MECHANISMS OF CARCINOGENESIS BY DICHLOROACETATE AND TRICHLOROACETATE

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FOREWARD

The AWWA Research Foundation is a nonprofit corporation that is dedicated to the implementation of a research effort to help utilities respond to regulatory requirements and traditional high-priority concerns of the industry. The research agenda is developed through a process of grass-roots consultation with members, utility subscribers, and working professionals. Under the umbrella of a Five-Year Plan, the Research Advisory Council prioritizes the suggested projects based upon current and future needs, applicability, and past work; the recommendations are forwarded to the Board of Trustees for final selection.

This publication is a result of one of those sponsored studies, and it is hoped that its findings will be applied in communities throughout the world. The following report serves not only as a means of communicating the results of the water industry's centralized research program but also as a tool to enlist the further support of the nonmember utilities and individuals.

Projects are managed closely from their inception to the final report by the foundation's staff and large cadre of volunteers who willingly contribute their time and expertise. The foundation serves a planning and management function and awards contracts to other institutions such as water utilities, universities, and engineering firms. The funding for this research effort comes primarily from the Subscription Program, though which water utilities subscribe to the research program and make an annual payment proportionate to the volume of water they deliver. The program offers a cost-effective and fair method for funding research in the public interest.

A broad spectrum of water supply issues is addressed by the foundation's research agenda: resources, treatment and operations, distribution and storage, water quality and analysis, toxicology, economics, and management. The ultimate purpose of the coordinated effort is to assist water suppliers to provide the highest possible quality of water economically and reliably. The true benefits are realized when the results are implemented at the utility level. The foundation's trustees are pleased to offer this publication as a contribution toward that end.

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EXECUTIVE SUMMARY

BACKGROUND

The haloacetic acids are now recognized as a pivotal class of disinfectant by-products. They have a variety of toxicological effects, including carcinogenic, reproductive and developmental toxicities, and neurotoxicity. These effects have only been observed at high doses, requiring in excess of 10 mg/kg body weight to be demonstrated in humans or animals. The key issue is how likely these effects are likely to be at the low levels in drinking water where daily doses to an adult human are unlikely to exceed 1 μg/kg body weight. The present project was aimed at examining the metabolic, physiologic and biochemical effects that underlie the carcinogenic response to these chemicals. The new proposed guidelines for Carcinogen Risk Assessment (EPA, 1996) emphasize the importance of such data in determining the risks such compounds pose at low environmental concentrations.

APPROACH

The intent of the project as it was initially conceived was to determine whether the carcinogenic activity of the haloacetic acids could be associated with peroxisome proliferation. Peroxisomes are subcellular organelles that have a variety of metabolic functions, the most widely recognized being the oxidation of lipids by a process that yields hydrogen peroxide as a by-product. Chemicals referred to as peroxisome proliferators increase the numbers of these organelles. This response appears to be confined to rodent species. Trichloroacetic acid (or more properly trichloroacetate, TCA) had been shown to be a member of this class of carcinogen. As the project was initiated, the work of DeAngelo and coworkers made it clear that peroxisome proliferation plays little, if any, role in the carcinogenic responses to dichloroacetate (DCA). Thus, the scope of the project in the peroxisome proliferation area was narrowed and two additional areas were suggested for exploration. These included 1) examination of how DCA and TCA influenced cell replication and cell death rates in normal liver and liver tumors and 2)

determining how the metabolism of DCA, TCA, and their metabolites might be modified by chronic exposure.

Peroxisome proliferation and liver cancer induction

The intent of this work was to associate the induction of oxidative damage within DNA of the liver with peroxisome proliferation induced by DCA and/or TCA. This would have provided a plausible link between increases in the number of peroxisomes and the induction of tumors since excess hydrogen peroxide could be converted to hydroxyl radical by the Fenton reaction. The major measurement of oxidative damage utilized was increases in the amount of 8-hydroxy-2-deoxyguanosine (8-OH-dG) in nuclear DNA of the liver.

While there was evidence of small increases in the levels of 8-OH-dG in mice give acute doses of DCA and TCA by gavage, this response was clearly not associated with peroxisome proliferation. Neither compound increased the steady-state levels of 8-OH-dG in animals maintained on drinking water containing sufficient concentrations to produce a large and consistent carcinogenic response for up to 71 days. This lead to the conclusion that the carcinogenic effects of TCA are probably still associated with some response to activation of the peroxisome-proliferator activated receptor, but it does not seem to be mediated through oxidative damage to DNA. Our work confirmed the observations of previous workers that the carcinogenic effects of DCA are not associated with its ability to induce peroxisome proliferation.

Effects on Cell Replication

Experiments on cell replication involved chronic treatment with DCA and TCA until sufficient pre-cancerous lesions and tumors developed. Then the high concentration was replaced with distilled water or a series of lower concentrations to determine whether an effect on the replication rates of normal and altered cell populations could be detected. These lower concentrations were maintained for two weeks, a marker substance for dividing cells injected, the experiment terminated, and the cells that divided counted in both normal and altered cells.

In the case of DCA, normal cell replication rates were inhibited at concentrations of 0.5 g/L and above, and at 2 g/L the rate of altered cell populations was approximately doubled. These effects were associated with the induction of a specific group of oncoproteins within the tumors, c-Jun and c-Fos.

Tumors induced by TCA did not display increased expression of the c-Jun and c-Fos oncoproteins, strongly suggesting that the two chemicals are acting by distinct mechanisms. TCA did decrease replication rates within normal hepatocytes at concentrations of 1 and 2 g/L in drinking water. However, the replication rates within tumors displayed no obvious dependence upon TCA treatment.

These data demonstrate that a major component of DCA's carcinogenic effects are attributable to effects on cell replication. Thus, it appears to be acting like a true tumor promoter. Such effects form the basis for non-linear risk assessment models. Risk assessments for TCA may take a different form, but still non-linear forms. Previous data suggests that peroxisome proliferators act by preventing damage cells from dying. If these damaged cells replicate there may some possibility of their assuming a neoplastic phenotype.

Effects on Metabolism and Pharmacokinetics

The metabolism and pharmacokinetics of DCA are drastically modified by dosing regimens that induce cancer in both mice and rats. Provision of as little as 0.2 g/L in the drinking water produced maximum inhibition of a high capacity low affinity pathway for DCA. This amounts to a daily dose of approximately 20 mg/kg. Similar doses have been shown by clinical investigators to inhibit metabolism in humans as well. Pretreatment had little effect on the metabolism of TCA in either mice or rats. In part, this is due to the fact that a smaller fraction of TCA is metabolized in either animals or humans.

The implications of these data are that as doses of DCA are increased in chronic treatments, the blood levels of DCA will increase dramatically. Thus, linear extrapolation of effects at high dose will grossly overestimate the risks associated with DCA at the concentrations found in drinking water.

CHAPTER 1
INTRODUCTION

Potential hazards associated with the disinfection of drinking water first surfaced in the early 1970's with the identification of the trihalomethanes as by-products of chlorination (Rook, 1974; Bellar et al., 1974). A cancer bioassay of chloroform (trichloromethane) was completed in the same general time frame (NCI, 1976) which indicated that it produced tumors in rats and mice. These findings served to focus attention on the identification of additional by-products, toxicological characterization of by-products, and the identification of alternative methods of avoiding or decreasing the formation of harmful by-products. As a result of the ensuing research efforts, there have been some suggestions of increased cancer risk in populations served by chlorinated supplies, many additional chlorination by-products have been identified and some have been identified as carcinogens (Bull and Kopfler, 1991). On the other hand, it has also become apparent that alternative forms of disinfection, such as ozone, chlorine dioxide and chloramine form by-products as well (Bull and Kopfler, 1991).

A large number of epidemiological studies of chlorinated water have been conducted following the identification of trihalomethanes as a by-products. This literature is much too extensive to be reviewed in detail in this project report. In summary, two tumor sites show a small but fairly consistent relationship with the use of chlorine; bladder and colorectal cancer (Craun, 1988; Morris et al., 1992; Bull et al., 1995). More recently, pancreatic cancer has been identified as a site (Ijsselmuiden et al., 1992), but this result has yet to be replicated. It is important to point out that although the incremental cancer risk associated with these tumor sites has been suggested as being fairly substantial (Morris et al., 1992), this is in part a function of how the population at risk is calculated (Murphy, 1993). There are several problems which underlie these data, two of which will be given particular mention here. First, the small odds ratios obtained in these studies make it difficult to rule out other contributors to these apparent risks. This is a particular problem since the best studies compared chlorinated surface water with non-chlorinated ground water. Second, the nature of by-products varies significantly by geographic location (Singer, 1995; Singer, 1996). These differences in by-product character could be responsible for the relatively small odds ratios and could account for some

inconsistencies that have been found in the types of cancers in different locations in more recent investigations (Bull et al., 1995).

Disinfection provides an important barrier to the transmission of waterborne infectious disease (Bull et al., 1995). Therefore, a method whose efficacy and safety appears established by long use should not be discarded lightly. While there are alternatives to chlorine that are as effective in killing organisms, the ability to maintain and residual (as either chlorine or chloramine) adds an important additional measure of safety. The application of chlorine requires less sophisticated operator training. Thus, it is much better suited for small systems. Consequently, it is critical that the magnitude of the cancer risk associated with by-products be characterized and considered in light of competing risks before chlorine is discarded as a disinfectant.

Parallel to the studies in humans have been advances in the ability to identify and measure by-products other than the trihalomethanes. This, in turn, has led to toxicological evaluations of additional classes of by-products, particularly the haloacetic acids, the haloacetonitriles and halogenated aldehydes and ketones (Bull and Kopfler, 1991).

The haloacetic acids are now recognized as by-products that are critical to evaluating the health hazards that might be associated with the chlorination of drinking water. Dichloroacetic acid (DCA) and trichloroacetic acid (TCA) are the two most widely measured and characterized members of this class. In general, DCA and TCA are found in chlorinated waters at concentrations ranging from a few to as high as 100 μg/L (Krasner et al., 1989; Singer and Chang, 1989; Singer 1995). This is the same concentration range in which the trihalomethanes are found to occur. Under conditions of low pH (e.g. pH 5-7), the haloacetates can significantly exceed the trihalomethanes in concentration (Singer et al., 1995). Since both of these chemicals are strong acids, they exist in all drinking water in the salt forms, as dichloroacetate and trichloroacetate, respectively. Because they are in the salt form, unlike the THMs they are not volatile.

DCA and TCA have received considerable attention in the recent past because they are significantly more potent as carcinogens than the strictly chlorine-substituted trihalomethane, chloroform (Bull and Kopfler, 1991). They also have greater potency and specificity for producing neurotoxic (Katz et al., 1981; Stacpoole et al. 1989), reproductive (Katz et al., 1981; Cicmanic et al. 1991; Toth et al., 1992; Linder et al., 1994), and developmental (Smith et al.,

1989; Smith et al., 1992) toxicities. Conventional application of the linearized multistage model (LMS) to the estimation of carcinogenic risks from the median concentrations of DCA and TCA found in chlorinated water (Bull and Kopfler, 1991) has focused regulatory attention most strongly on their carcinogenic properties.

The application of the LMS to estimating the human risk from the chlorinated acetic acids may not be appropriate for DCA and TCA. There are general issues that relate to the relative sensitivity of humans to the chemical relative to the experimental animal in which carcinogenic activity has been demonstrated. However, the key most important question arises from preliminary understanding of their modes and/or mechanisms by which the haloacetates induce cancer. These directly challenge application of the assumptions that underlie linear extrapolation of available data to low dose rates. While these distinctions are generally made implicitly in the development of regulations. EPA's newly proposed Cancer Assessment Guidelines (EPA, 1996) place much more emphasis on mode and mechanism of action in developing regulatory strategies for chemicals that have been shown carcinogenic. Therefore, the interplay between research results and decision-making is going to be much more explicit in the future.

TCA did not meet the International Agency for Cancer Research criteria (IARC, 1991) as a probable human carcinogen. While it is a reasonably potent inducer of liver cancer in mice (Herren-Freund et al., 1987; Bull et al., 1990), its activity is limited to this organ. Rats do not respond with liver cancer when treated with TCA in drinking water (DeAngelo et al., personal communication). Moreover, TCA belongs to a specialized class of carcinogens, the peroxisome proliferators. This class of carcinogen is characterized by an ability to induce the synthesis of a subcellular organelle in the liver of rodents, the peroxisome. It requires much higher concentrations of peroxisome proliferators to increase the numbers of peroxisomes in non-rodent species, including primates (Bieri et al., 1988; Lake et al., 1989). The relative responsiveness of isolated hepatocytes from these species is well maintained *in vitro* (Blaauboer et al., 1990; Cornu et al., 1992; Dirven et al., 1993). Isolated human hepatocytes are also much less responsive with respect to peroxisome proliferation (Blaauboer et al., 1990) than rodent hepatocytes. Because of this, it has been argued that peroxisome proliferators are not likely to induce liver cancer in humans. This early interpretation is now somewhat uncertain for a number of reasons. For example, a transcription factor referred to as the peroxisome proliferator activated receptor

(PPAR) has been identified in human as well as rodent tissues. However, activation of this receptor is indirect (Issemann and Green, 1990) and expression of responses is dependent upon other factors. The activity of the PPAR depends upon its dimerization with other members of the steroid hormone superfamily of receptors, most specifically the retinoid X receptors. This class is known collectively as the erbA-nuclear receptor superfamily, and includes the retinoic acid, glucocorticoid, thyroid hormone receptors (Motojima, 1993). The interactions of some members of these families served to enhance transcription while others inhibit transcription. In particular, the PPARα's activity as a transcription factor is stimulated by the RXR receptor, but inhibited by dimerization with PPARγ (referred to as hNUC1 when first identified in human tissues) (Jow and Mukherjee, 1995). Until the causal pathway between peroxisome proliferator administration and the induction of cancer is better established, this issue will remain controversial, as these examples amply show that responsiveness is not simply related to the presence of a receptor, but involves the interplay between all of them.

Several studies have been conducted of DCA and TCA's potential for inducing DNA damage and point mutations. Conventional Ames' test evaluations have been generally negative (Nestmann et al., 1980; Rapson et al., 1980; Waskell, 1978; Moriya et al., 1983; Meier and Blazek, 1991). Herbert et al. (1980) did report positive results in Salmonella tester strain TA98, however, these responses failed to meet the commonly accepted criteria of at least doubling the background mutation frequency. Initial studies by Nelson et al., (1988; 1989) indicated that DCA was capable of producing single strand breaks in the liver of rodents *in vivo*. Subsequent authors failed to confirm these results (Styles et al. 1991 ; Chang et al. 1992). More recently, it has been shown that there are small increases in the 8-hydroxy-2-deoxyguanine (8-OH-dG) content of nuclear DNA isolated from mice treated with DCA and TCA (Austin et al., 1996). These increases could account for the apparent increases in single strand breaks reported by Nelson et al. (1988). Bhunya and Behera (1987) reported that TCA-induced micronuclei in bone marrow and sperm abnormalities in mice. The more recent findings of Styles et al. (1991) indicate that the latter results arose from the administration of the free acid rather than salt forms.

There is one report indicating that DCA could be a weak inducer of point mutation (DeMarini et al., 1994). In this study, DCA and TCA were introduced into the test system as the free acids into a sealed system. As the free acids, these compounds do have a significant vapor

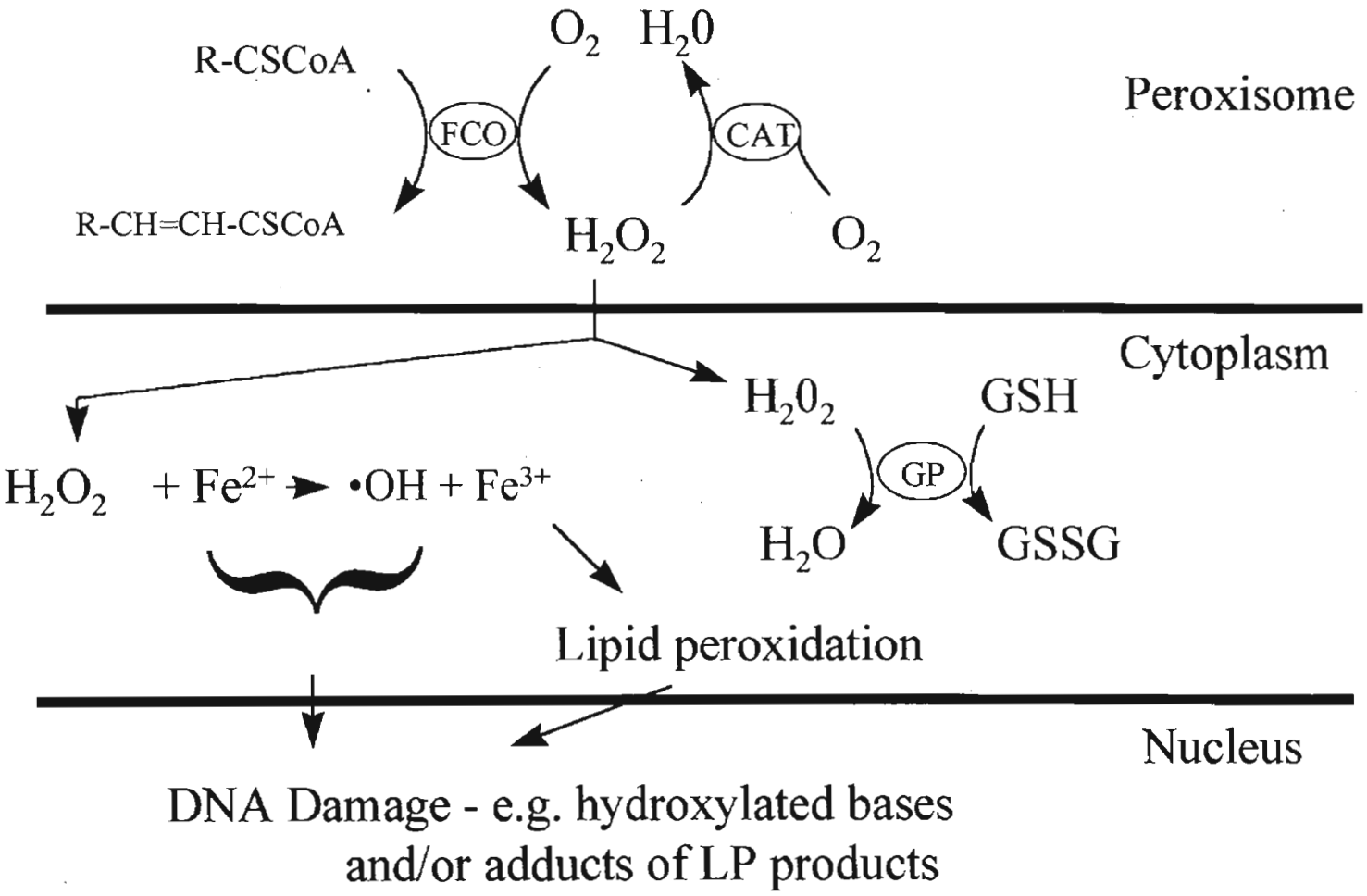
pressure, but this is not characteristic of the salt forms that would exist at drinking water pH because the pKa's of both compounds are well below 2. This method of administration potentially sets up a circumstance in which DCA and TCA would be trapped at high concentrations within the media. If this were to occur it is probable that substantial decreases in pH would occur. There was no indication that controls for acidification of the media were included in these experiments.

Unlike TCA, DCA does induce liver cancer in both mice and rats (Herren-Freund et al., 1989; Bull et al., 1990; DeAngelo et al., 1991; Richmond et al., 1995). While DCA does have some peroxisome proliferator activity (DeAngelo et al., 1989; Nelson et al., 1989), it induces liver cancer at doses lower than those required to increase peroxisome numbers (DeAngleo et al., 1989; Daniel et al., 1992).

Given the lack of direct evidence that either DCA or TCA can directly damage DNA, this project was sought to establish whether other mechanisms might account for their carcinogenesis. Such information is essential for arriving at alternative low dose extrapolation models to the LMS. The initial intent of the project was to determine if the induction of hepatic cancer could be associated with oxidative damage to DNA proposed to result from the increased hydrogen peroxide anticipated from the increased metabolism of fatty acids and other substrates by the higher amounts of peroxisomal enzymes. This direction to the research was suggested by the results of Kasai et al. (1989) and other investigators (Takagi et al., 1990a; 1990b; 1991) with other peroxisome proliferators. An hypothesis of how these activities act to induce damage in DNA was proposed by Reddy and coworkers (Fahl et al., 1984; Goel et al., 1986) and is outlined in Figure 1.1.

The measure of oxidative damage to DNA used in these experiments was the 8-OH-dG content of nuclear DNA in animals treated with DCA and TCA. Increases in cyanide-insensitive acyl-CoA activity and catalase were convenient and well-established markers of increased peroxisome numbers (Reddy and Lalwani, 1983). In addition, the induction of a cytochrome P450 family (IVA) that is known to be sensitive to peroxisome proliferators (Okita and Okita, 1992) was also measured. This enzyme is known for its capability to hydroxylate the terminal carbon of lauric acid (12-OH). The products of such oxidations have been implicated as intermediates in the induction of peroxisome proliferation. A clear association could be made

Figure 1.1. Oxidative stress hypothesis for linking peroxisome proliferation to cancer. Hydrogen peroxide is produced by fatty acid acyl-CoA oxidase (FCO) and detoxified by catalase (CAT) in the peroxisome. Glutathione peroxidase (GP) can break down hydrogen peroxide in the cytoplasm.



Adapted with permission from: Conway et al. (1987) CIIT Activities 7:1.

between these variables would be consistent with the hypothesis that the induction of cancer in rodents was directly dependent upon the increases in the number of peroxisomes. A corollary would be that if there was no evidence of peroxisome proliferation in human cells, then humans should not be at risk from a chemical whose carcinogenic effects are dependent upon increased numbers of peroxisomes.

As the project got underway, DeAngelo and coworkers informed the PI that they had dissociated the effects of DCA from peroxisome proliferation. Consequently, the project took a more exploratory direction based upon consensus opinions of the Project Advisory Committee (PAC). The original intent was to examine the oxidative damage from peroxisome proliferation in sensitive (mice and rats) and non-sensitive (guinea pigs) species. The scope of this work was narrowed to initial characterization of the responses in mice and to refocus some resources on identifying potential modes of action of DCA. Subsequently, the PAC concurred with additional changes in the scope of work as it became apparent that chronic treatment with DCA and TCA did not result in increases of 8-OH-dG even in the most sensitive species (mice).

Two specific areas of additional research were identified. The first was to obtain information on the ability of the haloacetates to modify cell replication rates, not only with normal cells in the liver, but those found in preneoplastic lesions (henceforth, altered hepatic foci, AHF, and hyperplastic nodules, HN) and tumors (hepatocellular adenomas and carcinomas). The second area were studies of the metabolism and pharmacokinetics of DCA and TCA, focusing particularly on changes in these parameters that would be induced with continuous exposure at concentrations in drinking water that induced liver tumors in experimental animals.

When the form of the chemical that is responsible for the toxicological effect becomes apparent, information on its metabolism and pharmacokinetics is very important for normalizing effective internal dose given comparable external exposures across species. Without such information, dose is scaled by surface area as clearance of chemicals appears to be related generally to the basal metabolic rate of a species which in turn is usually proportional to total body surface area. The practical consequence of this assumption is the inclusion of an additional uncertainty factor. Of particular interest in this study is that long-term treatments in animals were used to assess the potential harm that DCA and TCA would have to human health. This was viewed as a particularly important issue with DCA, as clinical use of repeated high doses of DCA

has been shown to modify clearance of the chemical from the blood (Curry et al., 1985). If the same effects were observed in animals, then the effective dose required to produce an adverse response at low exposures would be overestimated. In turn, this would result in an underestimate of the size of the administered dose that would be required to produce a particular effect. As a result the risk attributed to low exposures from drinking water would be significantly exaggerated without more accurate information on pharmacokinetics and metabolism.

Combined, these three types of data (mode of action, mechanism of action, and metabolism and pharmacokinetics) provide the basis for the construction of a biologically-based dose-response model that will more accurately normalize doses and predict responses in humans exposed to low concentrations of DCA and TCA in drinking water.

CHAPTER
METHODS

Oxidative Stress and Peroxisome Proliferation

Chemicals and Standards

Dichloroacetic acid (DCA) was obtained from Fluka Chemika-Biochemika, Ronkonkoma, NY. Trichloroacetic acid (TCA), HPLC grade methanol, sodium acetate, citric acid, chloroform, isoamyl alcohol, nuclease P1, ribonuclease A (Type II-A), ribonuclease T1, bacterial alkaline phosphatase (Type III), proteinase K, and 2'-deoxyguanosine were purchased from Sigma Chemical Co., St. Louis, MO. DCA and TCA were found to be $\geq$ 99% and DBA >97% pure by gas chromatographic analyses. Sucrose was obtained from Gibco BRL, Gaithersburg, MD

Chromatographic standards for 8-hydroxy-2-deoxyguanosine (8-OH-dG) were synthesized by the method of Kasai and Nishimura (1984) and chromatographically purified by the method of Floyd *et al.* (1986), except that the methanol in the mobile phase was reduced to 5%. UV and mass spectral characteristics of purified 8-OH-dG were in agreement with previously reported values (Kasai and Nishimura, 1984).

Animals

Seven-week-old male B6C3F$_1$ mice (20-22g) were obtained from Charles River Laboratories, Inc., Kingston, NY. The mice were housed 6 per cage in an environmentally controlled room at constant temperature (22-24°C), humidity (40-60%), and light cycle (12 h light/12 h dark). The animals were randomly assigned to one of treatment groups after a one week acclimation period. Solutions of dichloroacetic and trichloroacetic acid were prepared to the indicated concentrations and neutralized with sodium hydroxide to a pH between 6.8 and 7.2, and supplied to the mice *ad libitum* as their drinking water. Control groups of animals received double distilled water, pH 6.8 -7.2, in their drinking water.

For the 21 and 71 day experiments, mice were provided drinking water provided containing TCA or DCA at concentrations of 0, 0.1, 0.5 or 2.0 g/L or clofibric acid at a concentration of 1.25 g/L. Animals were sacrificed after 21 or 71 days by cervical dislocation. The livers of the animals were immediately excised, weighed, and snap frozen in polypropylene vials by immersion into liquid nitrogen where they were stored at -195 °C until isolation of liver nuclei.

