

chelator EDTA reduced the extent of CCl_4-induced hepatic necrosis without preventing CCl_4-induced increases of hepatic calcium content when administered 30 minutes after CCl_4 in a report published in abstract form (Schuman and Evans, 1982).

Based on the hypothesis that calcium chelation could be a mechanism of the observed therapeutic effects of cystamine/cysteamine on chemically-induced hepatic necrosis, a study of the therapeutic effects of chelating agents and cysteamine analogs on galactosamine-induced hepatic damage was performed. Putative therapeutic agents were administered 12 hours after the toxicant and hepatic damage was assessed 24 hours after galactosamine administration. The potent calcium chelators EDTA and EGTA, as well as cysteamine and its analog the chelating agent (D)-penicillamine had significant therapeutic effects on hepatic necrosis. EGTA, cysteamine and penicillamine, but not EDTA, also reduced hepatic calcium accumulation to some degree. The heavy metal chelating agents dimercaptopropanesulfonic acid (DMPS) and dimercaptosuccinic acid (DMSA) had no therapeutic effect on galactosamine-induced hepatic necrosis.

N-acetylcysteine and N-acetylcysteamine, cysteamine derivatives which have acetylated nitrogens on the carbon adjucent to the sulfhydryl carbon, reduced galactosamine-

induced hepatic necrosis. In contrast, thioproline, which contains a secondary amine nitrogen in the thiazolidine ring one carbon removed from a non-sulfhydryl sulfur, was not protective in this model. Cysteamine analogs which did not possess a free sulfhydryl, (taurine, S-methylcysteamine and 2-AET) had mixed effects on galactosamine-induced necrosis. S-methylcysteamine had no therapeutic effect while taurine with a highly oxidized sulfur and 2-AET which rearranges in vivo to form 2-mercaptoethylguanidine, which does possess a free sulfhydryl, did reduce galactosamine-induced hepatic necrosis.

Some of the protective agents did appear to reduce hepatic calcium accumulation to some degree and hence did not exactly mimic the therapeutic effects of cystamine on galactosamine-induced hepatic damage. However, hepatic calcium levels are known to be nearly maximal by 12 hours after galactosamine administration (Farber and El-Mofty, 1975 and Figure 11) the time when the protective agents were administered in this study. All determinations of hepatotoxic indices in this study were made 24 hours following galactosamine administration, 12 hours after treatment with the agents being tested, and therefore 12 hours after maximal hepatic calcium levels are reached. It is therefore possible that differences between groups

may actually be due to differences in the clearance of calcium from the livers of the treated animals, rather than a difference in the extent of calcium influx. This is an inherent weakness in examining only one time point. Differences in hepatic uptake, distribution, disposition, and clearance of these agents may all affect their measured effects on hepatic calcium levels at a single time point after administration. It is therefore important that time course studies be performed and that more information about the uptake and disposition of these agents be determined.

The therapeutic effectiveness of EDTA and EGTA indicate that free sulfhydryls are not necessary functional groups for therapeutic agents in this model. In fact DMPS and DMSA chelating agents which have two sulfhydryl functional groups each were ineffective as therapeutic agents, while cysteamine and D(-) penicillamine, chelating agents which have a primary amine and a sulfhydryl group on adjacent carbons, were effective. N-acetylcysteamine and N-acetylcysteine cannot act as chelating agents because of an acetylated nitrogen. However, these compounds can be deacetylated **in vivo** to yield cysteine and cysteamine (Lauterberg, et al, 1983). Thioproline, which was not an effective therapeutic agent, is probably not metabolized to a cysteamine-like

structure, since thioproline is not a substrate for the intracellular prolinase which is known to liberate cysteine from the thioproline analog L-2-oxothiazolidine-4-carboxylic acid (Williamson and Meister, 1982).