Isolation of Liver Nuclei and Genomic DNA

Liver nuclei were isolated by the method of Lynch *et al.* (Lynch *et al.*, 1970). Briefly, 1 g of liver tissue was thawed in 10 ml of 0.3 M sucrose solution and homogenized with 4 strokes in a glass/Teflon homogenizer. The resulting homogenate was passed through 4 layers of sterile gauze and centrifuged at 1500 x *g* for 20 minutes at 4°C. The nuclear pellet was resuspended in 20 ml of 2.0 M sucrose and centrifuged over a 10 ml layer of 2.2 M sucrose at 74,000 x *g* for 1 hour at 4°C. The supernatant was aspirated off and the purified liver nuclei was suspended in 8 ml of 0.1 M tris-EDTA (TE) buffer.

Genomic DNA was isolated from purified liver nuclei by a modification (Cattley and Glover, 1993) of a previously reported method (Marmur, 1961). Phenol was omitted to avoid artifactual formation of 8-OH-dG. Briefly, 80 µl of 10% SDS and 0.2% proteinase K were added to the nuclei suspension which was then placed on ice for 45 minutes. The resulting digest was

extracted twice with an equal volume of 5:1 chloroform:isoamyl alcohol (CIA). DNA was precipitated with 2 volumes of cold 2-ethoxyethanol and washed with 70% ethanol. The resulting pellet was dissolved in 2 ml of TE buffer overnight at 4°C. The pellet was purified further by incubating the sample with RNAse A and T1 for 1 hour at 37°C followed by a second CIA extraction and 2-ethoxyethanol precipitation step. Isolated genomic DNA was digested to the nucleoside level by incubation with nuclease P1 and bacterial alkaline phosphatase and stored on ice until HPLC-Electrochemical (HPLC-EC) analysis.

HPLC-EC Analysis

Analysis of DNA hydrolysates was performed using high performance liquid chromatography with simultaneous UV (254 nm) and electrochemical (5 na) detection as described by Kasai and Nishimura (Kasai and Nishimura, 1984) and Floyd *et al.* (Floyd *et al.*, 1986). The HPLC system consisted of a Shimadzu LC-10AD pump, SIL-9A automated injector, SPD-6AV UV detector, and a ESA Coulochem II electrochemical detector equipped with a model 5020 guard cell (400 mV) and a model 5010 analytical cell (E_1 = 100 mV, E_2 = 350 mV). The column used was a thermostatted (35°C) 4.6 x 250 mm Supelcosil LC-18S protected by a 4.6 x 20 mm Supelguard guard column (Supelco, Inc., Bellefonte, PA). In-line UV and EC detector responses were monitored on a Kipp and Zonnen model BD112 dual channel flatbed recorder. Base oxidation was determined by calculating the ratio of 8-OH-dG/10^5dG based on external standards. Sample sizes of 40 fmol in spiked tissue extracts could be repeatedly measured with less than 10% variance and with special care 10 fmol could be measured with a variance of approximately $\pm$ 30%.

Cyanide-Insensitive Acyl-CoA Oxidase Activity

Palmitoyl-CoA oxidase activity was measured by the method of Lazarow (Lazarow, 1981). This method spectrophotometrically measures the reduction of NAD to NADH that occurs at the third step of the β-oxidation spiral in the presence of palmitoyl-CoA. Separate experiments for DCA and TCA had concurrent control groups. The control values between

experiments and at the two sacrifice times varied as much as a factor of 2. Therefore, data were presented as percent of concurrent control values to simplify the presentation of the data.

Laurate Hydroxylase Activity

Differential centrifugation was performed on liver homogenates based on previously described methods (Okita and Okita, 1992). Livers are minced in homogenization buffer (10 mM phosphate buffer, pH 7.4, containing 250 mM sucrose and 1 mM EDTA) and then homogenized using 4 strokes of a glass/Teflon homogenizer. Total volume was adjusted to 3 ml buffer/g tissue and then spun at 4°C for 15 minutes at 7,700 x g to remove debris and unbroken cells. The supernatant was transferred to new tubes and first centrifuged for 15 minutes at 18,500 x g to pellet mitochondria and peroxisomes. The supernatant was again transferred to new tubes and centrifuged for 1 hour at 86,000 x g to pellet microsomes. The supernatant (cytosolic fraction) was saved and the microsomal pellet was resuspended in 10 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and 100 mM KCl. The resuspended pellet was centrifuged for 1 hour at 85,600 x g. Both the mitochondrial and microsomal pellets were resuspended in 50 mM phosphate buffer and 1 mM EDTA and stored at -70°C along with the cytosolic fraction until needed.

Total cytochrome P450 content was measured by the method of Estabrook and Werringloer (Estabrook and Werringloer, 1978). Cytochrome P450 4A activity was measured using a modified reverse-phase HPLC method using (1-14C)-laurate as substrate (Okita *et al.*, 1979). The modification consists of extracting once in methylene chloride in place of three times in ethyl acetate.

Cell Replication Experiments

Animals and experimental

Six week old male B6C3F1 mice were purchased from Simonsen Laboratories (Gilroy, CA), allowed to acclimate for one week, and randomly assigned to control and treatment groups. Mice were treated with 2.0 g/L DCA or TCA in their drinking water for pretreatment periods of

38 or 50 weeks, respectively. This was the period of time that was determined to be sufficient to allow detection of AHF and tumors in preliminary experiments. At the end of the pretreatment period, mice were then randomly assigned into groups of 12 receiving 0, 0.02, 0.1, 0.5, or 2.0 g/L DCA or TCA for an additional two weeks. This two week recovery period had been previously determined to be a sufficient amount of time to allow differences in cell replication to be detected between low and high dose recovery groups. Animals from the preliminary and main experiments were combined in the data analysis.

Food and water consumption were monitored and animals weighed weekly for the duration of the study. Three days, for DCA treated animals or five days, for TCA treated animals, prior to sacrifice mini-osmotic pumps, model 1003D or 2001 (Alza Corp., Palo Alto, CA) were implanted subcutaneously over the dorsal region of the mice, under Metofane™ anesthesia. Pumps continuously delivered 1 µl/hr 5-bromo-2'-deoxyuridine (BrdU) (Sigma chemicals, St. Louis, MO) for 72 or 120 hours. It should be noted that 5-day mini-osmotic pumps were used in the TCA experiment because it had been determined that background rates of cell proliferation would be detected more effectively at the low rates seen in the aged mice. In particular, it allows more accurate determination of the inhibition of division in the normal cell population that was observed in the previously completed DCA experiment.. The mice were killed by cervical dislocation and the livers were weighed, gross lesions measured, recorded, and removed. Representative sections were allowed to fix in 10% buffered neutral formalin for 24 hours and then moved to 70% ethanol until they could be paraffin embedded.

Tissue staining

Four sets of serial sections were cut from the non-involved portion of the livers of mice and rats treated with DCA and TCA and stained with Periodic acid-Schiff stain (PAS), anti-BrdU antibody (Becton Dickinson, San Jose, CA), and with hematoxylin and eosin (H&E). PAS sections were examined and glycogen-poor foci identified in and the cell proliferation rate within the foci counted in the next serial section for the DCA-treated animals. TCA-induced lesions were not visible upon examination of PAS, therefore, H&E counterstained, anti-BrdU stained sections were examined for eosinophilic and basophilic AHF. Lesions that were identified visually

from both TCA- and DCA-induced lesions were sectioned into a set of four serial sections, stained with the following antibodies, anti-c-Jun (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), anti-BrdU antibody and anti-c-Fos antibody (Oncogene Science, Uniondale, NY). Periodic acid Schiff stain (PAS) was applied to the fourth section to detect glycogen that remains after fixation as previously described (Bull et al., 1990).

The immunohistochemistry methods used were adapted from the method of Eldridge *et al* (1990). Briefly, liver sections were incubated for 1 hour with the primary antibody (anti-BrdU, c-Jun or c-Fos). After incubation with the primary antibody, biotinylated mouse or rabbit IgG was incubated for 30 minutes at room temperature, then incubated with an avidin-biotin peroxidase complex (Vectastain peroxidase Elite ABC Kit, Vector Labs, Burlingame, CA) for 30 minutes. BrdU incorporation was visualized with the chromagen 3-amino-9-ethylcarbazole (Biomedia Corp., Foster City, CA) and c-Jun and c-Fos expression was visualized with a Vector VIP substrate kit (Vector Labs, Burlingame, CA).

Labeling indices were determined by counting a minimum of 2000 hepatocytes in the non-involved tissue (NIT, i.e. meaning tissue that was not recognizable as a nodule or tumor) or the entire nodule or tumor when applicable.

Metabolism Experiments

Animals and pretreatment

Male B6C3F1 mice and F344 rats were randomly assigned to control and pretreatment groups. In the initial experiments, F344 rats were obtained with surgically implanted jugular cannulae from Hilltop Laboratories. Subsequently, non-cannulated animals were used. The pretreatment groups were placed on either 0.2 or 2 g/L of DCA of TCA in drinking water for 14 days. On the fifteenth day, all animals were administered an acute dose of a single dose of 5, 20 or 100 mg/kg of [^{14}C]-DCA or [^{14}C]-TCA (administered i.v. in initial experiments with rats, but p.o. in mice and comparison studies in rats) and were housed in air tight glass metabolic cages. Carbon dioxide was collected in 2 N NaOH traps and aliquots were taken periodically and used to measure cumulative conversion of DCA or TCA to CO_2.

Blood samples

Levels of radiolabel in blood was performed following the protocol of Kessler (1989). DCA and TCA in blood were analyzed by gas chromatography using ECD detection on a DB-WAX column, 30m X 0.537mm i.d.,1.0μm film thickness (J & W Scientific). Carrier gas Helium; makeup gas Argon/Methane (95/5). Methods of analysis were as described in Templin et al. (1994) except that they were stored frozen prior to acidification, methylation and extraction into ether. This avoids the artifactual breakdown of TCA to DCA when fresh blood is acidified.

Metabolites in urine

The methods used were described previously by Xu et al. (1995). Urine samples were treated to precipitate oxalate as calcium oxalate and counted (LSC). The remaining organic acid metabolites were extracted with diethyl ether, back extracted as sodium salts and were separated by HPLC using a ROA Organic Acids column (Phenomenex), where ion exclusion, ligand exchange, and adsorption mechanisms are all involved in the separation process. Fractions identified with authentic standards on the HPLC were quantified by LSC.

Statistical Evaluations of the Data

Since single measurements were made in individual animals (except in metabolism studies), experiments were individually assessed across dose using a one-way ANOVA. If the ANOVA was found to be significant ($P \leq 0.05$) separation of means was accomplished by Tukey's multiple comparison test. $P \leq 0.05$ was taken as the benchmark for rejecting the null hypothesis. Experiments involving only a single treatment and control group were evaluated using Students' t-test.

CHAPTER 3
RESULTS

General

Two sets of experiments were conducted that involved long-term treatment of animals with DCA or TCA. The first of these used concentrations of up to 2 g/L for up to 71 days. The subsequent experiment was a 40-week treatment with DCA or a 52-week treatment with TCA at 2 g/L in both cases. The longer treatment period for TCA was used because of previous observations of the generally longer latency period to tumor relative to that seen in the DCA-treated mouse (Bull et al., 1990). The adequacy of the treatment period for nodule/tumor induction was confirmed in a preliminary sacrifice of 5 animals. In neither experiment were there significant effects on body weights of mice (Figures 3.1 and 3.2), although terminal body weights of DCA-treated mice approached the point of being statistically different from their controls. This may be, in part, attributed to the tendency for the control group to be heavier than the treated group throughout the experiment.

As previously shown (Bull et al., 1990; Sanchez and Bull, 1990), both DCA and TCA produce significant increases in the size of the liver with both short and long-term exposure. The liver/body weight ratios from the 71 day experiments are shown in Figure 3.3. The liver/body weight ratios obtained in the chronic studies were: 40-week control, 4.9 ± 0.5; 40-week DCA, 9.7 ± 0.5; 52-week control, 5.5 ± 0.1; and 52-week TCA, 6.5 ± 0.7. These latter figures are significantly biased by the portion of the liver that included tumors in the DCA and/or TCA treated groups. Therefore, the data from the chronic study should not be viewed to be the simple result of hepatomegaly (enlargement of the liver) as is reflected in the 21 and 71 day data. There was not a statistically significant decrease in liver weight as a result of suspending treatment by either compound for 14 days.

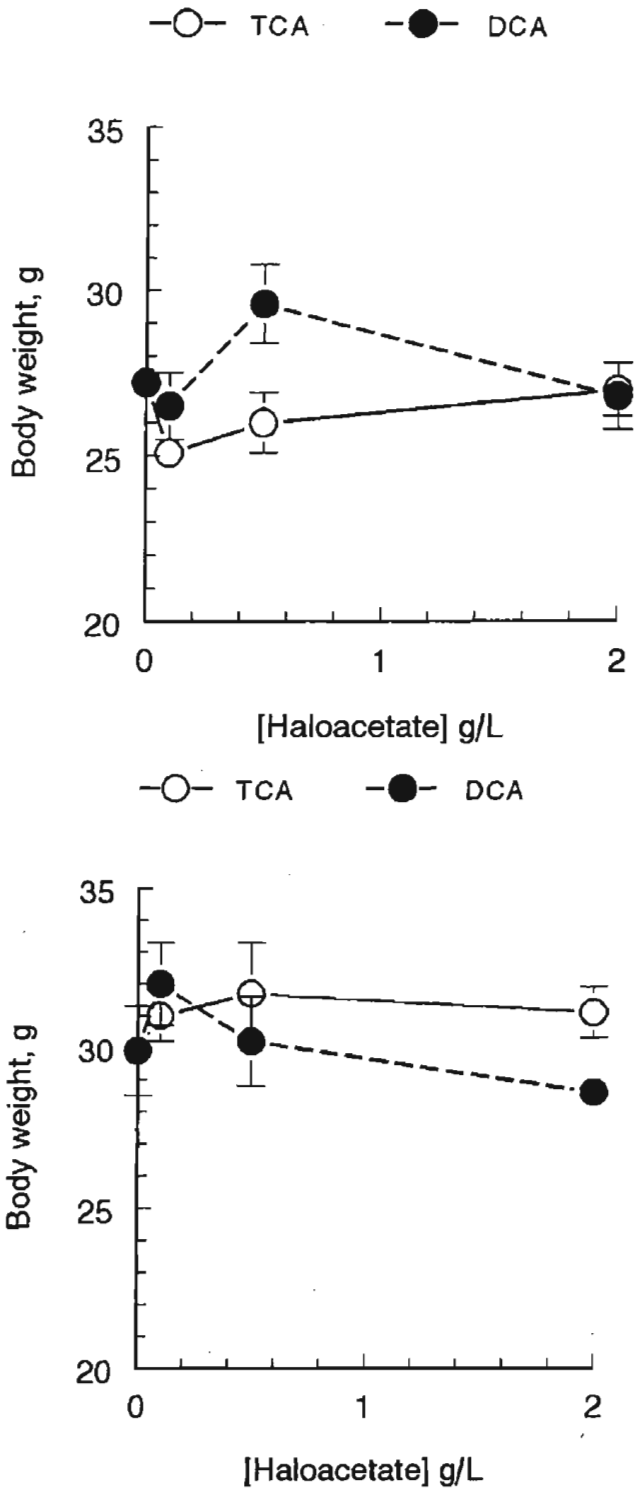


Figure 3.1. Mean body weights of male B6C3F1 mice maintained on treatments with dichloroacetate (DCA) and trichloroacetate (TCA) for 21 (upper panel) and 71 (lower panel) days of treatment. The haloacetates were administered at the indicated concentrations in the animals' drinking water. The vertical bars indicate the SEM.

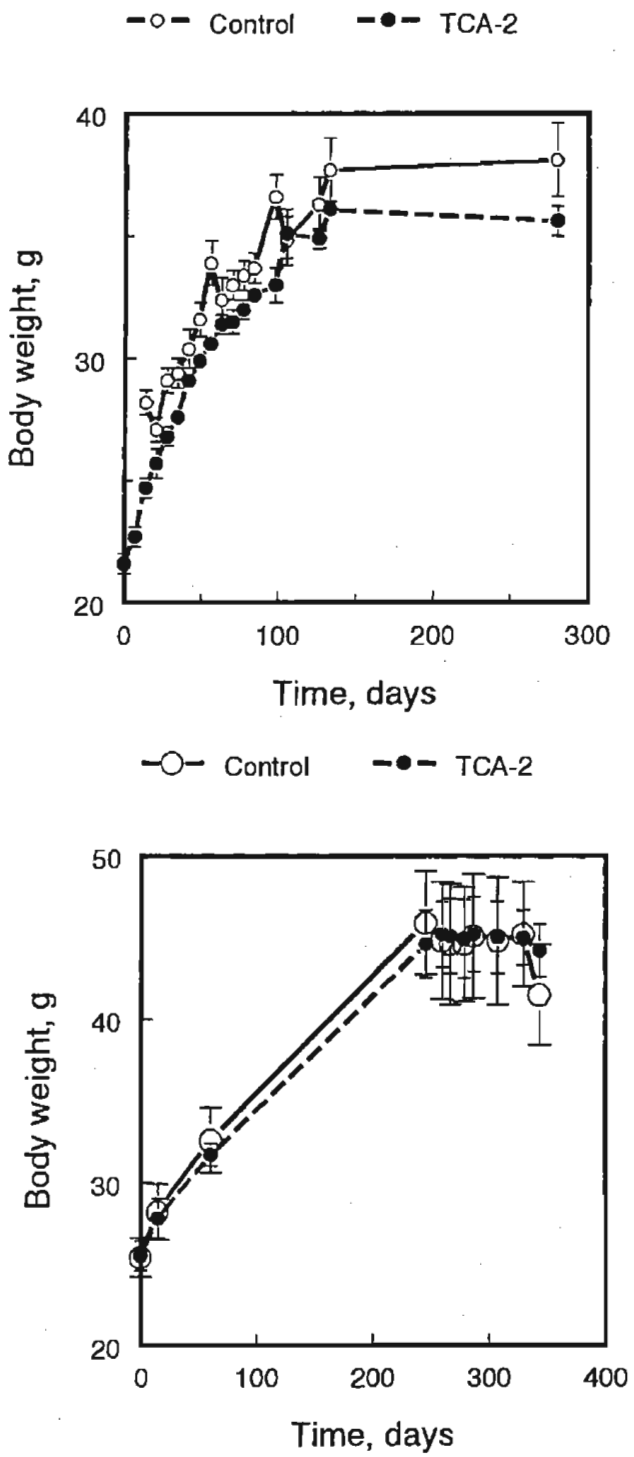


Figure 3.2 Body weight gains of mice maintained on dichloroacetate (DCA, upper panel) or trichloroacetate (TCA, lower panel) at 2 g/L in the drinking water for 40 or 52 weeks, respectively. The values show the mean ± SEM for 50 treated mice and 10 control mice in each case. The initial body weights were not available for the control mice in the DCA experiment. As the result of a mix-up in delivery of mice from the vendor, these mice were not obtained for two weeks after the experiment began. However, they had the same birth date as the experimental groups.

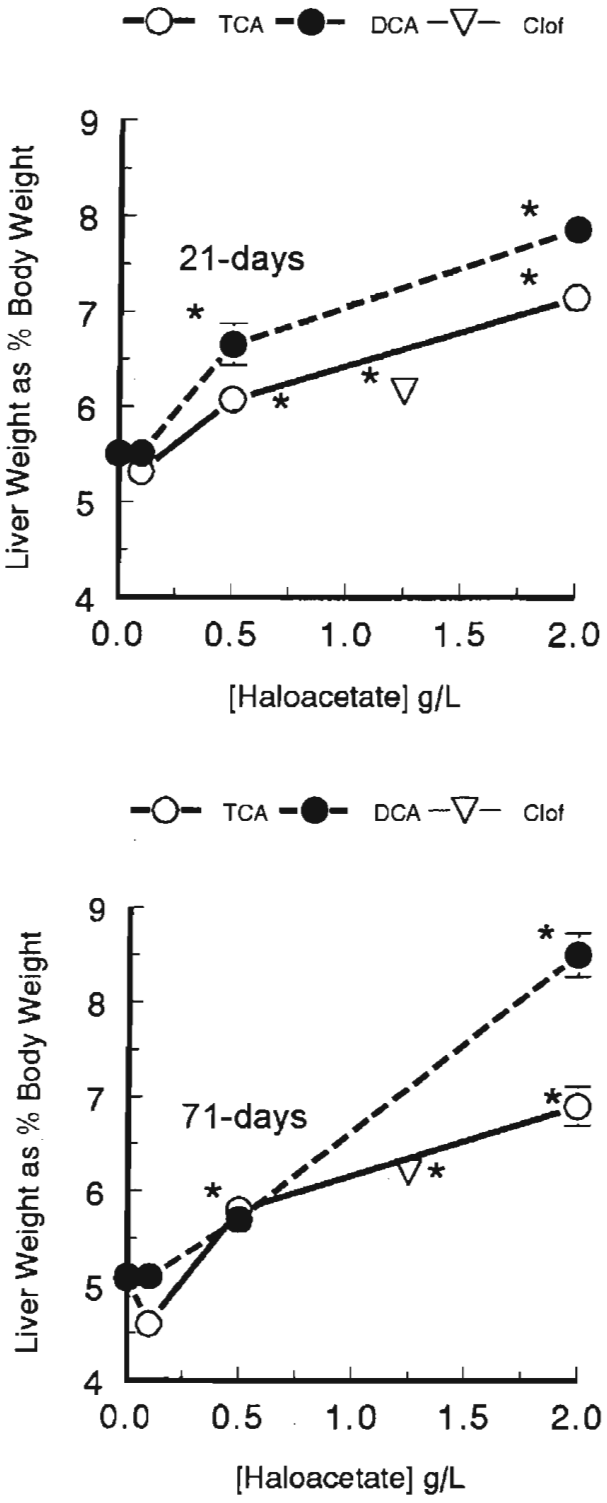


Figure 3.3 Liver/body weight ratios for mice maintained on dichloroacetate (DCA) or trichloroacetate (TCA) for 21 (upper panel) or 71 days (lower panel) of treatment. Each point represents the mean value for the indicated experimental group ± SEM. Asterisks indicate values that were significantly different from control at $P < 0.05$ by one-way ANOVA and Tukey's test. The triangle indicates the mean liver/body weight ration observed with the positive control, clofibric acid, administered at 1.25 g/L of drinking water.

Peroxisome Proliferation and Oxidative Damage to DNA

Figure 3.4 provides results of measurements of cyanide-insensitive acyl-CoA activity in the livers of DCA and TCA-treated mice. At 21 days of treatment, a significant increase in cyanide-insensitive acyl-CoA activity in the liver of mice treated with the highest concentration of TCA in drinking water (2 g/L). With continued treatment, the activity of the enzyme increased significantly at this dose and was elevated with treatments as low as 0.1 g/L at 71 days. In contrast, DCA produced no significant increases at 21 days. A very small, but statistically significant increase was observed 0.5 g/L at 71 days, but this was not increased further with the highest concentration of DCA in water. It is notable that the increase produced by DCA was significantly smaller than those produced by TCA. TCA actually increased acyl-CoA activity 9-fold of that observed in the livers of control mice. Clofibric acid was used as a positive control in these experiments and was observed to produce a 6-7 fold increase over control activities at 71 days.

Hydroxylation of laurate at the 12-position was also significantly increased by TCA treatment, but not DCA treatment (Figure 3.5). The effect of TCA was quite modest at 21 days of treatment, but was almost 3X control activity at 71 days. The actual activity of the enzyme observed in treated mice were very similar at the two time points. There was, however, an apparent decrease in the amount of the enzyme that was present in control mice between 21 and 71 days.

Catalase is localized in the peroxisome of the mammalian liver. Therefore, the modification of its level was examined in those mice treated with the highest levels of DCA and TCA (Figure 3.6). As would be predicted from the data on acyl-CoA oxidase activity, increases in catalase activity was observed with TCA-treatment. DCA produced a smaller response that was just marginally significant ($P \leq 0.05$).

The time course for changes in 8-OH-dG following DCA or TCA treatment was examined over the same time intervals previously shown to encompass maximal increases in lipid peroxidation as measured by increased levels of thiobarbituric acid reactive substances (TBARS) in the liver (Larson and Bull, 1992). Thiobarbituric acid reacts with aldehydes, especially malondialdehyde, to form a colored product that can be measured spectrophotometrically. The

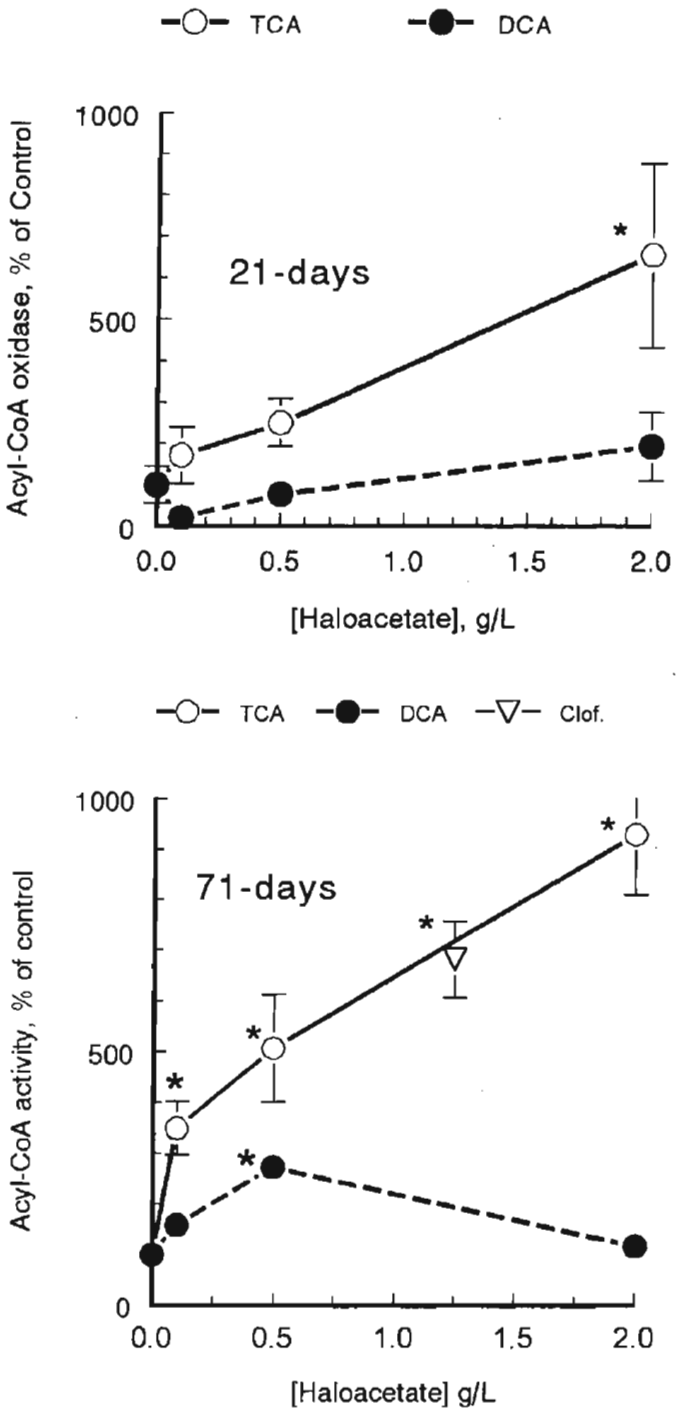


Figure 3.4 Induction of cyanide-insensitive acyl-CoA oxidase activity in the liver of male B6C3F1 mice by varying concentrations of dichloroacetate (DCA) or trichloroacetate (TCA) in drinking water for 21 (upper panel) and 71 days (lower panel). Points represent mean values ± SEM. Points marked with an asterisk were significantly different from control values by ANOVA and Tukey's test at $P \leq 0.05$.

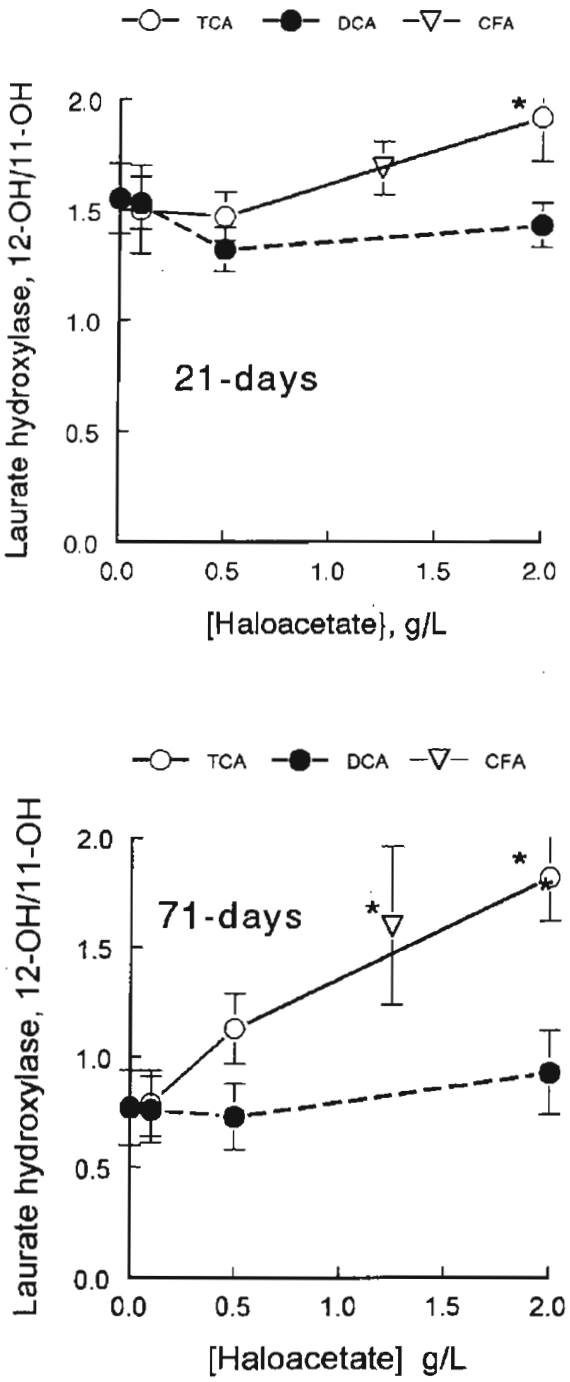


Figure 3.5. Laurate hydroxylase activity in the livers of male B6C3F1 mice treated with dichloroacetate (DCA) or trichloroacetate (TCA) in drinking water for 21 (upper panel) or 71 days (lower panel). The positive control was clofibric acid (CFA) Values are expressed as the ratio of 12- vs. 11-hydroxylase activity towards laurate. The vertical bars represent $\pm$ SEM. Values that are significantly different from control at $P \leq 0.05$ by one-way ANOVA and Tukey's test are marked by an asterisk.

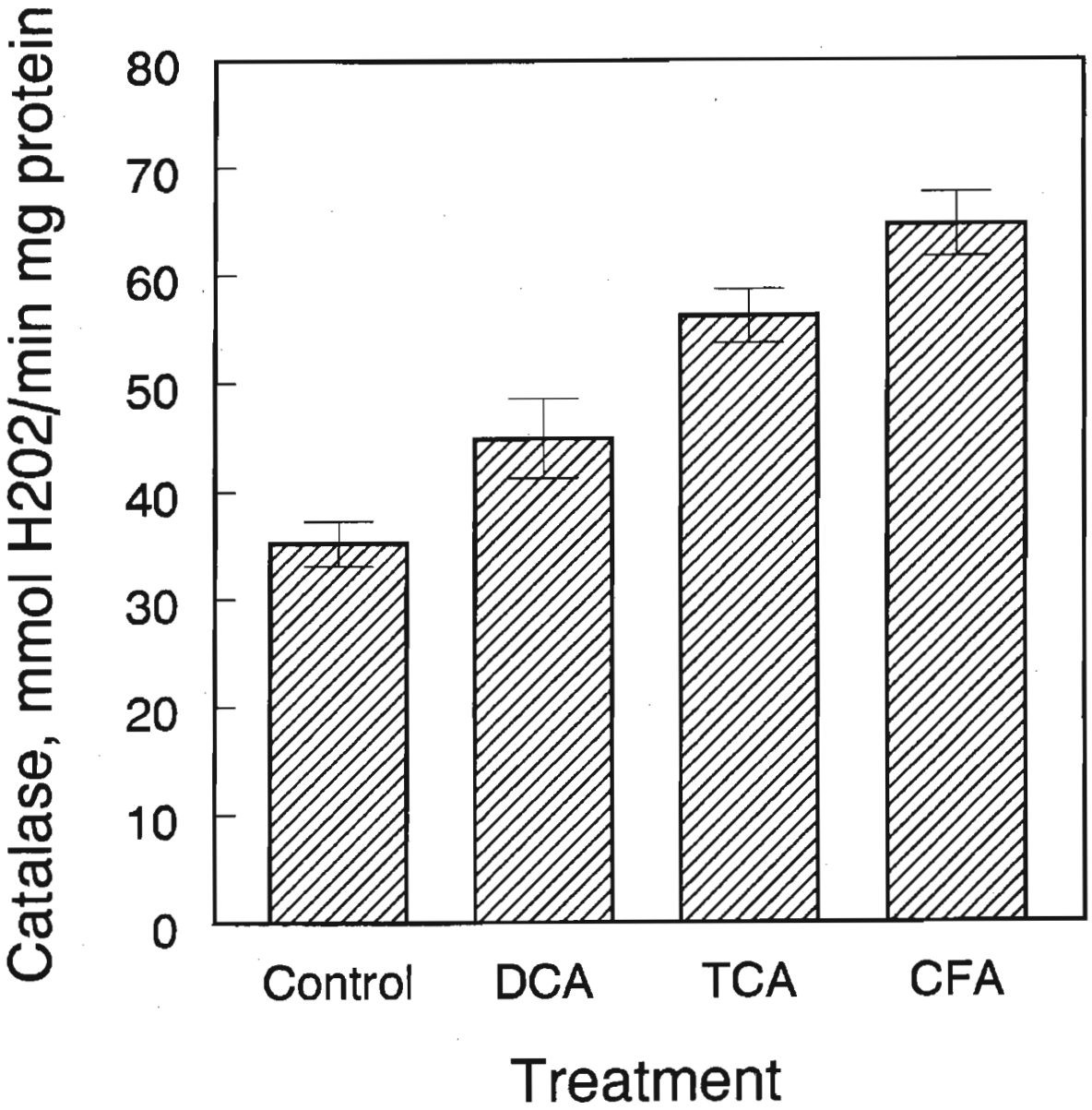


Figure 3.6. Catalase activity in the liver of male B6C3F1 mice treated with 2 g/L dichloroacetate (DCA), trichloroacetate (TCA), or clofibric acid (CFA) in drinking water for 21 days. The bars represent the mean values for 10 mice ± SEM.

ratio of 8-OH-dG to unmodified dG was observed to be at a maximum 6 hours after administration of DCA and 8 hours after the administration of TCA by stomach tube (Figure 3.7).

The critical question in these experiments, however, was whether oxidative damage to DNA produced by DCA or TCA could be correlated with measures of peroxisome proliferation. Peroxisomal numbers are unlikely to increase significantly in 6-8 hours, although there could be some increased levels of peroxisomal enzymes this early (Reddy et al., 1986). At the same 21 and 71 day treatment periods, there was little indication that either DCA or TCA administered in drinking water produced sustained damage of the type observed with acute doses administered by stomach tube (Figure 3.8). Small, but statistically significant increases in 8-OH-dG were observed at the two lowest doses of DCA, but this was not increased at the highest dose administered (2 g/L). It is notable that there was a significant increase in 8-OH-dG with age. In the interval between 21 and 71 days, the amount of 8-OH-dG increased by 50%.

Previous studies have reported increases in 8-OH-dG levels in hepatic DNA following treatment with peroxisome proliferators (Kasai et al., 1989; Takagi et al., 1990a; 1990b; 1991). Therefore, we examined the effect of isolating DNA from crude homogenates as had been the practice in these prior studies. As can be observed in Figure 3.9, there is a significant increase in the level of 8-OH-dG found in DNA of mice treated with TCA. If deferroxamine was added to the homogenate prior to beginning the extractions of DNA, the levels of 8-OH-dG were sharply decreased. This suggests that formation of 8-OH-dG results from Fenton chemistry occurring as a result the higher concentrations of hydrogen peroxide that would be anticipated in animals that had been treated with a peroxisome proliferator.

Effects of DCA and TCA on Cell Replication

In the course of the present study and previous experiments, measurements were made of cell replication rates of normal hepatocytes of control mice and those administered DCA and TCA (Figure 3.10). At a concentration of 2 g/L in drinking water, both DCA and TCA produce small, but statistically significant increases in the rate of cell replication during the first two weeks of treatment. At 4 weeks of treatment, the rates remain elevated with TCA, but the rates in DCA-treated animals are lower than control rates. The apparent inhibition of division of hepatocytes

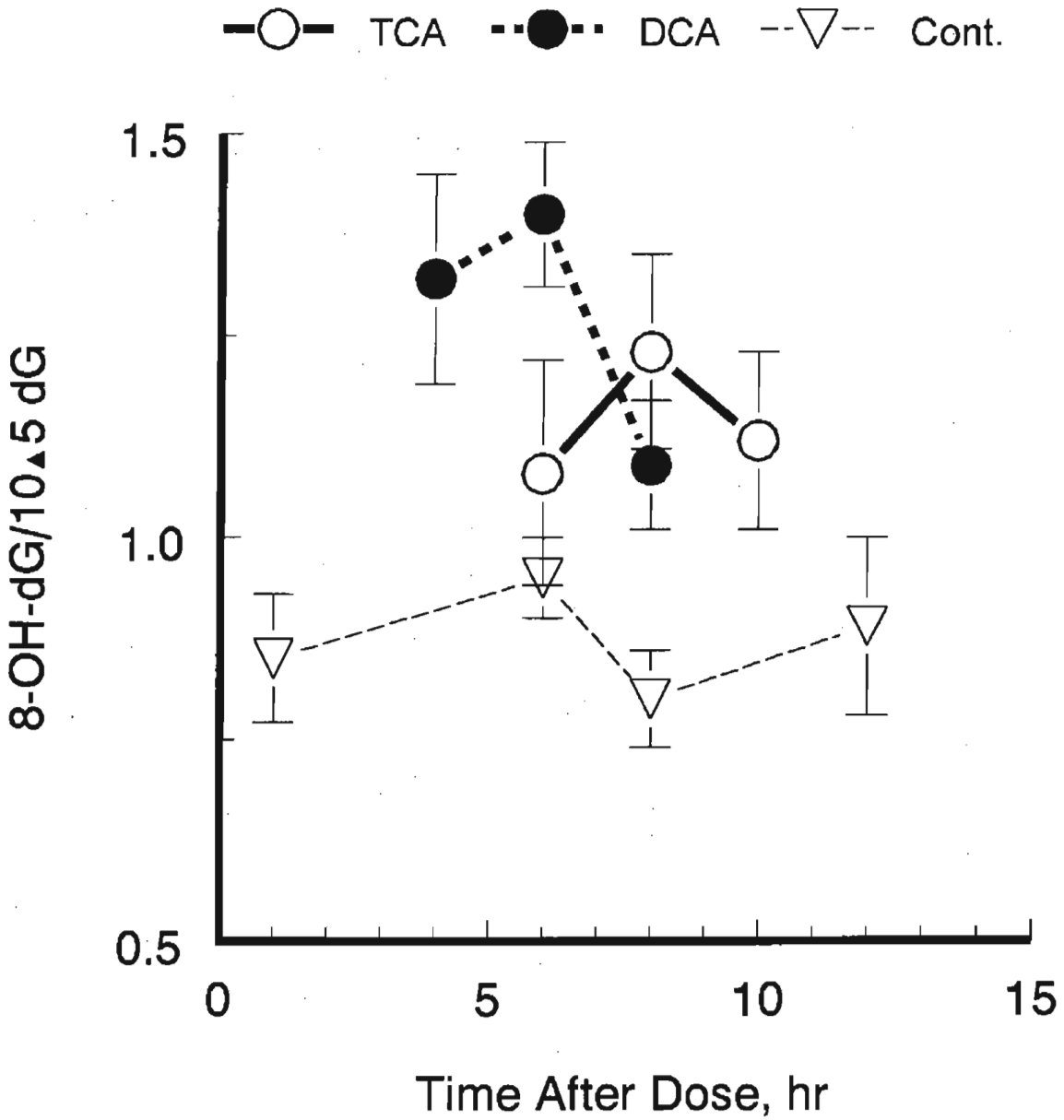


Figure 3.7. The time course of 8-hydroxy-2-deoxyguanosine (8-OH-dG) changes in nuclear DN of the liver of male B6C3F1 mice treated with a single 300 mg/kg dose of dichloroacetate (DCA) or trichloroacetate (TCA) by gavage. Each point represents the mean value from a minimum of 5 mice ± SEM

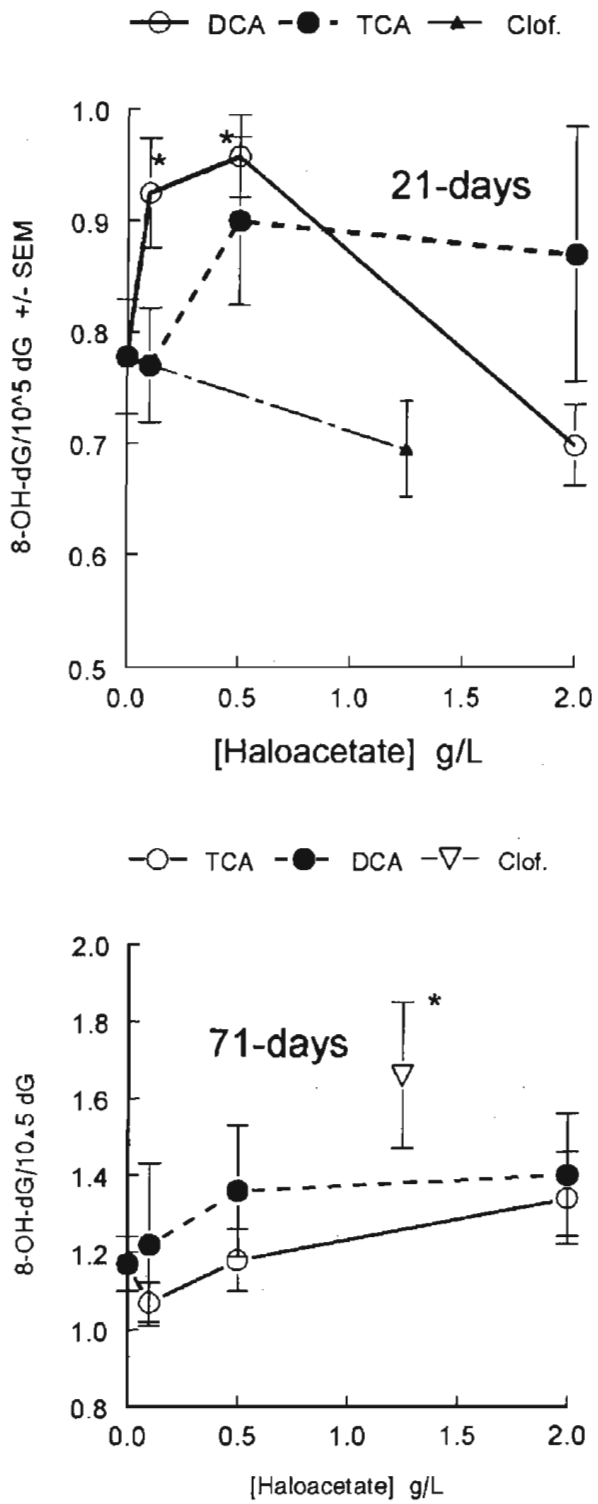


Figure 3.8. Levels of 8-hydroxy-2-deoxyguanosine (8-OH-dG) in nuclear DNA of the liver of male B6C3F1 mice treated with dichloroacetate (DCA), trichloroacetate (TCA), or clofibric acid (CFA) in drinking water for 21 (upper panel) or 71 days (lower panel). Each point represents the mean value from a minimum of 6 mice ± SEM. Asterisks indicate values that were significantly different from control at $P \leq 0.05$ by one-way ANOVA and Tukey's test.

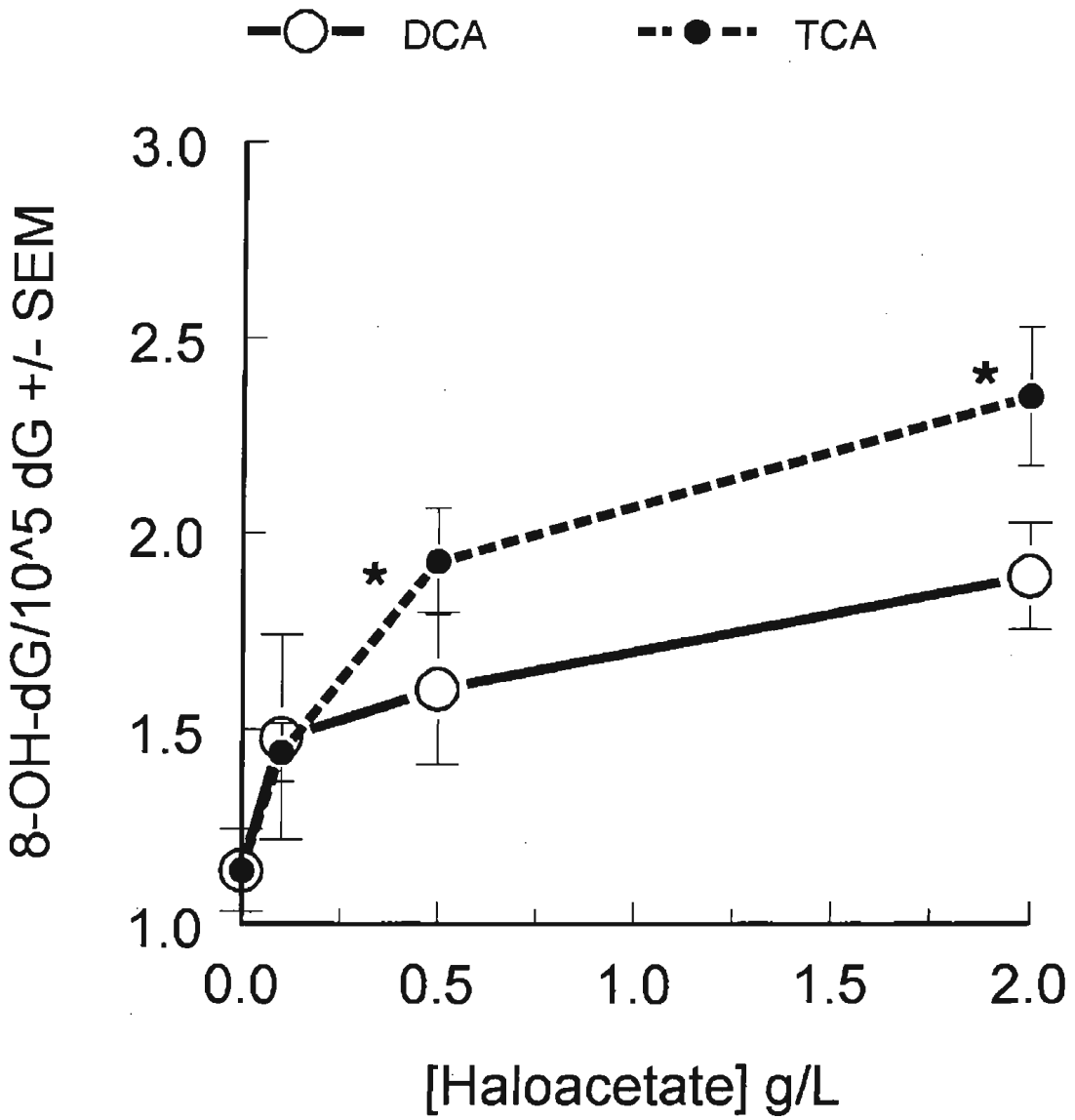


Figure 3.9. Measured levels of 8-hydroxy-2-deoxyguanosine (8-OH-dG) in hepatic DNA obtained without prior isolation of nuclei from male B6C3F1 mice treated with dichloroacetate (DCA) or trichloroacetate (TCA) in drinking water for 21 days (same mice used in Figure 3.8)) Each value represents the man value of a minimum of 6 mice $\pm$ SEM. Values that were significantly different from control at $P \leq 0.05$ by one-way ANOVA and Tukey's test are marked by an asterisk.

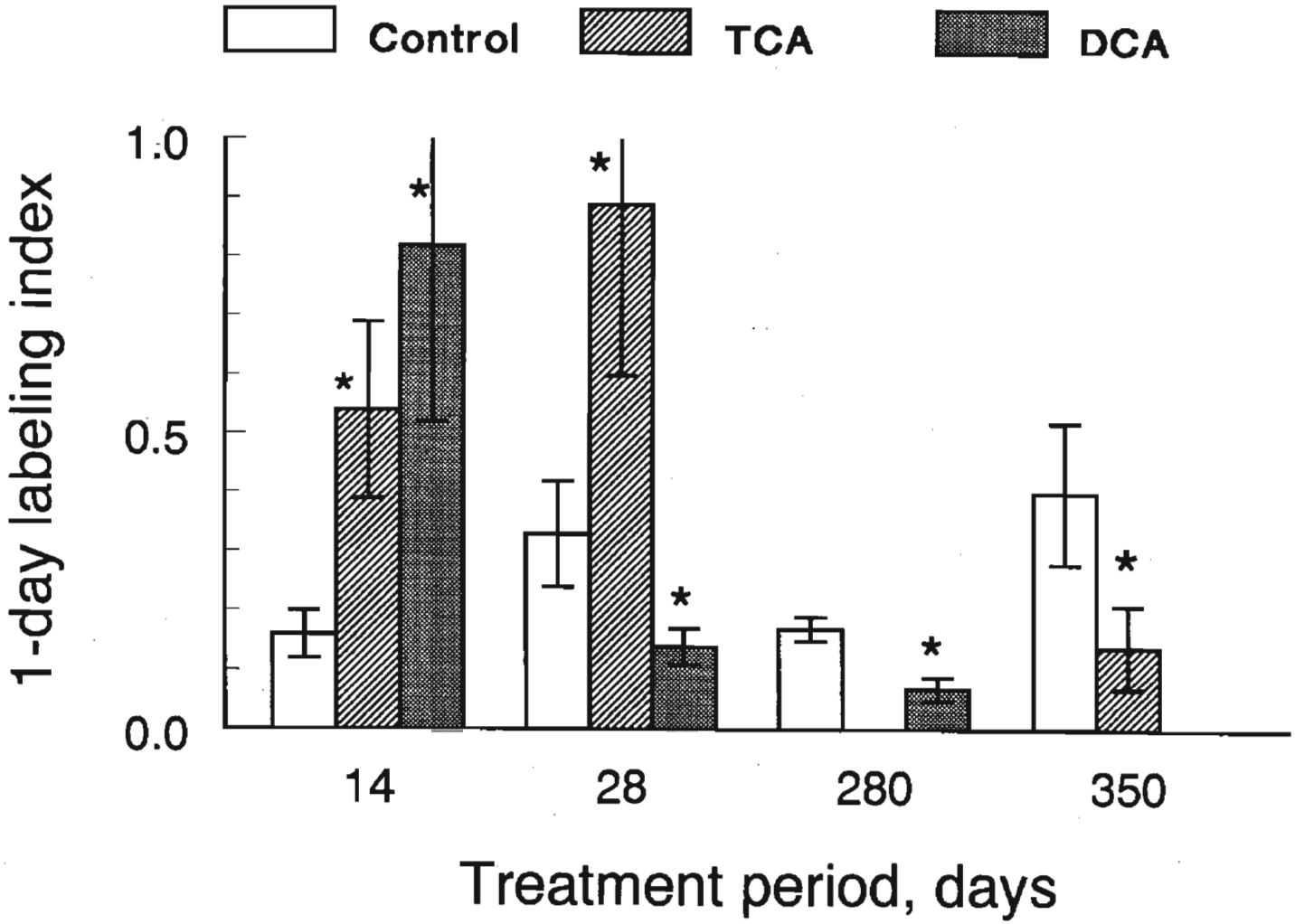


Figure 3.10. Effects of dichloroacetate (DCA) and trichloroacetate (TCA) treatments on the replication rates of normal hepatocytes of male B6C3F1 mice with different periods of treatment at concentrations of 2 g/L in the drinking water. Data at 14 and 28 days taken from prior experiments and represent the mean values of not less than 5 mice ± SEM. The longer term data are taken from the larger data set depicted in Figure 14. Values that are significantly different from their concurrent controls by Student's t-test were marked with an asterisk.

remains below the control levels with DCA and was also observed at one year of treatment with TCA.