The rearrangement of 2-AET **in vivo** also produces a cysteamine-like structure (Bacq, 1965). Of the compounds found to be effective therapeutic agents against galactosamine-induced hepatic damage only taurine lacked the structural ability to act as a calcium chelator or the ability to be metabolized to a chelating structure **in vivo**.

Oral administration of taurine 12, 16, and 20 hours after a hepatotoxic dose of CCl_4 has been shown to limit the toxicant-induced accumulation of hepatic calcium up to 24 hours after CCl_4 administration (Nakashima, et al, 1982 and 1983). This treatment also reduced the level of hepatic lipid peroxides observed 24 hours after CCl_4. It was suggested that a membrane effect limiting the influx of extracellular calcium into CCl_4-damaged cells could account for the observed therapeutic effect of taurine. However, there was no effect of taurine on galactosamine-induced hepatic calcium accumulation in this study (Table 22). This mechanism, however, may account for the therapeutic effects of other agents which did appear to limit galactosamine-induced hepatic calcium accumulation

observed 24 hours after the toxicant.

Taurine has also been shown to enhance the ability of mitochondria (Dolara, et al, 1973) and isolated sarcoplasmic reticulum (Huxtable and Bressler, 1973) to take up calcium. Taurine then may possibly have some membrane effects that influence intracellular calcium distribution and homeostasis that could account for its therapeutic effects in galactosamine-induced hepatic damage.

With the exception of taurine, EDTA and EGTA all of the agents found to have therapeutic effects meet the structural requirements to act as radioprotective agents (Bacq, 1965). Radioprotective effects, however, require the presence of the protective agent at the time of radiation exposure, which is clearly not the case for therapeutic effectiveness in galactosamine-induced liver damage.

In summarizing the structure-activity studies, it was found that with the exception of DMPS, DMSA, and taurine, only compounds with either known ability to chelate metals, the structural requirements necessary to do so, or compounds that could be metabolized **in vivo** to chelating structures were found to have a therapeutic effect against galactosamine-induced necrosis. The data do not prove that calcium chelation is the mechanism of

the observed therapeutic effect. Other possible mechanisms such as the mechanism of taurine's therapeutic effect should be explored. However, the structure-activity relationship for calcium chelating ability and cytoprotective effects in this model is consistent with the reduction of galactosamine-induced necrosis despite substantial hepatic calcium accumulation.

In total, the studies included in this investigation have developed quantitative animal models for the therapeutic effect of cystamine on chemically-induced hepatic damage. The animal models were then used to investigate possible mechanisms of the observed therapeutic effects. The results did not support cystamine-induced hypothermia, effects on hepatic regeneration, or hepatic protein synthesis as probable mechanisms of the therapeutic effect. Cystamine or cysteamine only transiently affected hepatic non-protein sulfhydryl levels and did not prevent the decrease to the low non-protein sulfhydryl levels observed 24 hours after galactosamine administration.

The unique observation that cystamine did not prevent hepatic calcium accumulation despite its therapeutic effect on chemically-induced necrosis appeared contrary to current knowledge and speculation about the role of calcium in cell death. This prompted further

investigations into the effect of cystamine on hepatic calcium disposition. Cystamine did not appear to affect subcellular calcium distribution in intoxicated rats, nor did it alter recovery of calcium sequestering ability of hepatic endoplasmic reticulum in CCl_4 intoxicated rats. The results of a structure-activity study of compounds suggested, but did not prove, that chelation may be a mechanism of the observed therapeutic effects. However, other biochemical or membrane effects may prove to be the actual therapeutic mechanism, since not all chelating agents nor only compounds with chelating ability had a therapeutic effect on galactosamine-induced necrosis.

Until the mechanism of cell death is better understood, it will be difficult to define exact therapeutic mechanisms. However, these agents, which can reverse certain events, or at least inhibit their progression, may be used as tools to help elucidate the critical events that lead to cell death. A better understanding these events may then lead to the rational development of better and more specific therapeutic agents.

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