The decreases in cell replication rates with chronic treatment were studied in more detail in the experiments where the treatment with of mice with 2 g/L DCA or TCA was suspended and concentrations ranging from 0 to 2 g/L provided for the last two weeks before the experiment was terminated. In the livers of mice treated with DCA, a substantial number of lesions were observed of varying size (Figure 3.11). These include a groups of cells in clusters of from 5-6 cells to more than 600 cells in cross-section that failed to display the very large increases in glycogen induced in normal hepatocytes by the DCA treatment that were seen only on microscopic examination of sections. These groups of cells were found to be eosinophilic, basophilic, or in a few cases had mixed basophilic/eosinophilic character and will be referred to as altered hepatic foci (AHF). In addition to these lesions were nodular growths that were observed macroscopically. Nodules were observed dependably as they approach 1 mm in diameter. In general, nodules observed macroscopically can represent anything from a simple hyperplastic nodules, hepatocellular adenomas and carcinomas. From prior experience very few of these lesions fall into latter category with treatment periods of less than one year. Therefore, we generally refer to them as nodules for the purpose of this report. Obviously, the division between AHF and nodules is arbitrary and in some respect represent a continuum. Nodules in the interior of the liver were frequently not be apparent until sections were made. The differing size of these lesions may depend simply upon how long they have been present. An alternative hypothesis is that the differing sizes reflect differing types of lesions whose growth rates have been differentially affected by the treatment. To differentiate between these hypotheses, the experiment was designed to determine if the treatment with DCA or TCA differentially affected replication within these lesions in a dose-dependent manner.

Where nodules were detected macroscopically on the surface of the liver, they could be easily identified, fixed, set in paraffin and sectioned so that serial sections could be examined. This allows association of patterns of cell replication with the expression of specific proteins within groups of cells within the lesion. The principal stains depended upon immunoreactivity to antibodies for the proteins c-Jun and c-Fos. These two proteins are components of the AP-1 transcription factor that is involved in triggering of the synthesis of additional proteins that are

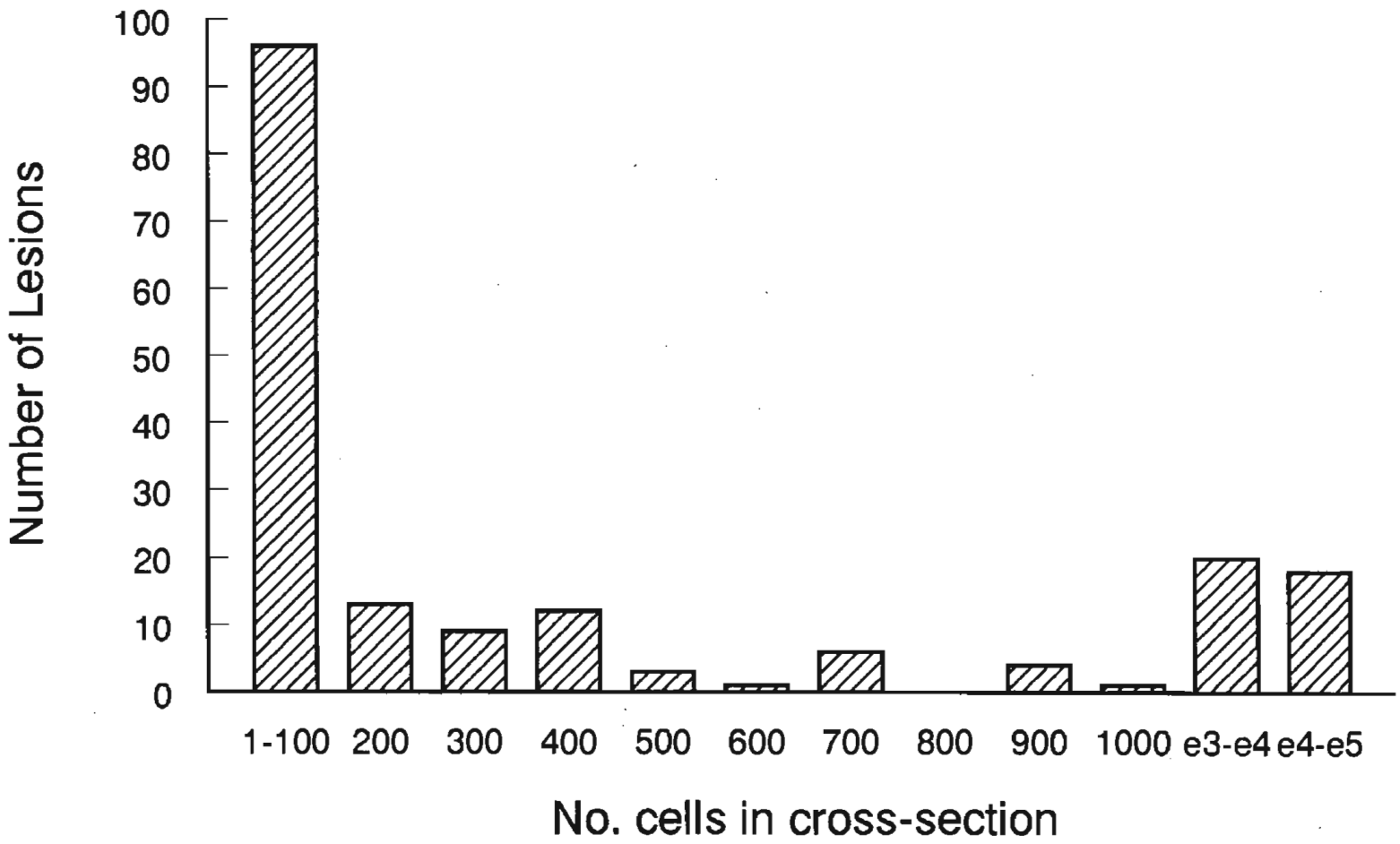
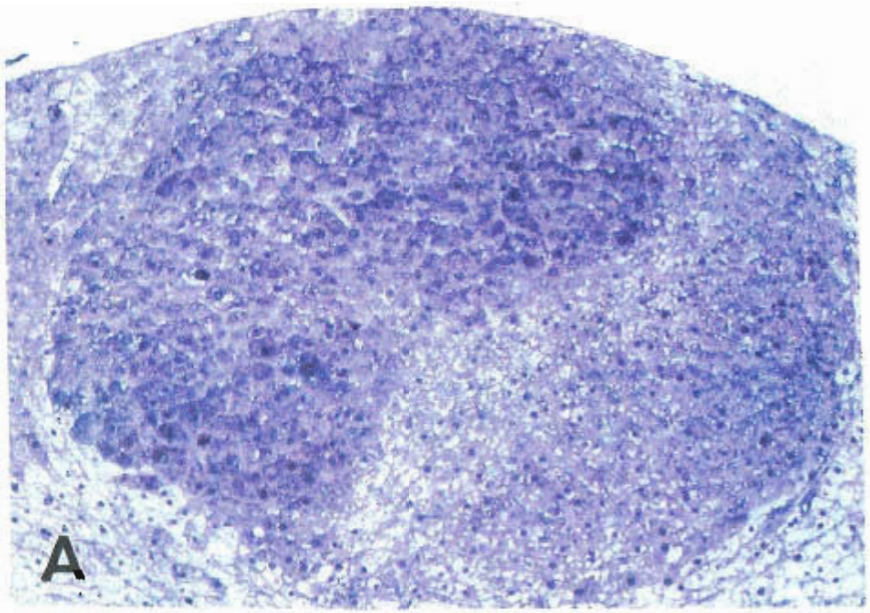


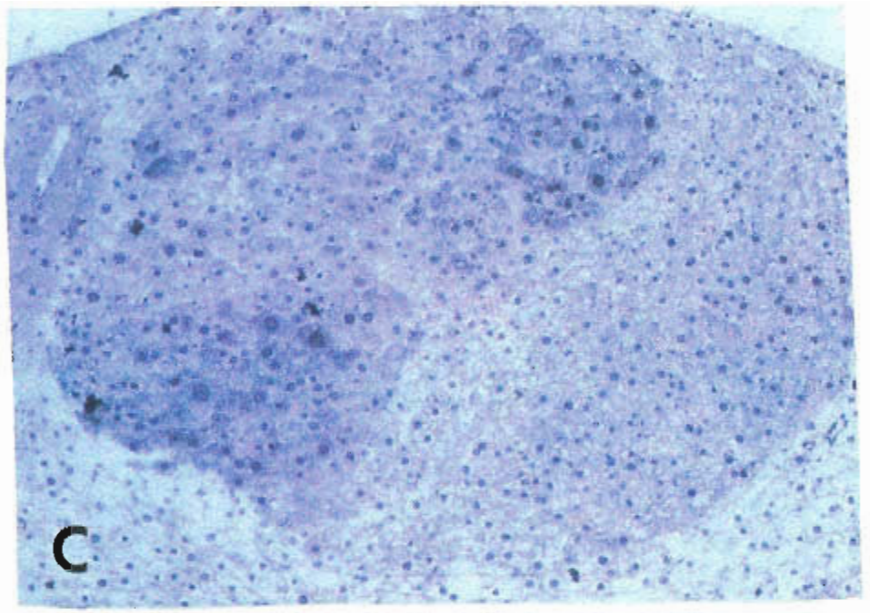
Figure 3.11. Size distribution of altered hepatic foci (AHF) and nodules identified in livers obtained from male B6C3F1 mice that had been treated for 38 weeks with 2 g/L dichloroacetate (DCA) in their drinking water. Lesions of up to 1000 cells in cross section were largely identified from four spaced sections taken through the central lobe of the liver. The larger lesions were identified macroscopically. Since the method of identifying the latter lesions is not random (i.e. they had to be seen on the surface of a liver lobe), the overall distribution should not be viewed as being representative of the occurrence of lesions of this size in the liver.

involved in the control of cell division (Angel and Karin, 1991). We chose these markers because prior work by Richmond et al., (1991) indicating that >90% of lesions induced by DCA in the liver of B6C3F1 mice expressed c-Jun at high levels relative to the surrounding normal tissue. Three serial sections of a particularly interesting lesion illustrates the analysis that was made. The section shown in the top panel of Figure 3.12 was stained with an antibody to c-Jun. As can be seen the lesion can be divided roughly into two parts, one which stains heavily for c-Jun and the other where staining is much lower. In the middle panel of Figure 3.12, a section of a tissue stained with an antibody to BrdU is shown. The staining with an antibody for BrdU allows the number of cells that have divided over this three day interval to be detected and counted. It is notable that most of the cells that have divided during this interval are to be found in the area stained heavily for c-Jun. In the bottom panel, the section was stained for c-Fos and it has more or less the same distribution as c-Jun.

The relationship between treatment concentration of DCA in drinking water and the cell replication rates in normal hepatocytes is shown in Figure 3.13. Cell replication in animals placed on water containing no DCA resulted in about 0.5% of the cells becoming labeled over the three-day interval that BrdU was administered. However, the replication rate for normal hepatocytes was significantly depressed at treatment concentrations of 0.5 and 2 g/L. The rate at 2 g/L was somewhat greater than observed at 0.5 g/L, but these rates are so low it is difficult to be certain that these differences are real although they are statistically different from one another at $P \leq 0.05$).

The rates of cell replication within AHF containing 100 or more cells in cross section were assessed separately from the larger nodules (Figure 3.14). A 100 cell minimum size was used to avoid the large variation in labeling indices that would be introduced by a difference of only one or two labeled cells within a lesion. However, the general shape of the dose-response curve is quite similar if 40 or 150 cells was used as the cutoff. The main conclusion that can be drawn from these data is that these cells are not sensitive to the inhibitory effects of DCA that are seen in the normal cell population. There appear to be more subtle variations in the cell replication rates at different doses that might be interpreted as stimulation of replication at low doses, with inhibition at 0.5 g/L and a secondary increase at 2 g/L. This question was deemed beyond the power of the present data to resolve. A conservative interpretation is that all lesions found in the





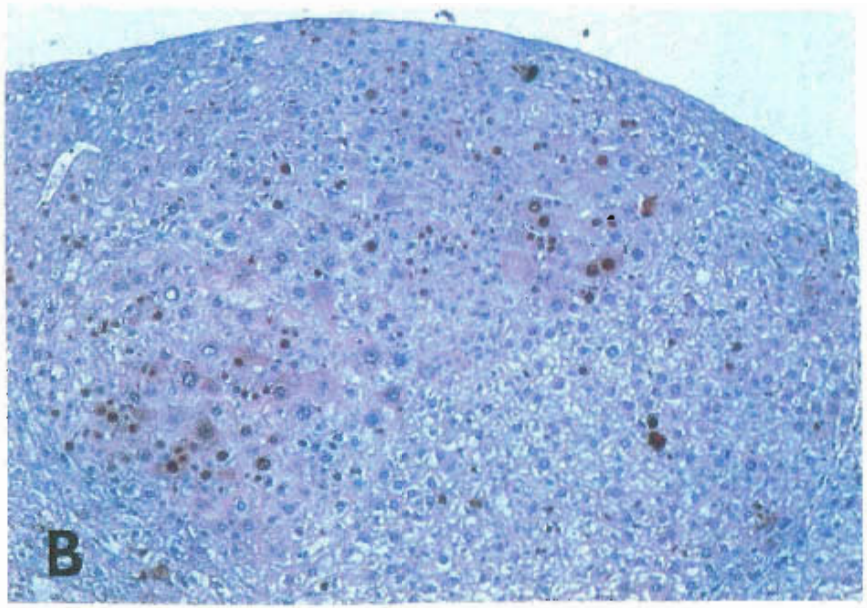


Figure 3.12. Serial sections of a nodule from a male B6C3F1 mouse that has been treated with 2 g DCA/L of drinking water for 40 weeks. The section in panel A was stained with a c-Jun antibody, panel B with an antibody to BrdU (and counterstained with hematoxylin) and the section in panel C with a c-Fos antibody. Note the congruence of areas of c-Jun and c-Fos staining and the areas where anti-BrdU stains indicate cells have divided.

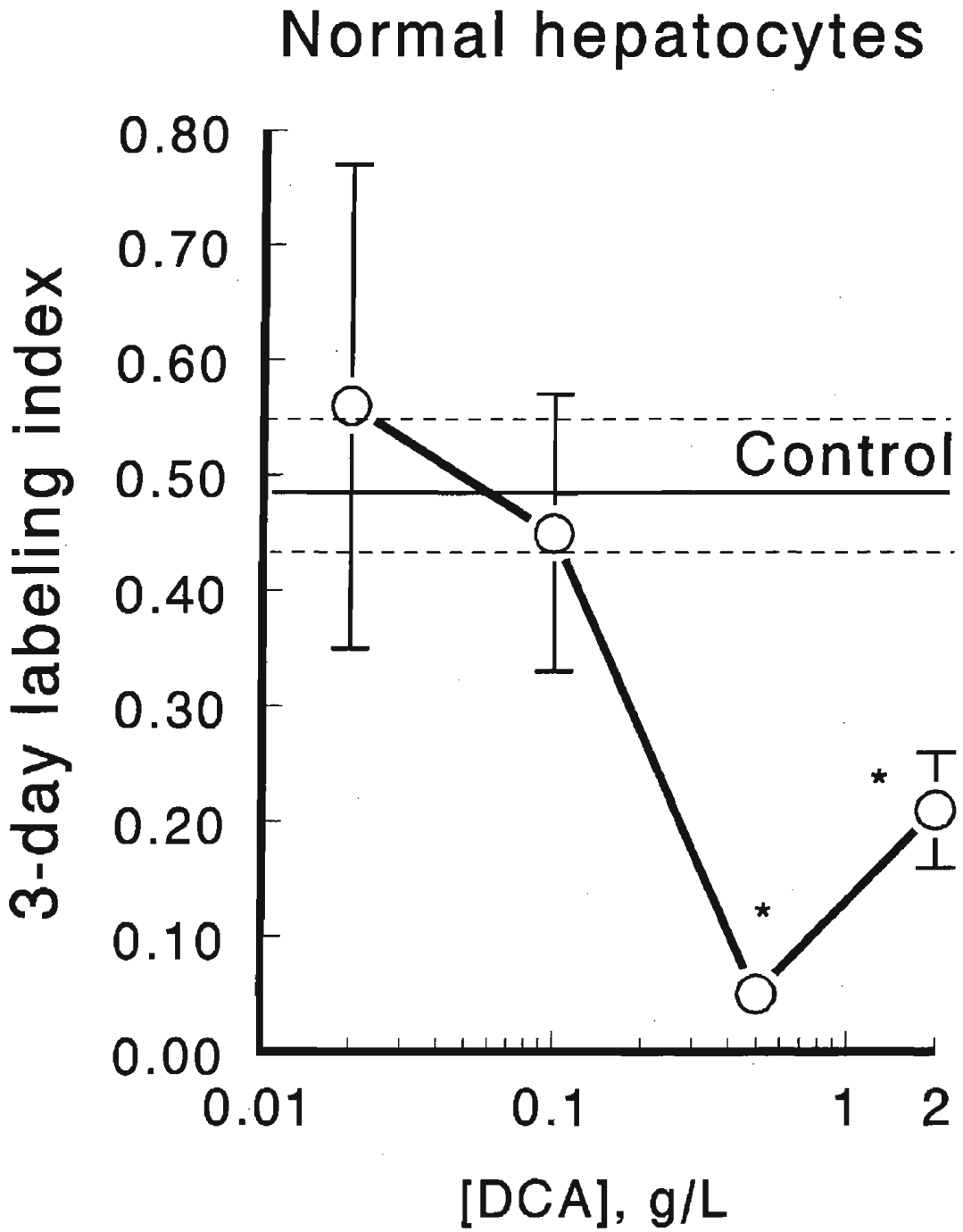


Figure 3-13. Replication rates of normal hepatocytes in male B6C3F1 mice that were first treated with dichloroacetate (DCA) at 2 g/L for 38 weeks followed by two additional weeks of treatment at the indicated concentrations. The solid bar, labeled control, indicates the rate measured in ten mice of the same age that were never exposed to DCA and the dashed lines indicate ± SEM. Values represent the mean of counting at least 2000 hepatocytes per mouse in 12 mice per treatment group. The vertical bars indicate ± SEM. Asterisks indicate rates that were significantly different from the rates in control mice.

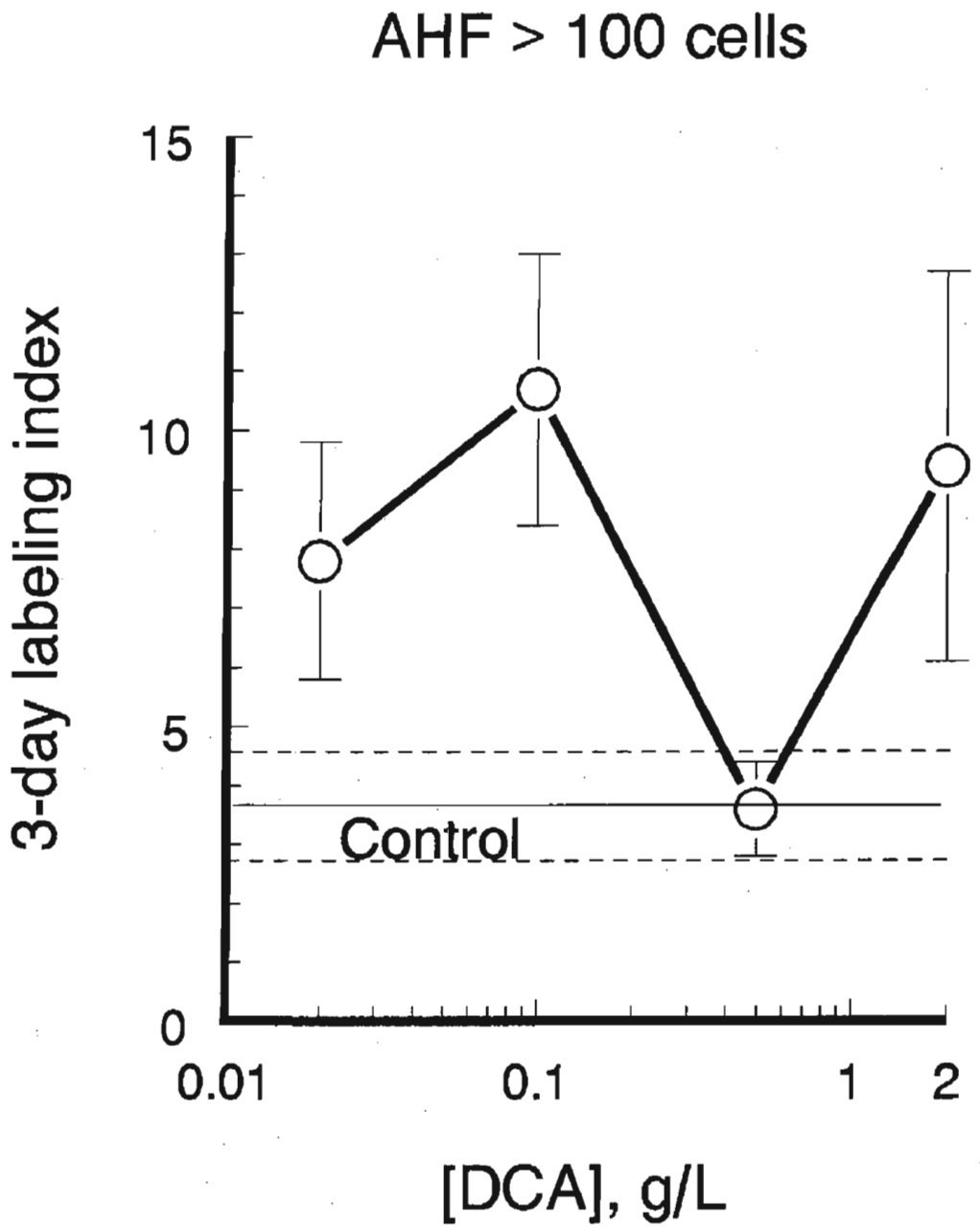


Figure 3.14. Replication rates of cells found within altered hepatic foci (AHF) identified as glycogen-poor in the liver of male B6C3F1 mice treated with 2 g/L dichloroacetate (DCA) in drinking water for 38 weeks, followed with two additional weeks of treatment at the concentrations indicated. The solid bar indicates the replication rates within the AHF of mice treated with 2 g DCA/L for 38 weeks and transferred to distilled water during the final two weeks of the experiment. All values represent means ± SEM.

liver of mice that had continued treatment with DCA were at or above the rates observed in mice transferred to distilled water. Therefore, this provides for the simpler conclusion that the inhibition of replication seen within normal hepatocytes was not apparent in AHF.

The effect of DCA on replication rates of AHF that were eosinophilic and basophilic were also evaluated. In this case, the number of BrdU-labeled cells were divided by the total number of cells (across animal and lesion) found with this two phenotypes. The actual counts of labeled and unlabeled cells in these categories are provided in Figure 3.15 to avoid over interpretation of these data. DCA does not appear to significantly affect the replication rate of the eosinophilic AHF. These lesions appear to have a replication rate that is generally lower than that seen in basophilic AHF. Within the eosinophilic AHF, except the lowest dose, the replication rate appears to closely approximate that observed within the same lesion taken from mice that had been transferred to 0 DCA in their drinking water. More complex effects on replication seemed apparent in the basophilic group of AHF. These data do suggest that DCA may exert some stimulatory effects on replication rates of basophilic cells, but the effect displays a complex dose-response relationship. In the 0.5 g/L treatment group, there appears to be some inhibition of replication rate within this population of cells such as that observed within the normal hepatocytes, although the basal rates within these AHF are much higher than normal hepatocytes and the magnitude of the inhibition is smaller. However, at the highest dose, the replication rate is twice that observed in AHF of mice that had been transferred to distilled water for two weeks. Such behavior could simply be an artifact of the small numbers of lesions examined. On the other hand, it may be explained by a heterogeneous population of basophilic AHF.

Where the lesions were sufficiently large to allow serial sections to be obtained, a more revealing association became apparent (Figure 3.16). Lesions in which c-Jun was expressed at higher levels displayed a dose-dependent increase in the rate of cell replication. This was true in nodules that stained heterogenously as shown in Figure 12 as well as in lesions that were uniformly stained for c-Jun, but only in the c-Jun+ area. This stimulatory activity was not apparent in cells that failed to stain for c-Jun. There were, however, a limited number of lesions within this size range that were c-Jun$^-$ so this should be interpreted with some caution.

TCA-induced liver lesions had different characteristics than DCA-induced tumors. The most obvious difference was that the lesions did not stain positive for c-Jun or c-Fos (Figure

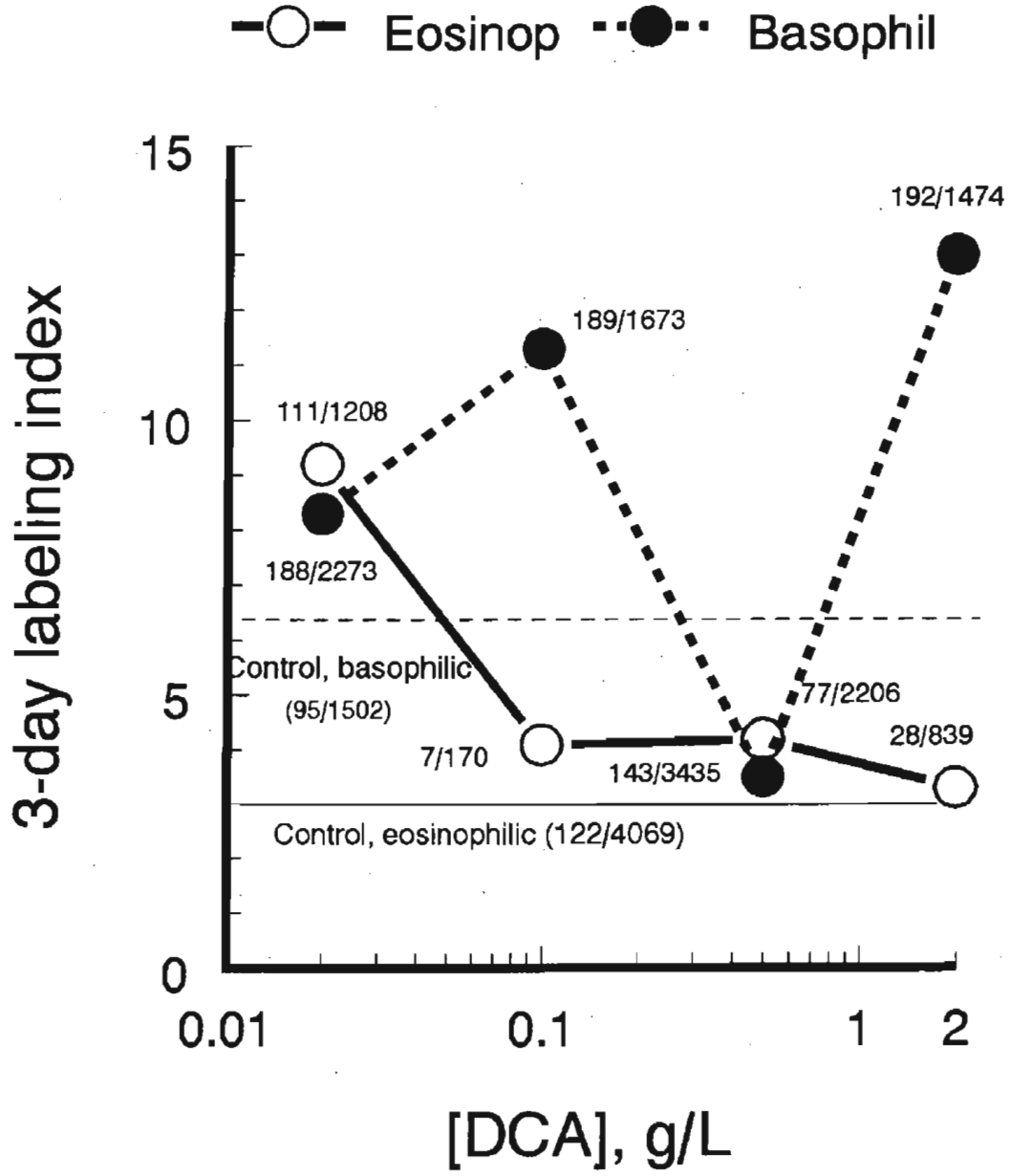


Figure 3.15. Effects of varying concentrations of dichloroacetate (DCA) on replication rates within eosinophilic and basophilic altered hepatic foci (AHF) induced by treatment with 2 g/L DCA for 38 weeks. Drinking water treatments were then changed to the concentrations indicated on the x-axis for an additional two weeks. These are the same population of AHF that were analyzed in Figure 3.14. A ratio of total cells labeled with BrdU are divided by the total cells within the AHF are provided for each point.

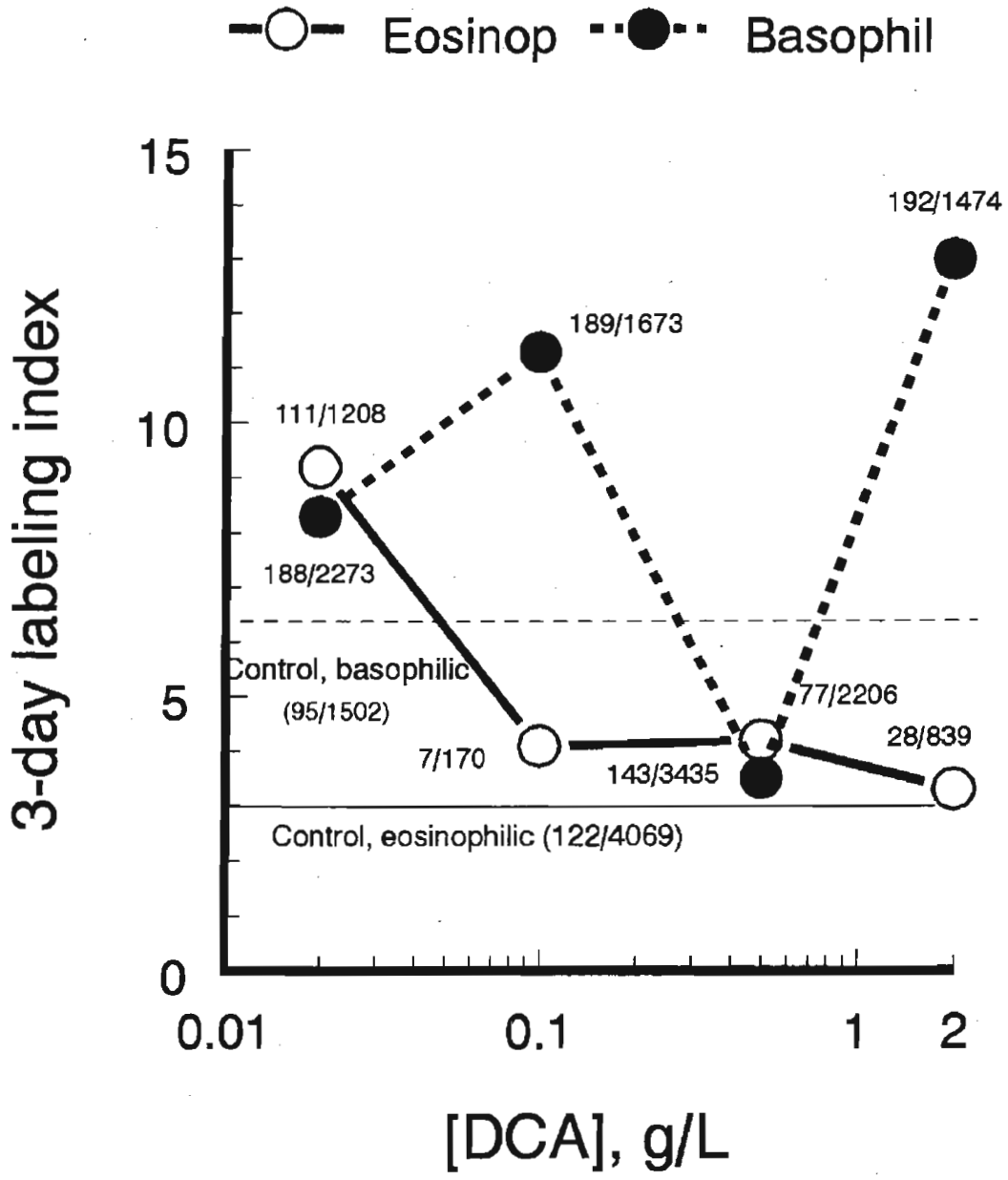


Figure 3.15. Effects of varying concentrations of dichloroacetate (DCA) on replication rates within eosinophilic and basophilic altered hepatic foci (AHF) induced by treatment with 2 g/L DCA for 38 weeks. Drinking water treatments were then changed to the concentrations indicated on the x-axis for an additional two weeks. These are the same population of AHF that were analyzed in Figure 3.14. A ratio of total cells labeled with BrdU are divided by the total cells within the AHF are provided for each point.

3.17). Examination of tissue taken from two of these animals with Western blots demonstrated that both c-Jun and c-Fos were expressed at higher levels in the normal tissues relative to liver from control mice at 52 weeks. However, these oncoproteins appear to be depressed within the tumors induced by TCA (data not shown) consistent with the immunohistochemistry results.

Figure 3.18 depicts the cell replication rates measured in normal hepatocytes in animals treated for 50 weeks with TCA at 2 g/L and then transferred to the indicated concentrations of TCA for the final two weeks of the experiment. The replication rates observed when lower concentrations of TCA were administered where quite variable. Although the means exceeded the control means at both 0.1 and 0.5 g/L, neither of these rates were statistically significant. Increasing the dose to 1 and 2 g/L did significantly($P \leq 0.05$) reduce the replication rate of normal hepatocytes, however, when compared to rates observed in the liver of animals never treated with TCA or those which had been treated for 52 weeks and transferred to distilled water for 2 weeks. This pattern is similar to that observed with DCA.

Figure 3.19 depicts the size distribution of AHF, nodules and tumors found in the livers of mice subjected to chronic treatment by TCA. It is important to note that the frequency that AHF were encountered was small relative to larger lesions (i.e. > 1000 cells in cross-section). This contrasts with the results presented in the same type of histogram for DCA-induced lesions (Figure 3.11). There could be several reasons for this. Basically, AHF were easier to detect in DCA-treated mice because of the cytomegaly that is produced by this compound in normal hepatocytes provides a contrast with the more normal size cells seen within AHF and nodules. However, such lesions are also readily recognized by eosinophilia and/or basophilia and there was no obvious difficulty in detecting this property in the larger lesions. Therefore, we have concluded that the number of AHF are simply fewer in TCA-treated mouse liver than in DCA-treated mouse liver. From a practical point of view, the small numbers of AHF in TCA-treated mice made quantitation of the replication rates within AHF even more tenuous than it was with DCA. Consequently, we did not analyze these data.

Replication rates with nodules and tumors visible in TCA-treated mice at the terminal sacrifice are shown in Figure 3.20. The results of this experiment are disappointing because the yield of actual lesions was smaller than had been anticipated from the preliminary experiment. The small number resulted in an unequal number of lesions between groups despite the fact that

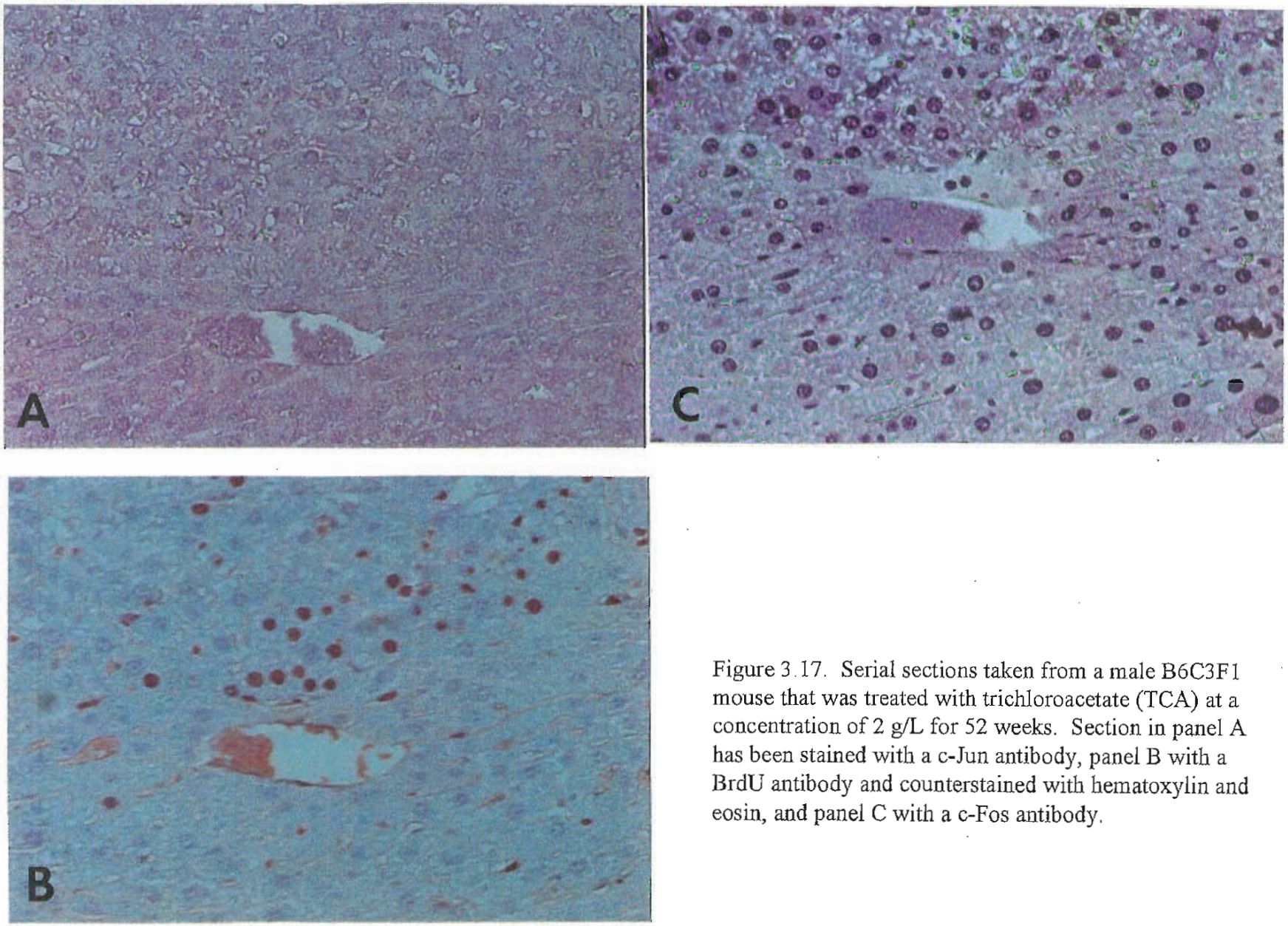


Figure 3.17. Serial sections taken from a male B6C3F1 mouse that was treated with trichloroacetate (TCA) at a concentration of 2 g/L for 52 weeks. Section in panel A has been stained with a c-Jun antibody, panel B with a BrdU antibody and counterstained with hematoxylin and eosin, and panel C with a c-Fos antibody.

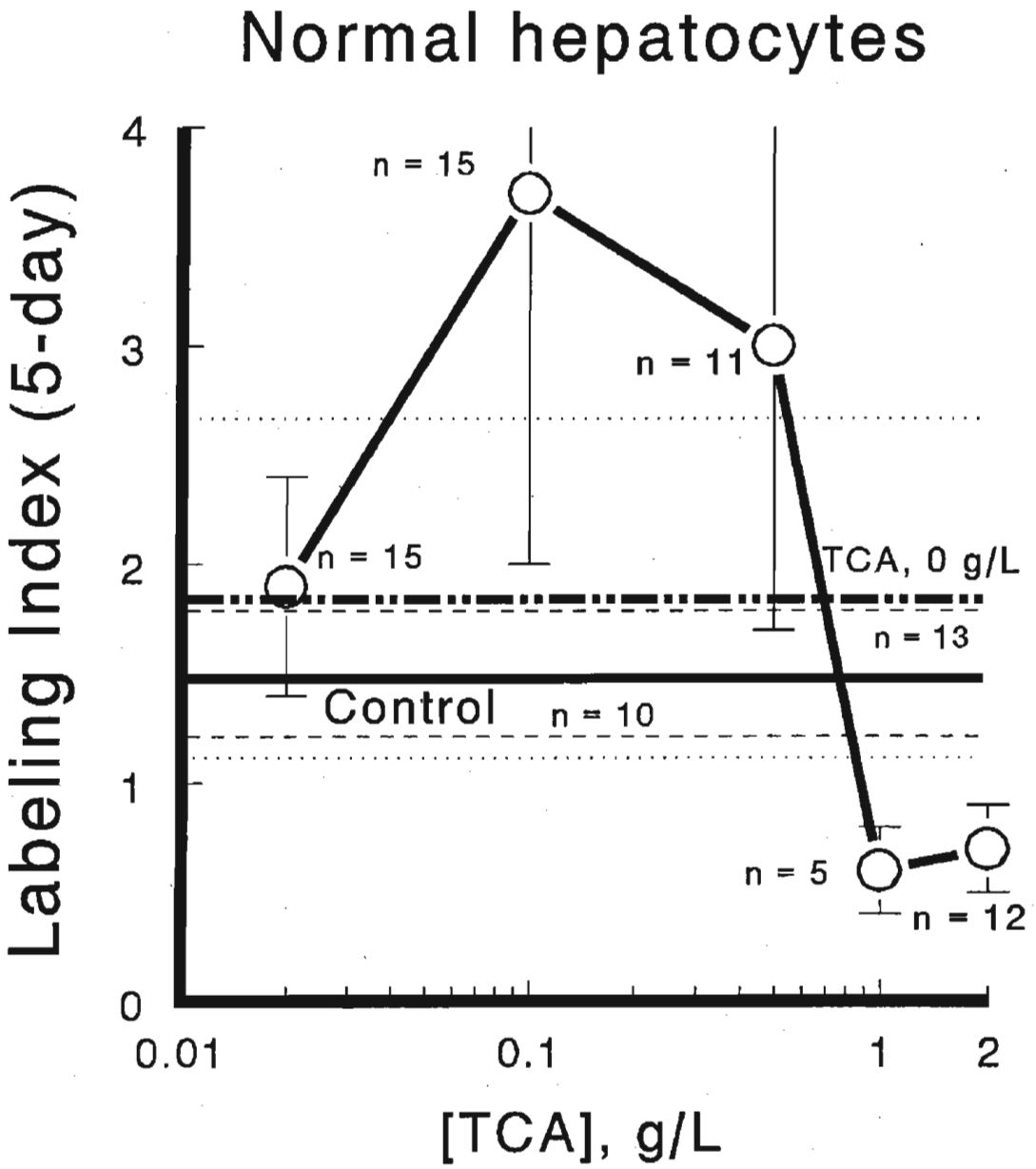


Figure 3.18. The effect of varying concentrations of trichloroacetate (TCA) in drinking water o the replication rates of normal hepatocytes of male B6C3F1 mice. All mice, except those indicated as controls (solid horizontal line), were treated with TCA at 2 g /L for 50 weeks. At that time, the mice were transferred to drinking water containing TCA concentrations shown on the x-axis for an additional two weeks. The heavy broken line reflects the mean replication rates of normal hepatocytes of mice that were transferred to distilled water for the final two weeks of the experiment. The numbers of mice represented by each point are indicated on the graph. The vertical bars, light dashed line and dotted line represent $\pm$ SEM for each point, controls and mice transferred to distilled water, respectively.

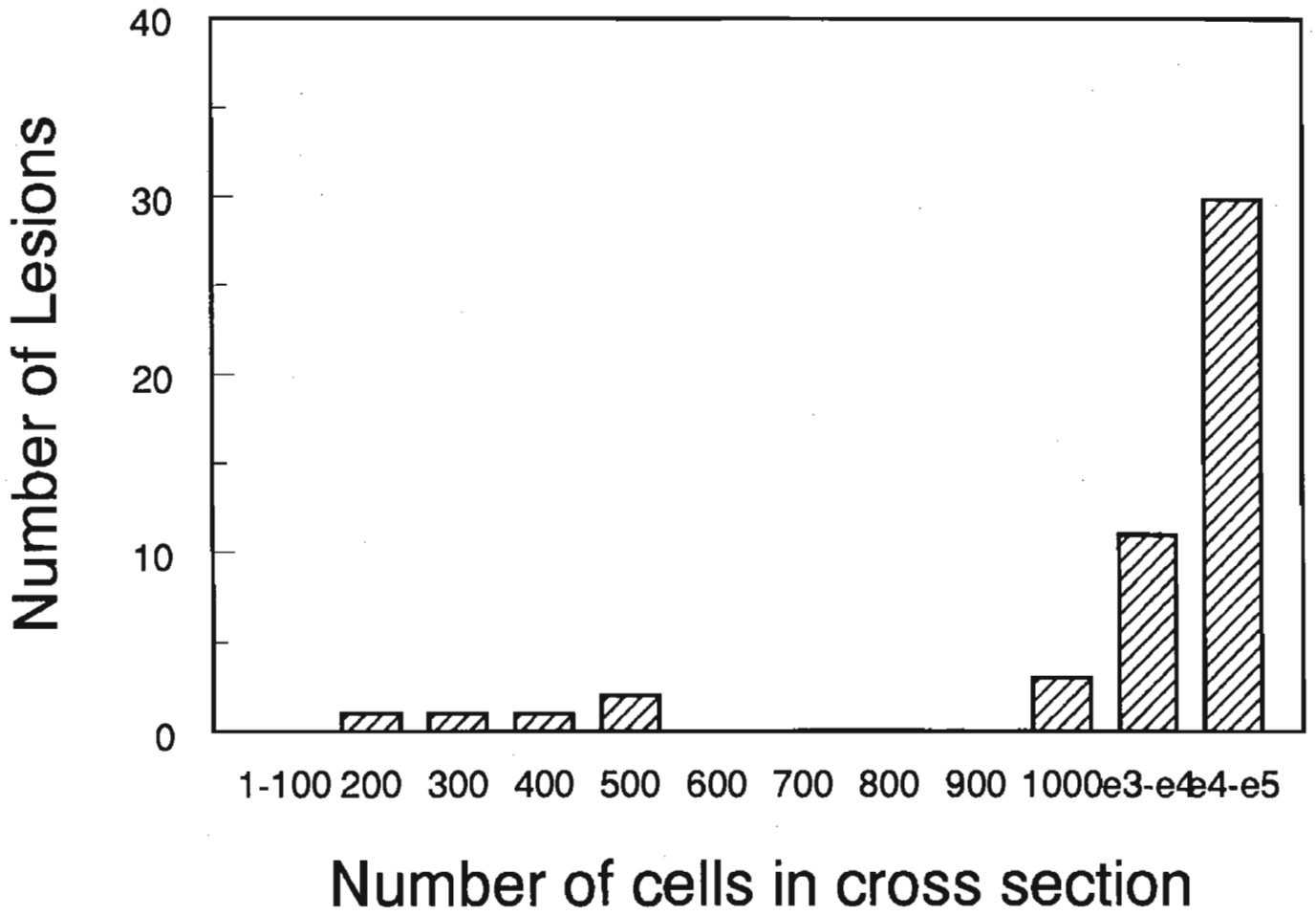


Figure 3.19. Size distribution of altered hepatic foci (AHF) and nodules identified in livers of male B6C3F1 mice that have been treated for 50 weeks with 2 g/L of trichloroacetate (TCA) in their drinking water. Lesions of up to 1000 cells in cross section were identified from 4 non-contiguous sections through the central lobe of the liver. The larger lesions were identified macroscopically. Compare with the distribution produced by DCA (Figure 3.11)

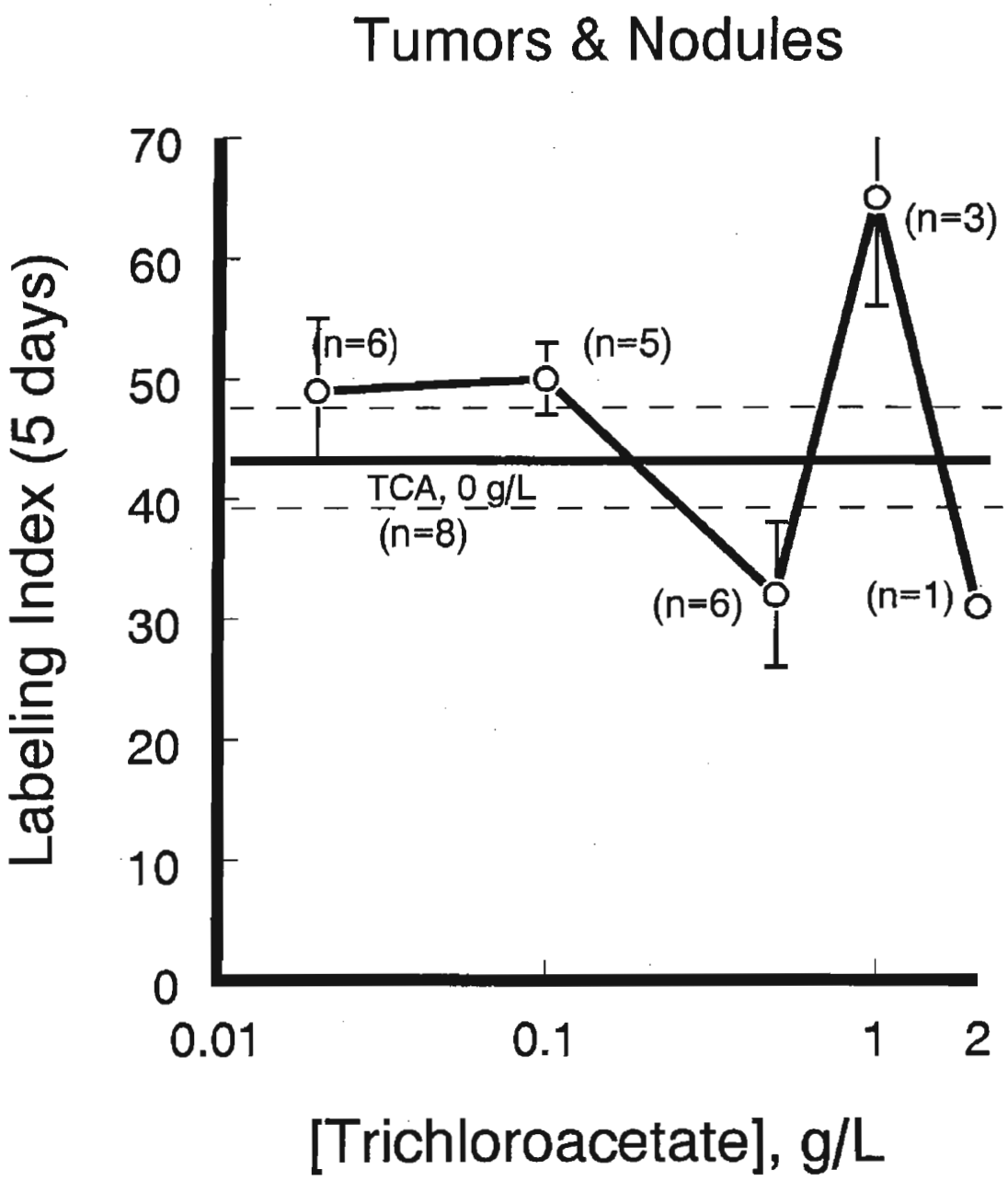


Figure 3.20. Effect of continued treatment with trichloroacetate (TCA) on replication rates observed in tumors and nodules. All mice were treated with 2 g TCA/L of drinking water for 50 weeks and then transferred to the concentrations indicated on the x-axis for two additional weeks of treatment. The solid horizontal line identifies the rate observed in a control group of mice that were treated for 50 weeks and then transferred to water containing no TCA. Vertical bars and dashed lines represent $\pm$ SEM for each treatment group and the control group, respectively. The numbers in parenthesis indicate the number of nodules/tumors identified in each experimental group.

their assignment was done randomly. Only a single mouse assigned to the high dose group was found to have a tumor, although 3 of 5 animals assigned to the 1 g/L concentration had tumors (this group arose from a dosing error committed in the preliminary experiment where 5 animals per dose group were sacrificed to make sure that we were in fact observing tumors at 52 weeks of treatment). As a consequence, a definitive statement cannot be made to the question of whether TCA, itself, affects the replication rates with the lesion it induces. However, these data made clear that the basal rate of cell replication with TCA-induced nodules and tumors were significantly higher than those observed with DCA-induced tumors.

The replication rates in lesions from the DCA and TCA experiments are normalized in Figure 3.21 (since 3-day minipumps were used in the DCA experiment whereas 5-day minipumps were employed in the TCA experiment). This bar graph illustrates that the rate of replication of lesions induced by DCA were only about one-half those of TCA-induced lesions. When the high dose of DCA-treatment was maintained, however, the replication rates were quite similar to that observed in TCA-induced tumors. However, there was no convincing evidence that continued treatment with TCA in anyway influenced the rate within TCA-induced tumors when the data from the two highest doses were combined (recognizing the limited value of a n = 4 in the combined data).

Metabolism and Pharmacokinetics

The initial experiments on the effect of DCA pretreatment on its metabolism were conducted in rats fitted with jugular cannulae. Considerable initial success was experienced with the protocol of pretreating the rats for 14 days at 0.2 or 2 g/L of DCA in drinking water and then administration of the ^{14}C-DCA in the morning of the 15th day. However, difficulties arose in maintaining the cannulae in animals prepared for more recent experiments despite the fact that they were obtained from the same supplier. To complete the project, we have opted to perform some comparison studies to establish that the same phenomena could be observed utilizing oral doses of [^{14}C]-DCA. This was found to be the case. Therefore, all studies with TCA were conducted were conducted utilizing stomach tubes to administer the compound. All work in the mouse utilized the oral route.

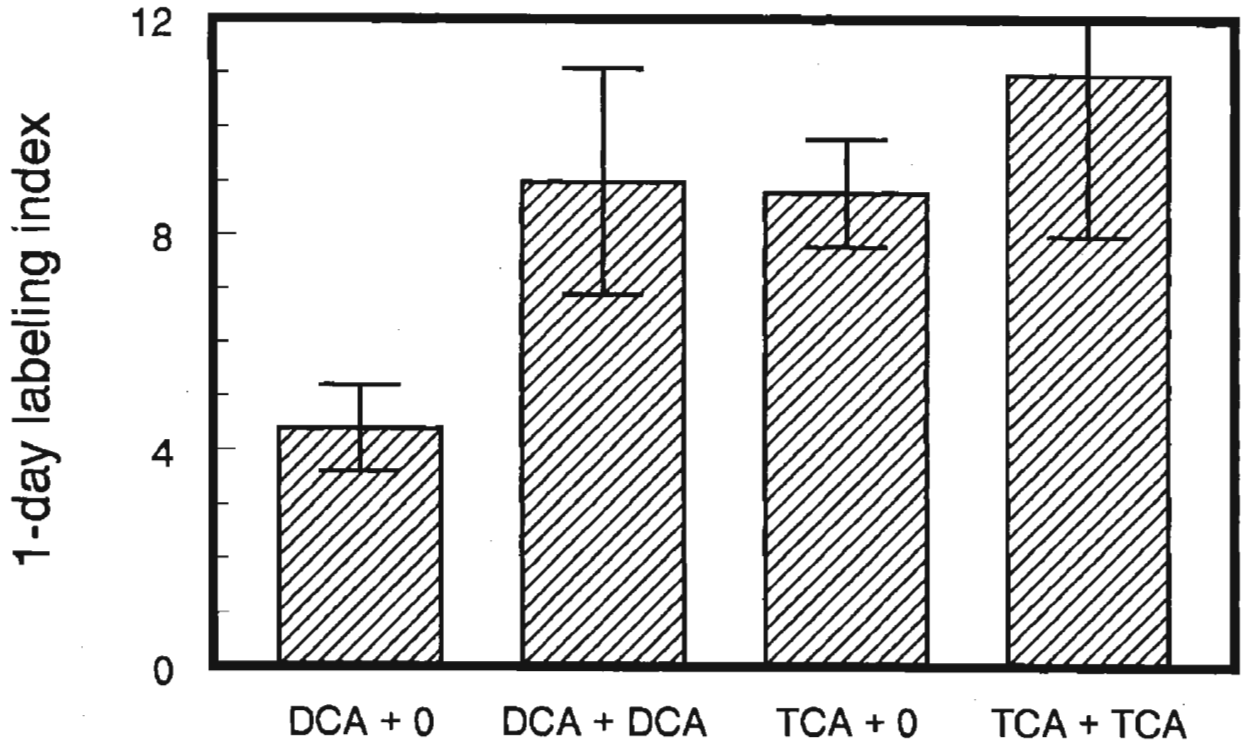


Figure 3.21. Normalized rates of replication within nodules and tumors induced by dichloroacetate (DCA) and trichloroacetate (TCA). Rates provided for DCA are those within c-Jun$^+$ areas. Bars labeled DCA + 0 and TCA + 0 represent rates obtained from livers of mice treated 38 and 50 weeks respectively and followed by removal of the treatment for two weeks. Bars DCA + DCA and TCA + TCA represent results from mice that were maintained on 2 g/L DCA and 1 or 2 g TCA/L for the final two weeks, respectively.

Figure 3.22 depicts the effect of DCA pretreatment of male F344 rats with 2 g/L of drinking water for 14 days on the conversion of doses of 5, 20 and 100 mg/kg [^{14}C]-DCA administered intravenously to CO_2. The pretreatment very substantially decreased the conversion of DCA to CO_2 at all three doses. A double reciprocal plot (Figure 3.23) indicates that a low affinity, high capacity metabolic pathway to CO_2 has been largely, if not completely eliminated by the pretreatment. A pathway with an apparent affinity about five-fold higher, but with much more limited capacity to CO_2 remains.

The pretreatment leads to substantial prolongation of the blood levels DCA in rats (Figure 3.24). At a [^{14}C]-DCA dose of 100 mg/kg, the half-life of DCA was increased from approximately 2 to 10 hours. It is instructive to examine the relationship of the blood levels of DCA determined utilizing gas chromatography relative to the total radioactivity seen in the blood over the time course. The total radioactivity from DCA actually was higher in the blood of control animals. However, if the equivalence of DCA measured by gas chromatography was subtracted from the total radioactivity seen in the blood, it is clear that the higher radioactivity seen in blood from control animals was due to metabolites (Figure 3.25). Much of this radioactivity has been previously shown to be found in metabolically incorporated glycine and serine of blood proteins (Stevens et al., 1992). These appear to arise from transamination of glyoxylate, an established metabolite of DCA (Larson and Bull, 1992).

The changes in blood levels of DCA and its metabolites is clearly reflected in the distribution of metabolites that are measured in the urine. In Table 3.1, the percent of the administered dose that is eliminated in the urine in rats pretreated with DCA relative to control animals is shown for doses of 5, 20 and 100 mg/kg. At all doses there was a substantial increase in total radioactivity that is eliminated in the urine of the pretreated rats. The changes in the amount of radiolabel that was eliminated in the feces was small and not clearly affected by pretreatment. There was a significant decrease in the percentage of the dose that is eliminated in the urine as the dose of [^{14}C]-DCA increases. Finally, the net effect of pretreatment on elimination of radioactivity in the urine was relatively constant across dose (i.e. an increase of about 6-12% of the total dose was found in the urine as a result of pretreatment). However, in relative terms, the effect was exaggerated as the dose increased (i.e. 1.6, 2.1 and 2.7-fold increases, respectively).

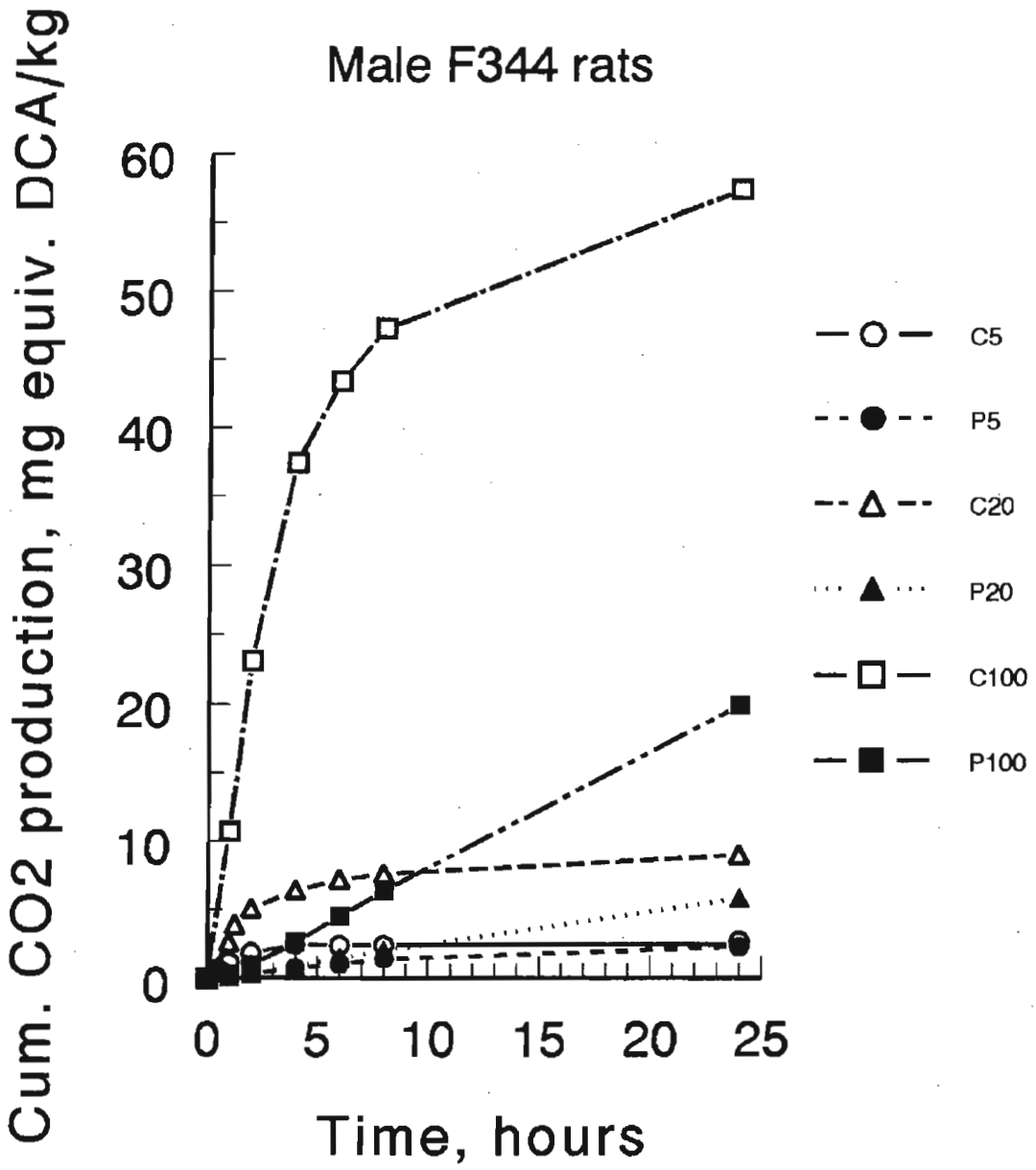


Figure 3.22. The effect of pretreating male F344 rats for 14 days with dichloroacetate (DCA) at 2 g/L in drinking water on the conversion of [^{14}C]-DCA to [^{14}C]-CO_2. Doses of [^{14}C]-DCA were 5, 20, and 100 mg/kg. Control, C, and pretreated, P, followed by number indicate test dose size. Each line graph represents the mean ± SEM of at least 5 rats.

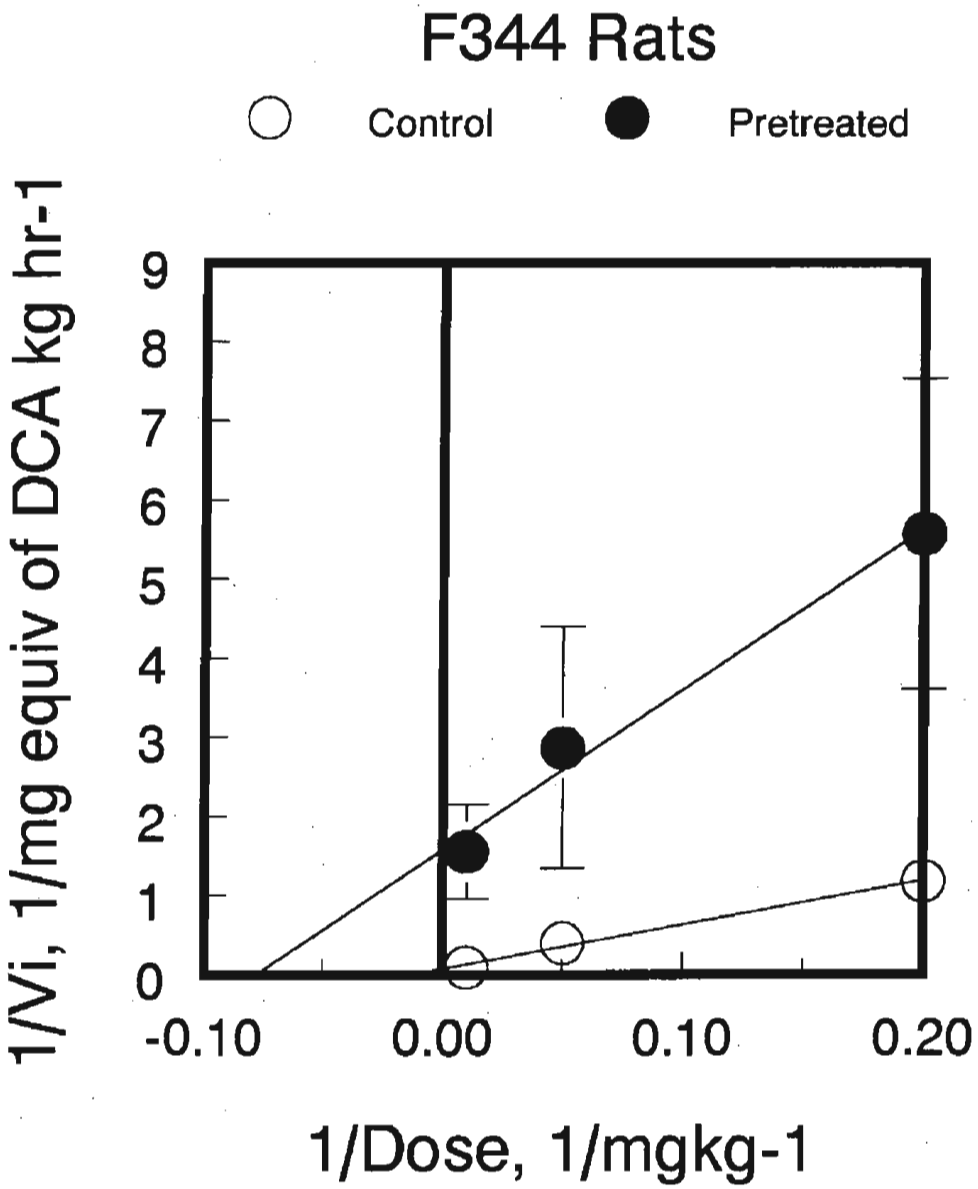


Figure 3.23. A double reciprocal plot of the data provided in Figure 3.22. Note that the pretreatment substantially decreases the apparent K_m as well as the altering the maximum rate of conversion of DCA to CO_2.

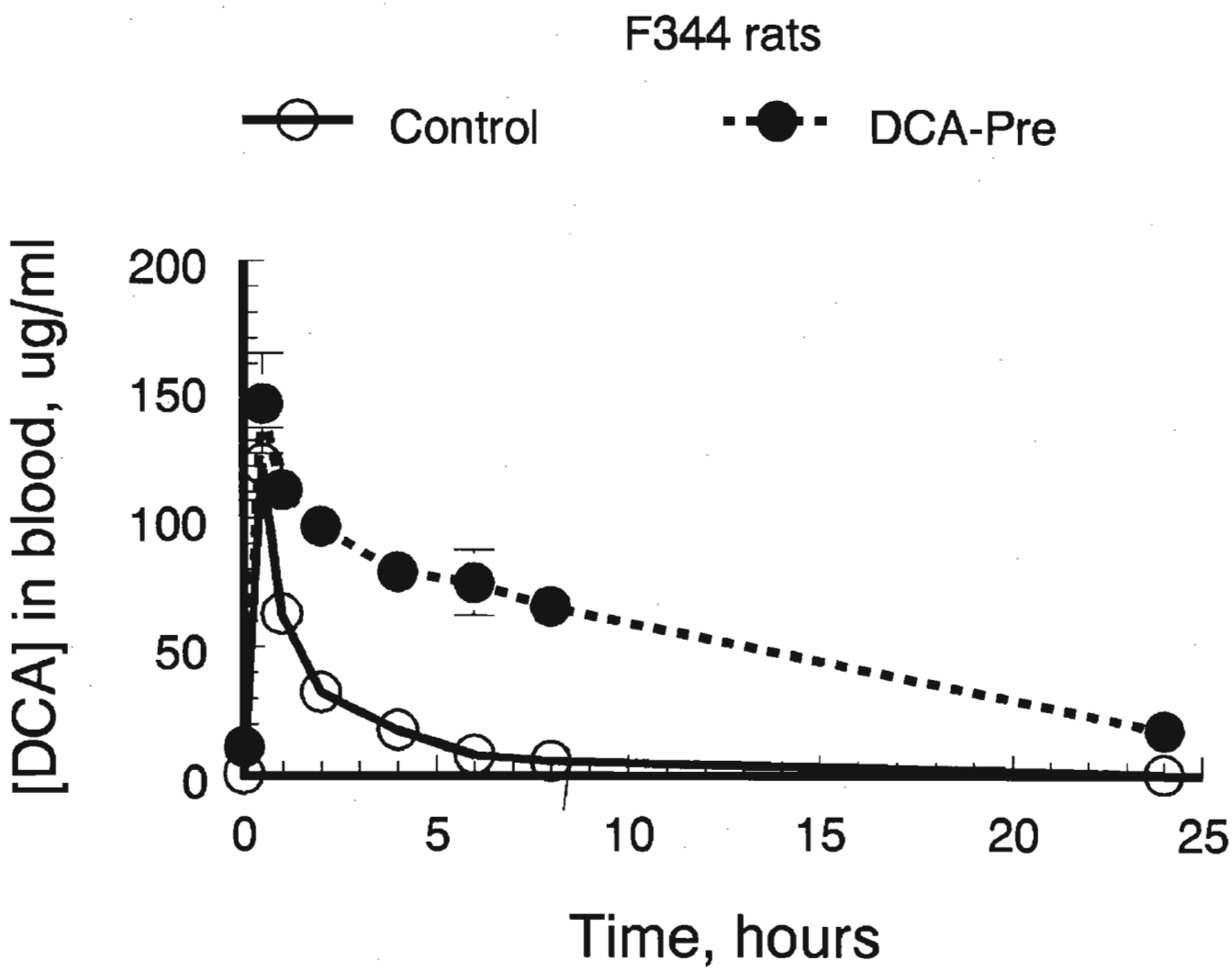


Figure 3.24. The effect of dichloroacetate (DCA) pretreatment on clearance of DCA from the blood of F344 rats following an intravenous dose of 100 mg/kg. The open circles provide mean values for control rats, the filled circles those of rats pretreated with 2 g DCA/L of drinking water for 14 days. DCA-containing water was replaced with distilled water 16 hours prior to administration of the test dose. The curves represent the results from a minimum of 6 rats. The vertical bars represent ± SEM and are not visible if smaller than the symbol.

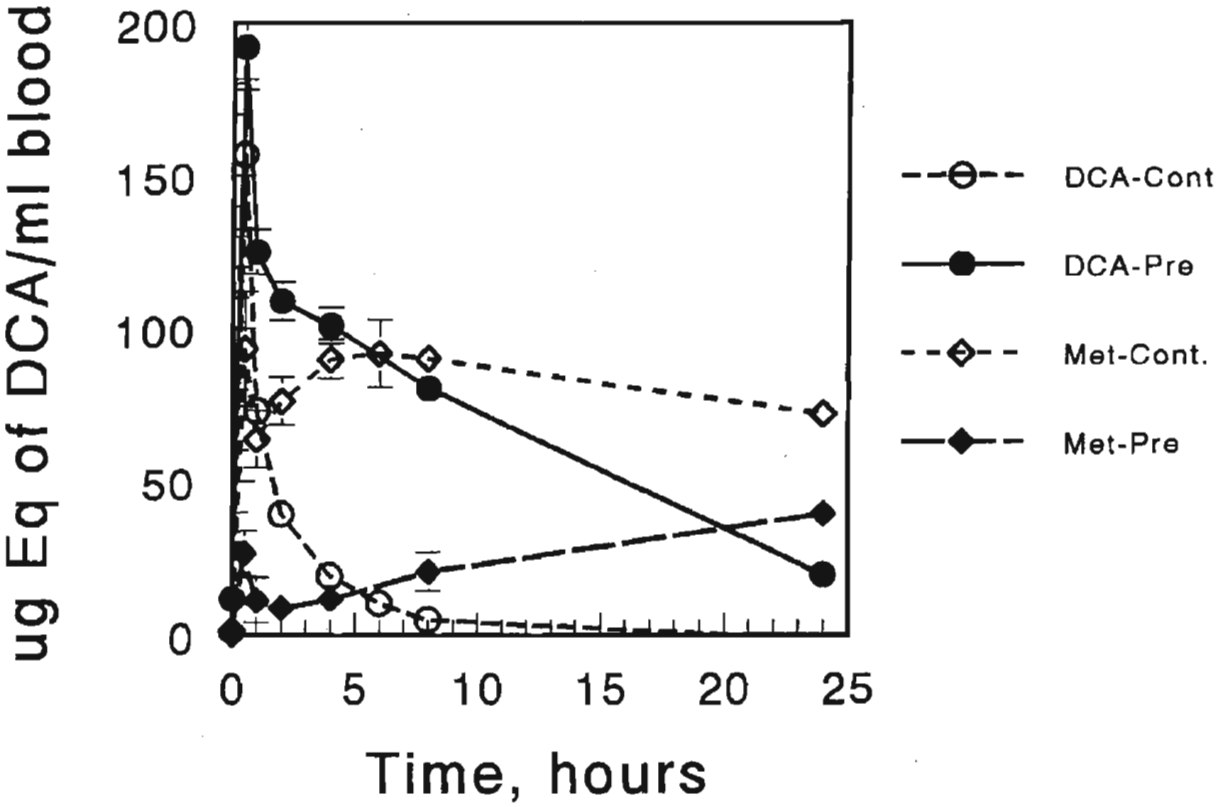


Figure 3.25. The time course of dichloroacetate (DCA) and "metabolite" concentration changes in the blood of male F344 rats that have been pretreated (filled circles) with DCA relative to naïve controls (open circles). An intravenous dose of 100 mg [^{14}C]-DCA/kg was administered as described in Figure 3.24. "Metabolite" concentrations were obtained by subtracting the amount of DCA measured by gas chromatography from the total ^{14}C measured in the same volume of blood. Vertical bars indicate $\pm$ SEM.

Table 3.1. Effect of pretreatment with DCA on the elimination of radioactivity into urine and feces of male F344 rats given 100 mg/kg [^{14}C]-DCA by gavage.

Dose mg/kg	Urine		Feces	
	Control	Pretreated	Control	Pretreated
5	11.4 ± 1.0[a]	17.9 ± 0.9	1.2 ± 0.9	1.0 ± 0.5
20	10.9 ± 1.9	23.3 ± 4.1	1.0 ± 0.6	1.6 ± 1.1
100	3.8 ± 2.2	10.7 ± 3.7	0.20 ± 0.06	0.59 ± 0.59

[a] Expressed as percent of administered [^{14}C]-DCA

Table 3.2. Effect of pretreatment by Dichloroacetate (DCA) on metabolites from a 100 mg/Kg dose of ^{14}C-labeled DCA recovered in the urine of male F344 rats in 24 hours.[a]

Treatment	**DCA**	**MCA**	**Glyoxylate**	**Glycolate**	**Oxalate**	**Acetate**
Control	1.18 ± 0.09[b]	0.04 ± 0.01	0.010 ± 0.003	0.47 ± 0.20	2.73 ± 0.76	0.085 ± 0.017
Pretreated[c]	15.1 ± 2.1	0.13 ± 0.02	0.026 ± 0.009	4.36 ± 1.5	1.88 ± 0.17	0.19 ± 0.04
Pretreated/ Control	12.2	3	2.5	9.3	0.7	2.3

[a] Total mg equiv. DCA/kg recovered as urinary metabolites: Control = 19.8 ± 4.5; Pretreated = 36.5 ± 11.8

[b] Mean ± SEM of values obtained from the urine of 5 control and 6 pretreated rats, expressed as mg DCA equiv.kg body weight.

[c] Mean ± SEM of values obtained from the urine or 6 rats pretreated with DCA, 2 g/L X 14 days

The differences in the elimination of label caused by pretreatment can be largely accounted for by the nature of the metabolites that are observed. Table 3.2 provides the results of an analysis of the urinary metabolites of DCA in rats administered a dose of 100 mg/kg. The increase in excreted radioactivity was largely accounted for by DCA. However, this analyses only accounts for 4.5% of the dose or about 22% of the label found in the urine in control animals. Because of the increase in DCA concentrations in the urine almost 60% of the urinary metabolites could be accounted for the pretreated animals. Thus, the uncharacterized fraction of the radioactivity in urine decreased from 78% of the amount found in the urine to 43% in the urine from pretreated rats. Based upon the behavior of "metabolites" seen in the blood we suggest that these uncharacterized metabolites may be largely derived from turnover of label that has been incorporated into protein. The pattern observed with these "metabolites" in the blood of pretreated rats reflects a rise in concentration with time, a behavior that is not consistent with the behavior of direct metabolites in a single metabolic compartment. To explain this behavior, the label would have to be incorporated into a pool that is turning over more slowly, such as proteins. In this regard, it is of particular note that one metabolite of DCA is also increased significantly in the urine, glycolate. While it does not achieve the concentrations observed with DCA, the fold increase in its elimination is almost as great (9.3 vs. 12.2 for DCA). The continued metabolism conversion of glycolate occurs slowly in mammals because of the reducing conditions found in most cells *in vivo*. Thus, elimination of glycolate in urine probably competes well with metabolism.

It should be recognized that the radioactivity observed in glycolate, glyoxylate and oxalate are not true measures of their concentrations. There are several natural sources of these compounds from normal metabolism and glyoxylate, in particular, has alternative pathways for its metabolism and conversion to CO_2. Therefore, the radioactivity in these metabolites represent the relative amount of DCA reflected in the measured pool (whether in blood or urine) at the time the sample was taken. Because of its more dynamic metabolism, glyoxylate levels are more susceptible to dilution than those of glycolate and this is the likely explanation of why radioactivity in glyoxylate was not as enriched by pretreatment as it was in glycolate.

Because of difficulties that were encountered with maintaining jugular cannulae, the effect of different pretreatment levels of DCA in drinking water on the conversion of [^{14}C]-DCA to

$^{14}CO_2$ was determined with orally administered [^{14}C]-DCA as well. These data are shown in Figure 3.26. Qualitatively, the effects of pretreatment were quite similar and the net size of the difference was very similar with the 100 mg/kg dose. The 0.2 g/L concentration in drinking water affected the conversion to the same extent as 2 g/L. As would be predicted, there are differences in the initial rates of metabolism because of the slower accumulation of DCA in the systemic circulation. Thus, the intravenous doses more effectively unmask the higher affinity, but low capacity system by avoiding the absorptive phase.

DCA pretreatment has very little effect on the conversion of [^{14}C]-DCA to CO_2 in mice (Figure 3.27). At 100 mg/kg dose of the labeled compound, the conversion to CO_2 is essentially identical. No effect was observed on the conversion to CO_2 even if the dose was increased to 1000 mg/kg (data not shown)

DCA pretreatment did, however, modify the blood concentrations of DCA in mice (Figure 3.28). While the effect was dramatic in terms of the maximum concentration of DCA that was achieved with a 100 mg/kg dose (130 μg/ml in pretreated mice compared to 2.1 $\pm$ 2.2 μg/ml in control mice). Thus, the peak concentrations of DCA in mice pretreated with DCA approximate those observed in the pretreated rat. However, the clearance of DCA from blood at this dose is significantly more rapid in mice, resulting in a significant difference in the area under the blood concentration vs. time curve (AUC).

The fraction of the total dose of radioactivity eliminated in the urine of mice administered 100 mg[^{14}C]-DCA/kg was significantly greater than observed at the same dose in rats (Table 3.3 vs. Table 3.1). As in the rat, the total radioactivity in mice was also increased by pretreatment. However, only a small fraction of the radioactivity was DCA. Therefore, unlike the rat, the difference between pretreated and control excretion is not accounted for by DCA. The small amount of urine available precluded more extensive analysis. Based on data obtained in rats and the much higher rate of DCA metabolism in mice even in pretreated animals, it was probable that the difference is accounted for by increased elimination of glyoxylate, glycolate and oxalate. Although the differences in the production of CO_2 was not statistically significant, the decrease in mean values could provide for about 60% of the increased label found in urine after pretreatment.

Pretreatment of rats with TCA at 2 g/L in drinking water failed to significantly affect conversion of [^{14}C]-TCA to CO_2 (Figure 3.29). Limited parallel experiments with non-

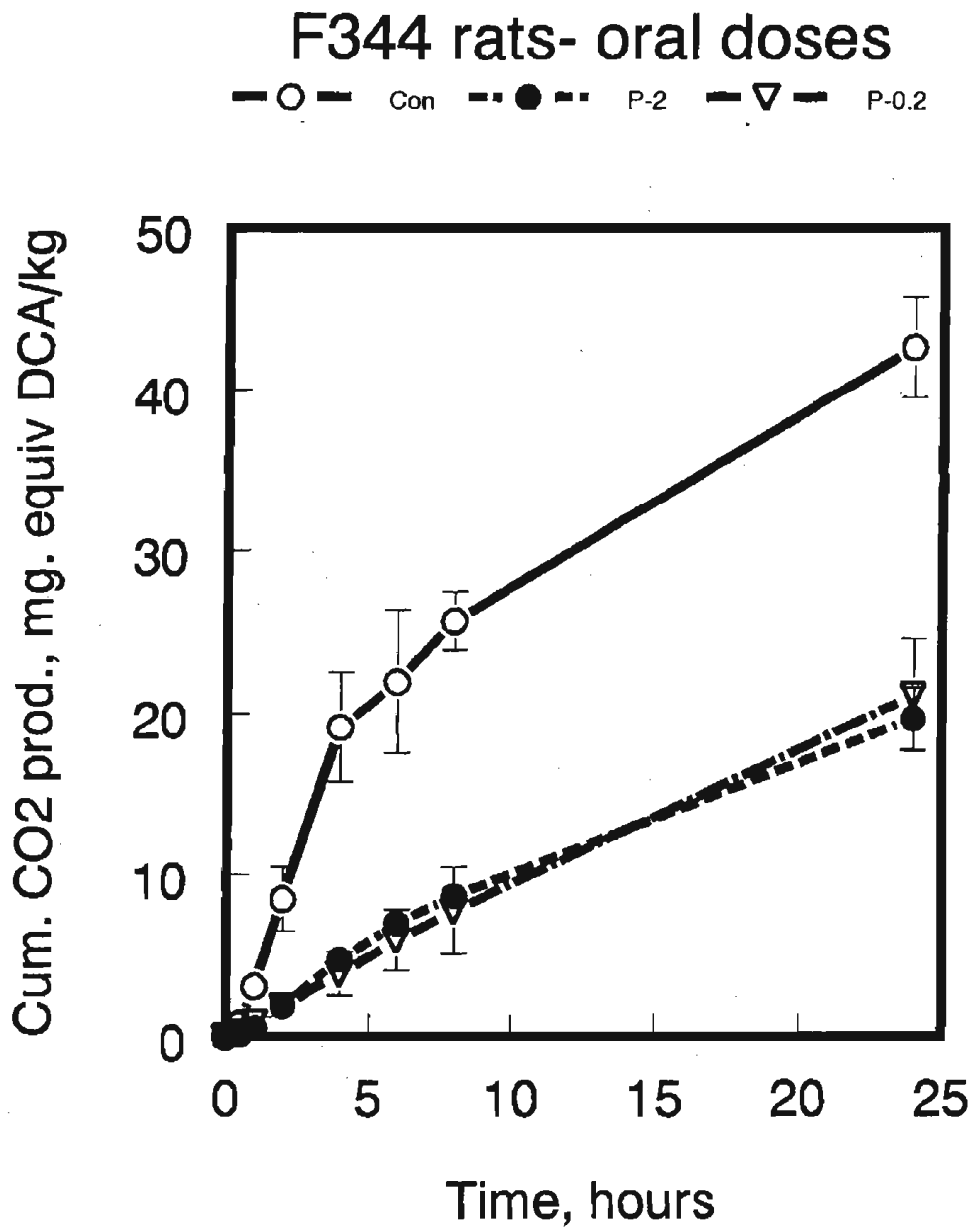


Figure 3.26. The effects of pretreating male F344 rats on the conversion of oral doses of [^{14}C]-DCA to [^{14}C]-CO_2. Open circles represent control rats; filled circles, rats pretreated with 2 g DCA/L; and open triangles, rats pretreated with 0.2 g DCA/L of drinking water for 14 days. Each point represents the mean value obtained from at least 5 rats ± SEM.

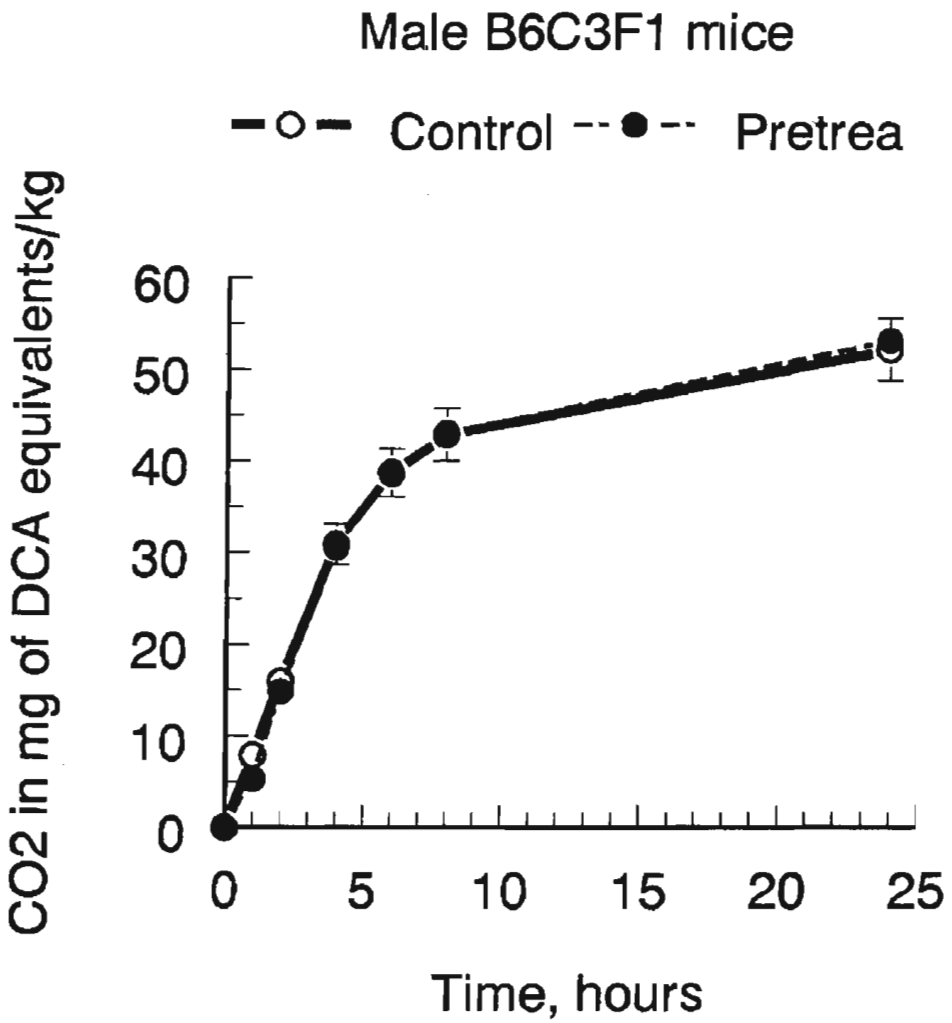


Figure 3.27. The effect of pretreating male B6C3F1 mice on the conversion of oral doses of [^{14}C]- DCA to [^{14}C]-CO_2. Open circles represent control rats; filled circles, rats pretreated with 2 g DCA/L of drinking water for 14 days. The DCA in drinking water was replaced by distilled water 16 hours before administering the test dose of [^{14}C]-DCA. Each point represents the mean value obtained from at least 6 mice $\pm$ SEM.

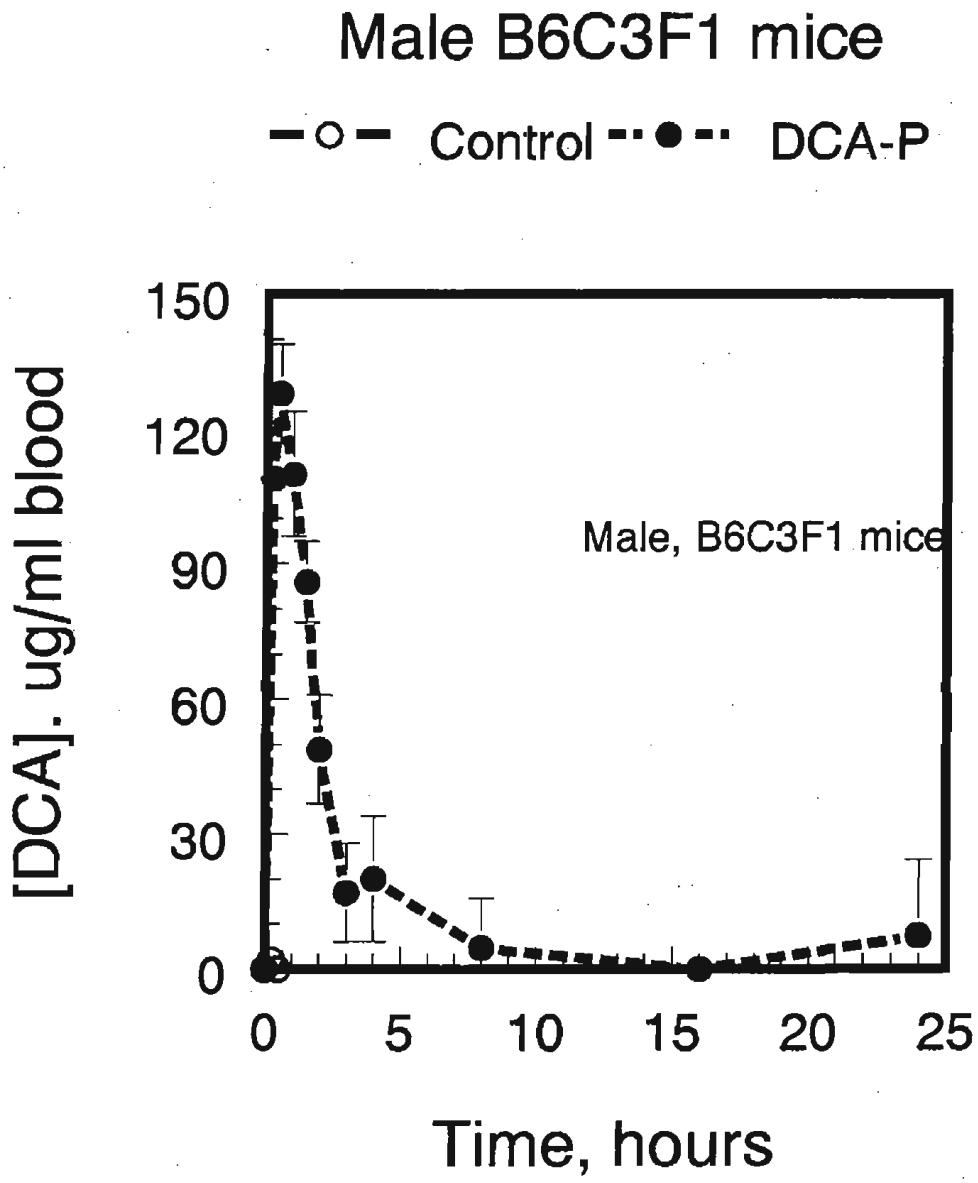


Figure 3.28. The effect of pretreating male B6C3F1 mice with 2 g DCA/L of drinking water for 14 days on blood concentrations following an oral, 100 mg DCA/kg dose. Control (naïve) mice are represented by open circles, pretreated mice by the filled circles. The DCA in drinking water was replaced by distilled water 16 hours before administering the test dose of [^{14}C]-DCA. Each point represents the mean value obtained from at least 5 mice ± SEM.

Table 3.3. Effect of dichloroacetate (DCA) pretreatment on the elimination of DCA and metabolites from a 100 mg/kg test dose of [^{14}C]-DCA administered by gavage to male B6C3F1 mice.[a]

	Urine		Feces	CO_2
	^{14}C	DCA	^{14}C	^{14}C
Control	10.9 ± 0.6[b]	0.11 ± 0.02	2.3 ± 1.0	54.4 ± 7.1
Pretreated	18.9 ± 3.6	0.58 ± 0.04	2.3 ± 0.6	49.5 ± 2.2

[a] Pretreated mice received drinking water containing 2 g/L DCA for 14 days. DCA was removed from water 16 hours prior to administering the test dose.

[b] mg equiv. DCA/kg body weight ± SEM excreted within 24 hours. Results obtained from 5 control and 7 pretreated mice

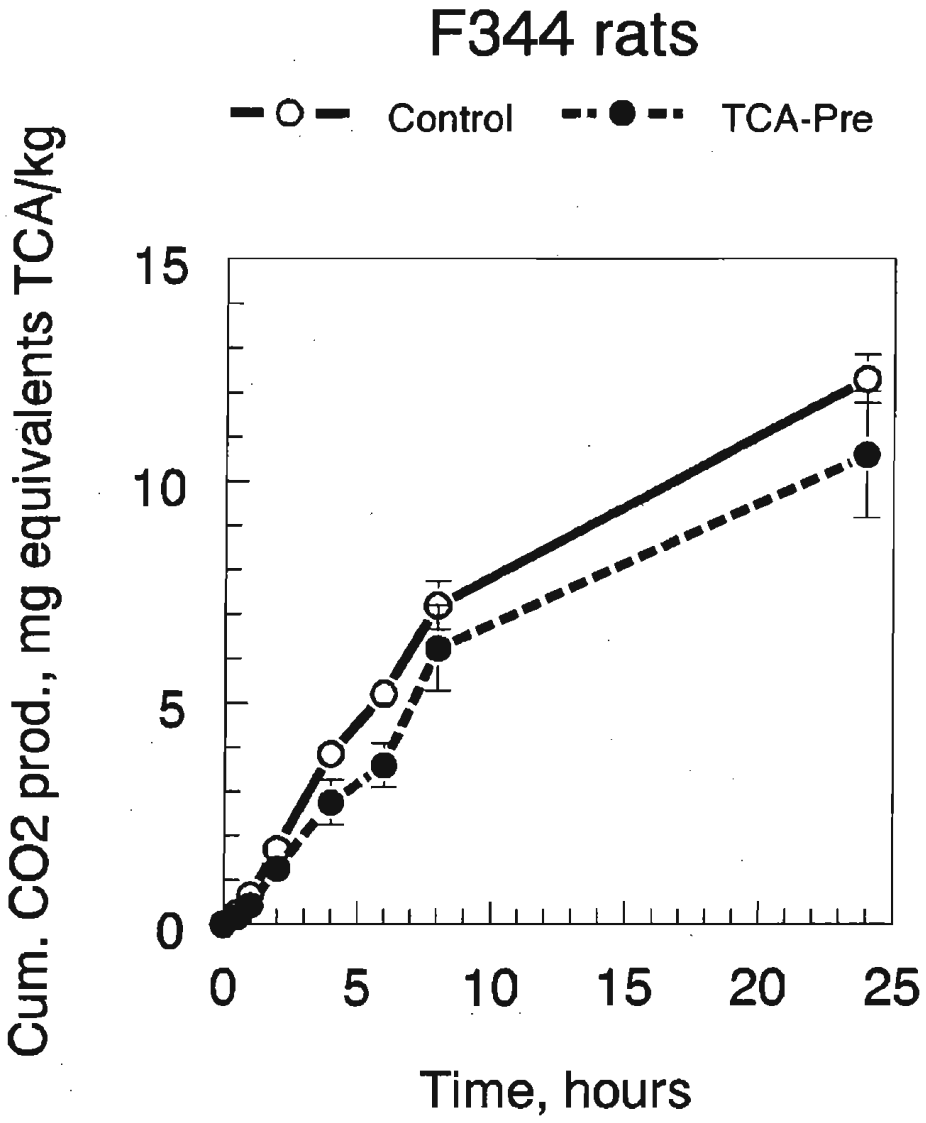


Figure 3.29. The effect of pretreating male F344 rats with 2 g TCA/L of drinking water for 14 days on conversion of [^{14}C]-TCA to [^{14}C]-CO_2. TCA was removed from the drinking water 16 hours prior to administration of the test dose by gavage. The line graphs represent the mean ± SEM of repeated samples from 6 control (open circles) and 7 pretreated rats (filled circles).

radiolabeled TCA, indicated that the elimination of TCA from the blood was not decreased after pretreatment (Figure 3.30). While repeated failure of cannulae prevented inclusion of more animals in this experiment, the trend is clearly in the opposite direction.

DCA was detected in the blood of rats treated with TCA. There appeared to be a small, but statistically significant increase in the blood concentrations of DCA that was observed. However, the levels were in the range of 1-5 μg/ml, close to the detection limit in the analysis that was scaled for TCA concentrations. Because of the limited numbers of animals that were examined, these data have not been included in the report.

CHAPTER 4
DISCUSSION

General

As been our previous experience, mice tolerate high doses of DCA and TCA quite well. Until concentrations of DCA or TCA exceed 2 g/L there are minimal effects on water and food consumption and the mice gain body weight quite normally. The major sign of toxic effect is the hepatomegaly that begins to become apparent within the first few days of treatment and the hepatic tumors that begin to appear after a few months treatment.

Despite the apparent consistency of the tumorigenic responses that have been reported for DCA and TCA, the present studies add further support to the hypothesis that there are some significant differences in the way in which these two chemicals produce cancer. It is important that these differences be understood since they could significantly affect how risks from these two chemicals would be viewed at the low concentrations found in drinking water. Moreover, it is important to understand how these differences in mechanism of action carry over into other members of the haloacid class of disinfectant by-products (e.g. brominated dihalo and trihaloacids, including those longer than 2-C in length).

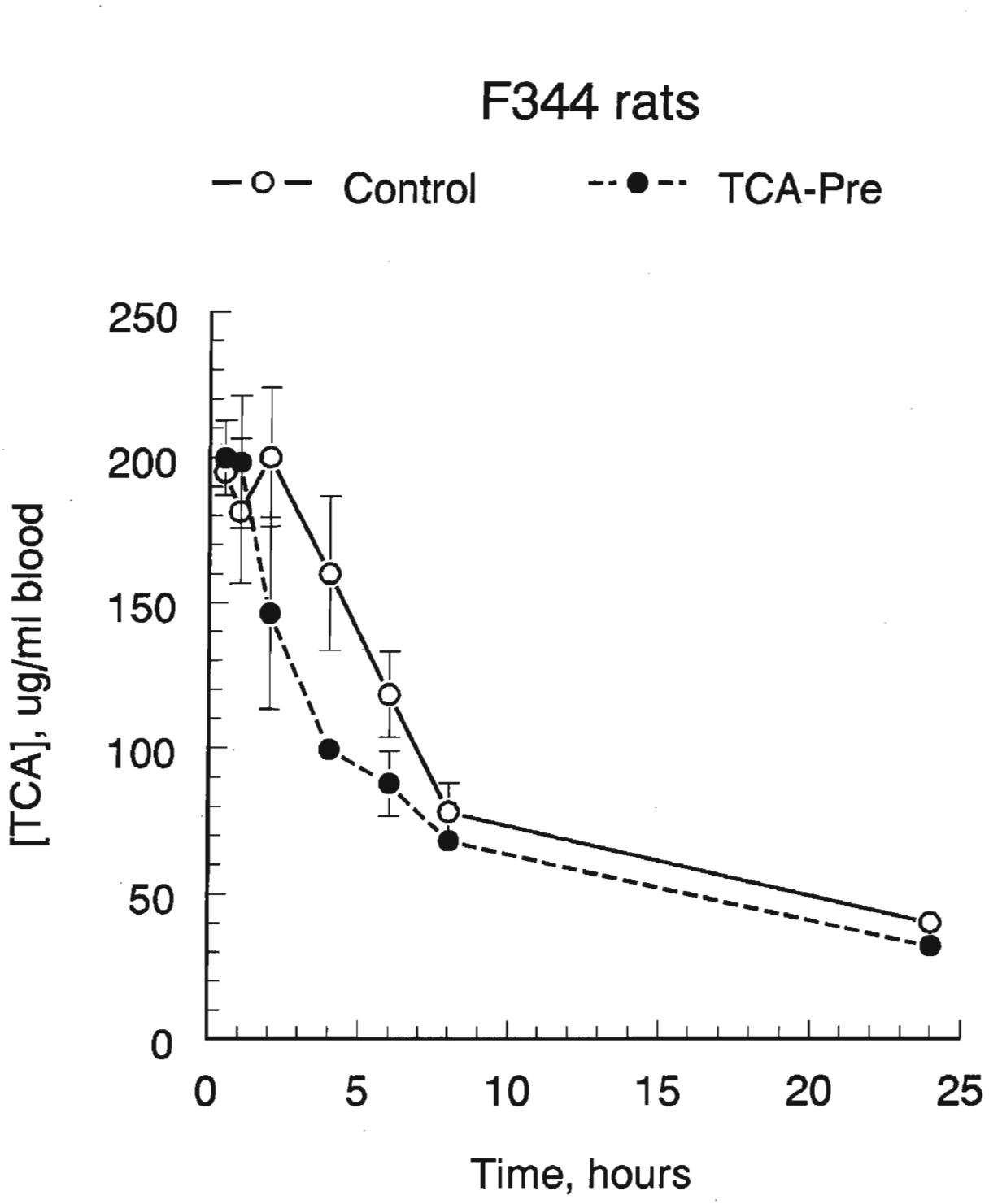


Figure 3.30. The effect of pretreating male F344 rats with 2 g TCA/L of drinking water for 14 days on clearance of an intravenous test dose of 100 mg TCA/kg from blood. TCA was removed from the drinking water 16 hours prior to administration of the test dose. The line graphs represent the mean ± SEM of repeated samples from 3 control (open circles) and 2 pretreated rats (filled circles).

Peroxisome proliferation and Oxidative Damage to DNA

Consistent with our previous results and those of others, the present study places TCA clearly into the class of peroxisome proliferators. In mice, some evidence of this response was observed at concentrations in drinking water as low as 0.1 g/L (as measured by increased cyanide-insensitive acyl-CoA and laurate hydroxylase activity). Consistent with this observation, hepatic tumors have been found in B6C3F1 mice as low as 1 g/L (Bull et al., 1990) after only one year of treatment. Peroxisome proliferators are known to produce hepatomegaly (Marsman et al. 1992). Therefore, all of the effects observed in these experiments with TCA can be associated with this response.

A key question is whether peroxisome proliferation can be more closely associated with the carcinogenic response. Since the peroxisome proliferator activated receptor (PPAR) and related proteins have been identified in human tissues, one cannot assume that the carcinogenic response is simply attributed to the increased numbers of peroxisomes. The hypothesis that has coupled increased numbers of peroxisomes to the carcinogenic response has been the oxidative stress hypothesis outlined earlier. This hypothesis would predict increases in lipid peroxidation and oxidative damage to nucleic acids (Fahl et al., 1984; Goel et al., 1986). When TCA is administered chronically, lipofuscin does accumulate within the normal cells within the liver, but not in tumor cells (Bull et al., 1990). Lipofuscin is generally regarded as an indicator of chronic oxidative stress (Chio et al., 1969) and is seen with many peroxisome proliferators (Conway et al., 1989). Recent experiments with other peroxisome proliferators have also reported increases in 8-OH-dG in hepatic DNA from treated animals (Kasai et al., 1989; Takagi et al., 1990a; 1990b; 1991). This provided a potential causal relationship between oxidative damage resulting from hydrogen peroxide generated within the peroxisome and initiation of cancer. However, while levels of this mutagenic base (Cheng et al., 1992) are easily measured, the actual level observed was found to be subject to a wide variety of artifacts in the isolation of DNA from the tissues. Cattley and Glover (1993) reported that if nuclei were isolated from cells prior to digestion of DNA, that treatment with clofibric acid, another well known peroxisome proliferator did not significantly increase the amount of 8-OH-dG found in nuclear DNA. It has been hypothesized that homogenization of tissues containing increased numbers of peroxisomes could increase the

amount of 8-OH-dG as a result of continued metabolism of peroxisomal metabolites with concomitant release of hydrogen peroxide. Metals released by this process could contribute to the formation of hydroxyl radical and the closer contact between these ordinarily spatially segregated compartments of the cell as a result of the homogenization of the tissue may be responsible for the artifactual formation of oxidatively-damaged bases.

The experiments described in this report failed to demonstrate increases in the amount of 8-OH-dG in nuclear DNA of mice treated with either DCA or TCA chronically. Small, but consistent, increases in the levels of this modified base was induced by administering the single doses of the compounds by gavage. The lack of significant increases in these modified bases with chronic treatment in drinking water casts significant doubt on their playing a role in TCA or DCA-induced hepatic cancer. These bases are produced at a relatively high rate by natural metabolic processes and a very rapidly repaired by glycosylases (Bessho et al., 1991). Therefore, alternative mechanisms for the carcinogenic responses must be sought, perhaps other effects that are mediated through the PPAR.

Modification of Rates of Cell Division

There are two important points related to the effects of DCA and TCA on the division of normal hepatocytes. First, the increases in cell division produced early in the treatments were very small and not at all similar to the very large increases that are induced by chemicals acting through a cytotoxic mechanism. Thus, the effects of DCA and TCA are quite distinct from those observed with carcinogenic doses of chloroform where substantial cell killing occurs within the liver resulting in very high rates of replication (Larson et al. 1994).

TCA and DCA both induce hepatic tumors in mice. Despite their chemical similarity there are reasons to suspect that the mechanisms and perhaps even modes of action are different. The stimulation of replication rates within c-Jun+ AHF and nodules was consistent with the promoting activity of 2.6 g/L DCA for diethylnitrosamine-initiated hepatic adenomas in mice reported by Pereira (1995). However, Pereira's data failed to account for the tumors observed at 0.5 g/L (Daniel et al., 1992). Our data suggest the differential suppression of normal cell replication by these doses were the basis of the tumors which develop with greater latency (Herren-Freund, et

al., 1987; Bull et al., 1990; DeAngelo et al., 1991; Daniel et al., 1992). In addition, demonstration that high doses of DCA select lesions that were immunoreactive to c-Jun was consistent with findings that DCA induces a H-ras codon-61 mutation frequency that was quite similar to background (Anna et al., 1994; Nakano et al., 1994). DeAngelo and coworkers (personal communication) have found that virtually all tumors induced by DCA have either a H-ras mutation or expressed elevated levels of H-ras. Activation of Ha-ras would be expected to increase c-Jun expression. The AP-1 activation that would occur as a result would be expected to induce a variety of other tumor markers such as GST-Pi (Nguyen et al., 1994).

Mutation frequencies in H-ras codon 61 in liver tumors induced in B6C3F1 mice by peroxisome proliferators are much lower than those induced by DCA or those found in spontaneous tumors (Fox et al., 1990; Hegi et al., 1993; Stanley et al., 1994). Therefore, GST-Pi has been found very infrequently in tumors that are induced by peroxisome proliferators (Grasl-Kraupp et al., 1993). Other phenotypic variations are observed between peroxisome proliferator-induced AHF and nodules (i.e. they are gammaglutamyl transpeptidase negative) relative to those seen with other tumor promoters, such as phenobarbital (Preat et al., 1986; Rao et al., 1986). This is consistent with our observation that c-Jun is not frequently overexpressed in TCA-induced tumors.

From these data and other literature (Anna et al., 1994; Hegi et al., 1993, Grasl-Kraupp et al., 1993), we conclude that DCA encourages the growth of a tumor phenotype that is distinct from those induced by peroxisome proliferators. While the present studies did not document the lack of GGT and GST-Pi expression in TCA-induced tumors, the lack of c-Jun expression in these tumors is consistent with that interpretation. The only data that appears inconsistent with this view is the recently reported H-ras mutation frequency reported in TCA-induced hepatic tumors in mice (Gerreira-Gonzalez et al., 1995). These authors reported 5/11 tumors had mutations in codon 61 of H-ras. This frequency is about twice that reported with other peroxisome proliferators (Fox et al., 1990; Hegi et al. 1993; Stanley et al., 1994). However, only 11 tumors were examined in this study and the dose utilized was significantly higher than employed in the present study (4.5 g/L).

Another important difference is the fact that the replication rates in the TCA tumors are constitutively higher than those induced by DCA. When DCA treatment was removed, the rate of

replication in c-Jun+ areas halved. Replication rates in other lesions induced by DCA were all lower than observed in these areas. The replication rates within lesions induced by TCA were two times those seen in the c-Jun+ areas in DCA-induced tumors and appeared not to be affected significantly by continued TCA-treatment (although the data are extremely limited on this last point). It is suggested that this behavior was consistent with the observation that while DCA-induced lesions remained proportional to total dose, they progressed slowly if treatment was suspended (Bull et al., 1990). In this same study, we observed that TCA-induced tumors appeared to regress when treatment was suspended, but what remained tended to be malignant. This behavior with TCA is consistent with the observation that some tumors induced by peroxisome proliferators tend to regress in stop experiments, even diagnosed hepatocellular carcinomas. Release from suppressed apoptosis (programmed cell death) has been suggested responsible for this effect of peroxisome proliferators (Bayly et al., 1994). Restoring high rates of apoptosis could offset the very high rates of cell replication observed in TCA-induced lesions. However, it was probable that by the time the lesions were observed as nodules or tumors, that many of them have progressed to the point of being malignant (Bull et al., 1990). At that late stage, the tumor would be autonomous from further treatment.

In contrast, while lesions induced by DCA seem not to progress in stop experiments (Bull et al., 1990), the lesions induced with treatments with 20 weeks or less at very high doses (i.e. where the stimulatory effect of DCA becomes apparent) do carry a high likelihood of progressing to carcinoma if the holding time increases (DeAngelo, personal communication). Consequently, even short term stimulation of the rate of division of the c-Jun+ lesions that occurs at high doses of DCA (3.5 g/L in DeAngleo's studies) may carry some probability of inducing cancer. However, it is unlikely that this would occur at lower doses which act primarily to inhibit division of normal cells. As doses are reduced below this point, it is difficult to believe a cancer hazard would exist because progression seems to require a substantial population of such cells for there to be sufficient probability of its occurring within a lifetime. It is very important to note that these same general phenotypes (i.e. ras+ or ras-expressing) appear to be largely responsible for the spontaneous tumors that are observed in B6C3F1 mice (Richardson et al., 1992). These mutations are present in very young mice (Moulds and Goodman, 1994). As one examines the mutation frequency produced by DCA, however, it becomes differentiated from the spontaneous

tumors. DCA either initiates tumors with the CAA - CTA transversion or it provides this particular mutation with a selective advantage. This transversion represents a much greater departure from the normal amino acid coded for normally by codon 61 (glutamine - leucine) as opposed to the other mutations within this codon (glutamine - lysine or glutamine - arginine). While this mutation is very effective in producing a transformed phenotype (Chuang et al., 1994), there may be some subtle differences in its interactions with proteins involved in subsequent steps in the signal transduction pathway would be an obvious potential explanation for such a selection.

Since TCA can be metabolized, in part, to DCA, there is a possibility that DCA could contribute to the tumorigenicity of DCA. This possibility cannot be completely ruled out from the present study alone. However, it is considered unlikely for several reasons. First, the nodules and tumors produced by TCA were uniformly different from those induced by DCA with respect to the expression of c-Jun. Second, the metabolic data indicate that the levels of DCA produced in the blood of mice by TCA was very modest compared to that seen with carcinogenic doses of DCA. Finally, the data on H-ras mutation spectra for the two compounds was quite different (Anna et al., 1994; Ferreira-Gonzalez et al., 1995). Therefore, if there is a contribution, it must be very small.

Several things must be kept specifically in mind when considering the carcinogenic potential of TCA. First, it is a very weak peroxisome proliferator, with approximately 8 mM concentrations being required to activate the PPAR *in vitro* (Issemann and Green, 1990; James and Roberts, 1994). Such concentrations are, in fact, approximated in the blood of both rats and mice at carcinogenic doses (Larson and Bull, 1992). Second, it only produces tumors in mice, failing to produce tumors in rats at very high doses in drinking water (DeAngelo, personal communication). As will be discussed further below, the blood levels of TCA at equivalent doses would be at least as high in rats than observed in mice based on data from single doses (Larson & Bull, 1992) and would be predicted even chronic studies based on our data with repeated exposure. Therefore, there appears to be some significant species differences in the sensitivity to TCA-induced liver cancer that go beyond questions of delivered dose. This may be simply because TCA is a less effective peroxisome proliferator in rats than in mice (DeAngelo et al., 1989) when it is administered in the drinking water. Early work with TCA had involved its administration by gavage in corn oil also resulted in modest increases in acyl-CoA activity in rats

(Goldsworthy and Popp, 1987). Consequently, it is reasonable to conclude that TCA is simply too weak to be active as either as an effective peroxisome proliferator or carcinogen in the rat at the systemic blood concentrations that can be achieved by treatment in drinking water. This issue could be easily pursued by the construction of a physiologically-based pharmacokinetic model that relates concentrations of TCA achieved in blood in the gavage studies (Goldsworthy & Popp, 1987) and drinking water studies (DeAngelo et al., 1989).

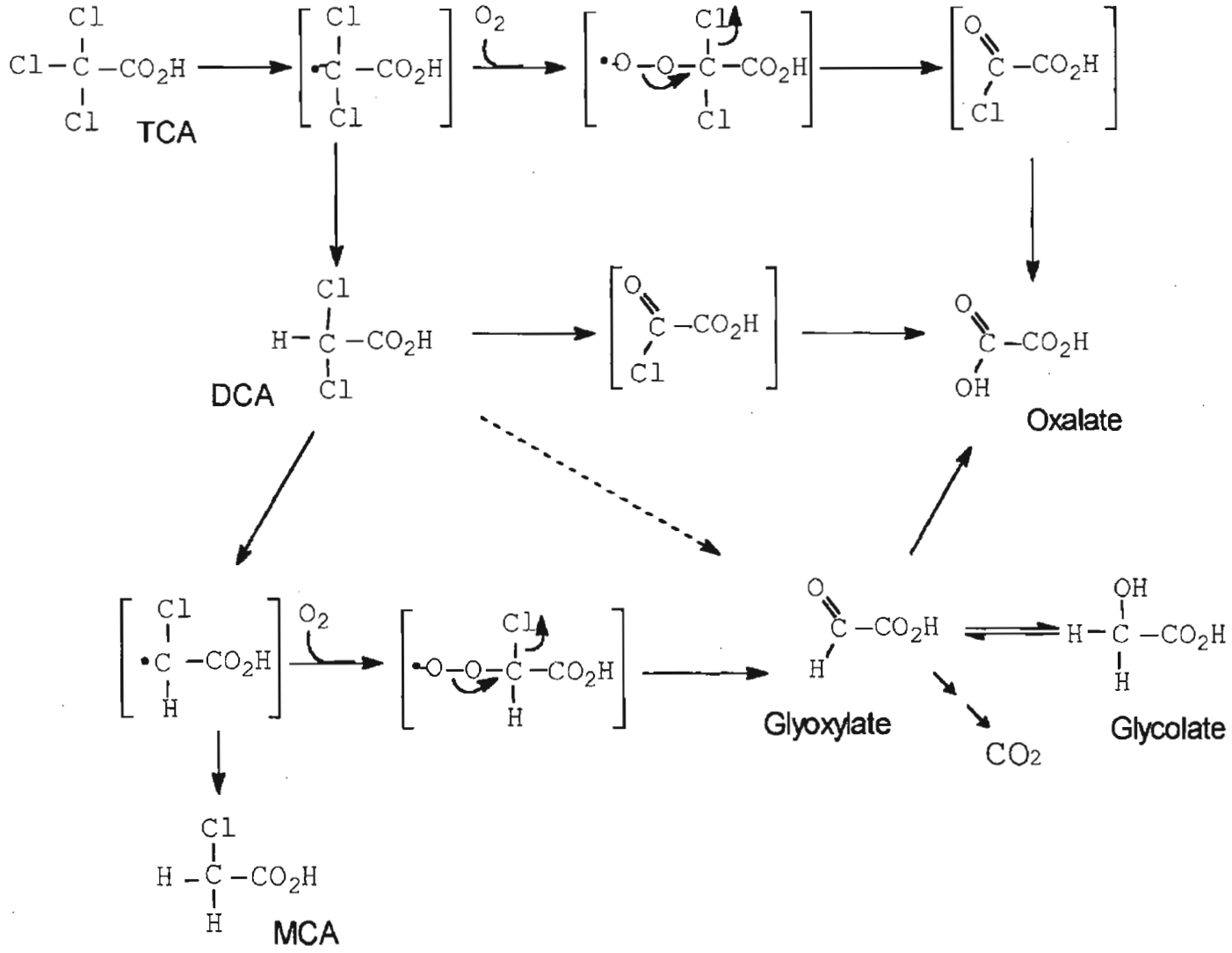
Metabolism and Pharmacokinetics

There appear to be substantive differences in the metabolism of the haloacids in mice and that are not apparent in the naive animal. A proposed outline of the metabolism of potential pathways in the metabolism of DCA are provided in Figure 4.1.

The fact that pretreatment with DCA greatly inhibits the conversion of subsequent doses to CO_2 in the rat, but not in the mouse suggests significant species differences within the pathways that lead to CO_2. It appears that there must be a substantially greater capacity to catalyze the initial steps of DCA metabolism in the two species. The blood levels of DCA are significantly increased by pretreatment in both mice and rats, however, the effect was much more sustained in rats. Therefore, it is probable that the mouse must have considerably more flexibility (i.e. in capacity) in the pathways that lead to CO_2. Insofar is known, the key intermediate in the metabolism of DCA to CO_2 is glyoxylate. In mammals, the glyoxylate can be converted to CO_2 through the carboligase pathway (Chalmers and Lawson, 1982) or be transaminated to glycine, converted to serine, then pyruvate to move into the Krebs' cycle. We are unaware of literature that would suggest that mice or rats are fundamentally different in routing glyoxylate down these two basic pathways.

There are three pathways that can be responsible for the initial metabolism of DCA; reductive dehalogenation, oxidative dehalogenation and hydrolysis. The latter pathway has only been demonstrated in plants and microorganisms (van der Ploeg et al., 1991). Of these pathways, only the oxidative dehalogenation step would fail to yield CO_2. Oxalate is not generally metabolized further in mammalian species. In other studies, we have found that the mouse produces significantly more oxalate at equivalent doses than the rat (6.8 ± 1.1 vs. 2.8 ± 0.2% of

Figure 4.1. A proposed metabolic scheme that includes both dichloroacetate (DCA) and Trichloroacetate (TCA).



an oral dose of 100 mg/kg found in the urine, respectively). Moreover, as much as 10% of lower doses of DCA are converted to oxalate when coadministered with bromodichloroacetate (BDCA), suggesting that the capacity for this pathway in mice is even larger when faced with competition from other related chemicals (Xu et al., 1996).

The greater sensitivity of conversion to CO_2 in the rat, however, does not come simply from a greater conversion to glyoxylate. In previous studies we have found that the mouse eliminates about 5.3 $\pm$ 1.7% of a 100 mg/kg oral dose of DCA in the urine as glycolate in 24 hours, whereas the rat eliminates only about 0.5 $\pm$ 0.1%. Moreover, utilizing iv dosing in the present study, the percent of the dose eliminated as glycolate goes up more than 9-fold in the pretreated rat. While the increases in blood concentrations of DCA indicate that its initial metabolism has to be sharply inhibited in the pretreated rat, the accumulation of glycolate in the urine would suggest that conversion of glyoxylate to CO_2 may be blocked to some extent as well. Considering the profound effects DCA has on intermediary metabolism at high doses (Stacpoole, 1989), this effect may not be simply related to direct inhibition of glyoxylate oxidation. However, the extension of this effect to doses as low as 5 mg/kg would be more consistent with a direct effect of DCA, since doses in the 50 mg/kg range are utilized therapeutically (Stacpoole, 1989).

From the toxicological point of view, large increases in the area under the concentration vs. time curve (AUC) for DCA was observed as a result of DCA-pretreatment in both species. This is important because it demonstrates clearly that pharmacokinetic behavior of DCA in naive animals is not predictive of what occurs with the chronic treatments used in producing the carcinogenic, neurotoxic, spermatotoxic and developmental toxicities of DCA. Second, the AUC was significantly longer in rats than in mice, primarily because of a large terminal half-life of DCA after pretreatment. This is of interest since the rat appears to be more sensitive to all of these effects than the mouse. At low doses, DCA is more potent as a hepatocarcinogen in rats and mice. Moreover, the neurotoxicity becomes so marked that experiments at doses greater than 2 g/L in the drinking water have had to be terminated because of peripheral neuropathy (DeAngelo, personal communication: Bull et al., unpublished observations). Reproductive and teratologic effects of DCA have not been reported in mice. From our personal experience, mice do not develop the hind limb weakness that is clearly displayed in rates with concentrations of > 1 g/L

DCA in the drinking water. Thus, it would appear that differences in metabolism may largely account for the species differences in toxicological responses to DCA.

Comparisons of the effect of pretreatment on the peak blood concentrations achieved is complicated by the use of two different routes of administration for the test dose of DCA. A small increase of C_{max} was observed in the rat, but the level seen in the mouse increased by more than 40-fold. While this is representative of what would happen if the chemical were administered orally by gavage, it does not allow for a clear comparison with the rat. It does indicate, however, that there is a huge first pass of DCA metabolism in the liver before it reaches the systemic blood in the mouse. Thus, inhibition of DCA metabolism in this first pass leads to very high levels of DCA in the blood relative to what was observed in control mice.

It was remarkable that the pretreatment effect of DCA on its metabolism was fully observable in rats with DCA concentrations in drinking water as low as 0.2 g/L. This indicates that chronic dosing with as little as 20 mg/kg (i.e. assuming a drinking water consumption of one-tenth the body weight) has a large effect on a high capacity pathway for DCA metabolism. Since this is still a maximal effect, it is likely to extend to somewhat lower exposures. Lipscomb et al., (1995) has shown that the bulk of DCA's metabolism occurs in the cytosol with only minor activity in microsomes. This activity has been poorly characterized, but has a K_m that is consistent with the pathway that is inhibited in these studies. It is clearly a quite different system than the suicide inhibition of the microsomal metabolism of carbon tetrachloride, however.

Unfortunately, the resources available to this project did not allow for the development of a formalized physiological model for DCA and TCA pharmacokinetics. Therefore, our conclusions have to be based primarily upon the descriptive data. Nevertheless, these data do indicate that it is extremely important to understand the metabolism and kinetics of these compounds in the chronically treated animal, as well as the naive animal. No pharmacokinetic model devised to date has any application to animals treated repeatedly with high doses of DCA. The second conclusion that can be made from these data is that interactions can be aniticipated in the metabolism of the different haloacetates that occur in drinking water. For example, data generated with other support indicates that high levels of DCA are produced from bromodichloroacetate (BDCA). If DCA preadministered or coadministered with BDCA, one

could anticipate sustained high levels of DCA in these animals as well and a significant contribution of DCA to BDCA induced liver tumors.

CHAPTER 5
SUMMARY AND CONCLUSIONS

The results in this report indicate that:

1. Induction of oxidative damage to DNA appears to play an insignificant role in the carcinogenic effects of DCA or TCA at low doses.
2. There is no consistent link between the ability of TCA to induce peroxisome proliferation and oxidative damage to DNA. Thus, we were unsuccessful in attempting to establish a causal link between increases in peroxisome numbers and the induction of cancer.
3. DCA and TCA provide selective advantages to different types of preneoplastic cells in the liver of B6C3F1 mice.
4. DCA affects cell kinetics in two distinct ways in the dose ranges that induce liver tumors in mice: a) Inhibition of normal cell division at concentrations of $\cong$ 0.5 g/L. Since normal rates of cell division are very low in the liver, this suppression provides a relatively small stimulus for outgrowth of preneoplastic cells. This accounts, at least in part, to the long latency for liver tumor development at this concentration. b) A selective stimulation of the replication of a specific phenotype at concentrations of $\geq$ 1 g/L. This behavior could easily account for the very rapid development of tumors observed at higher doses.
5. Tumors induced by TCA appear to express a phenotype similar to that induced by other peroxisome proliferators.
6. There was little evidence that TCA directly influences replication rates within tumors. This suggests that its major effect may be in providing an environment in which damaged cells that would ordinarily eliminated by apoptosis can survive and replicate. Tumors made up of such cells have been shown to disappear when treatment with other peroxisome proliferators has been suspended.

7. Large modifications in the metabolism of DCA is observed when the treatment goes beyond a single dose. These modifications are manifested differently in different species. They will have to be taken into account in arriving at effective doses between species at concentrations found in drinking water.
8. Effective doses of DCA at environmental levels will be poorly predicted from high dose studies of its metabolism and pharmacokinetics naive animals. Considerably more attention needs to be placed on identifying the point at which prior treatment or exposure to DCA modifies its metabolism in humans as well as in experimental animals.
9. These data need to be used to develop formal pharmacokinetic models for the haloacetic acids.
10. Based on data from independently funded work, these pharmacokinetic models should be expanded to included consideration of the effects of bromine substitution on the metabolism of this important class of disinfection by-products.
11. It is important to recognize how these models will applied to describe the metabolic pathways and kinetics of the corresponding haloacetaldehydes (chloral hydrate, bromodichloroacetaldehyde, etc).

In combination, these results show that extrapolation of risks from high dose studies of the haloacetates in animals to humans are probably not appropriately estimated by the LMS and the default assumptions about metabolism used previously by the U.S. Environmental Protection Agency. In particular, the carcinogenic activity of DCA seems to be largely, if not completely, accounted for by inducing selective changes in cell replication behavior within normal and preneoplastic cell populations. In addition, the metabolism of these chemicals at doses that have been shown to induce cancer and other adverse health effects (e.g. developmental and neurotoxic effects) is very non-linear and substantially modified by chronic exposure. Therefore, better estimates of risks will be obtained by specific consideration of the mode of action and the compound's metabolism and pharmacokinetics, as provided for in the proposed guidelines for cancer risk assessment (EPA, 1996).

Moreover, these and previously published data (Larson and Bull, 1992; Xu et al., 1995) indicate that there are substantive interactions in metabolism of haloacetates. Therefore, a reliable

estimate of risks from mixtures of the haloacetates found at low concentrations in human drinking water are going to be difficult to derive from simple study of these mixtures at the high that have been employed in animal studies. Further exploration of these metabolic and pharmacokinetic interactions are essential.

CHAPTER 6
RECOMMENDATIONS

The present project made significant progress in identifying critical problems for assessing the risks associated with haloacetates produced during the disinfection of drinking water. The data can be used to drive the development of alternative methodologies for assessing the risks, particularly for DCA. Even in this case, however, there are specific questions that require some attention to further narrow uncertainties and confidence intervals of these estimates of risk. Perhaps of greater importance, is the fact that this research provides a clear direction for research that should be conducted with other members of the haloacetate and related by-product classes (in particular, brominated haloacetates, haloacetaldehydes and longer chain haloacids). The following recommendations cover only the most obvious of these research needs:

1. The concentrations (doses rates) at which prior exposure to DCA begins to modify its metabolism needs to be identified in humans and experimental animals.

2. The metabolism of other dihalo- and trihaloacetates needs to be examined over dose ranges approximating those resulting from drinking water (generally between 1-100 μg/L) to those that produce adverse effects in experimental animals (0.2-3 g/L).

3. Interactions in the metabolism of haloacetates needs to be examined over the same range of doses identified in no. 2 in humans and in species where adverse health effects have been identified.

4. Some attention should be placed on potential metabolic interactions between the trihalomethanes and haloacids, in particular the trihaloacids.

5. Pharmacokinetic models should be developed which allow prediction of the contribution that metabolism of trihaloacetates (most specifically bromodichloroacetate, dibromochloroacetate, but also tribromoacetate if its occurrence appears to warrant such investigation) to the corresponding dihaloacetate (i.e. DCA, bromochloroacetate and dibromoacetate) contributes to the production of adverse health effects. Such data can take advantage of any biologically-based dose response model that is developed for DCA.

6. Stop experiments of the general design employed in the present work should be used to evaluate the quantitative contribution of suppressed apoptosis to the tumorigenic effects of TCA and DCA. It is anticipated that this effect will be more important with TCA than DCA.

7. Consideration should be given to development of an *in vitro* selection test to determine if the selection of phenotypes observed by DCA or TCA *in vivo*, can be duplicated *in vitro*. If so, such a test could be used to evaluate other haloacetates and related by-products for their mode of action.

8. Utilizing the *in vitro* selection technique suggested in no. 7 and/or a modification of the stop protocol used in the present study, each haloacid and other major by-products (i.e. the THMs) should be categorized by its major mode of action. Such studies would provide a direct method for studying the effects of DCA and TCA in human liver cells and provide a much more accurate estimate of the risks associated with chlorinated drinking water.

9. Based upon the metabolism and pharmacokinetic studies and mode of action studies described above, specific hypotheses should be developed about how these chemicals would interact to induce liver cancer. Selected hypotheses should be subjected to direct

test using the *in vitro* selection technique or a modification of the stop protocol utilized in the present study.

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