AD-A214 501

DTIC

REPORT DOCUMENTATION PAGE

1a RESTRICTIVE MARKINGS	
2 AD-A214 501	3 DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited.
2b DECLASSIFICATION/DOWNGRADING SCHEDULE	
4 PERFORMING ORGANIZATION REPORT NUMBER(S)	5 MONITORING ORGANIZATION REPORT NUMBER(S): AFOSR-TR- 89-1325
6a NAME OF PERFORMING ORGANIZATION: College of Pharmacy, Washington State University	6b OFFICE SYMBOL (If applicable):
6c ADDRESS (City, State, and ZIP Code): Pullman, WA 99164-6510	7b ADDRESS (City, State, and ZIP Code): Building 410, Bolling AFB, DC 20332-6448
8a NAME OF FUNDING/SPONSORING ORGANIZATION: AFOSR	8b OFFICE SYMBOL (If applicable): NL
8c ADDRESS (City, State, and ZIP Code): Building 410, Bolling AFB, DC 20332-6448	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: AFOSR-86-0284
	10 SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO 61102F; PROJECT NO 2312; TASK NO A5; WORK UNIT ACCESSION NO

11 TITLE (Include Security Classification)
IMPORTANCE OF DICHLOROACETATE AND TRICHLOROACETATE TO THE HEPATOCARCINOGENIC RESPONSE TO TRICHLOROETHYLENE IN B6C3F1 MICE

12 PERSONAL AUTHOR(S)
Richard J. Bull

13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Final	FROM 09/15/86 TO 08/14/89	10/15/89	46

16 SUPPLEMENTARY NOTATION

17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Trichloroethylene; trichloroacetate; dichloroacetate; hepatotoxicity; carcinogenicity; B6C3F1 mice; (*)

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

This project was aimed at determining the extent to which the metabolism of trichloroethylene (TCE) to trichloroacetate (TCA) and dichloroacetate (DCA) was responsible for its hepatotoxic and hepatocarcinogenic effects in B6C3F1 mice. An attempt was made to use coadministration of ethanol to selectively decrease the production of TCA. These effects were observed consistently only at high doses and were transitory, suggesting the modification to be non-specific. The project shifted to determining whether DCA and TCA could account for the hepatotoxic and hepatocarcinogenic effects of TCE in quantitative terms. It was established that both DCA and TCA were capable of inducing tumors in B6C3F1 mice at much lower doses with a shorter latency than TCE. DCA was very hepatotoxic, and tumorigenic response appeared to be dependent upon these effects. The tumorigenic effects of TCA were linear with dose (unlike DCA) and were closely associated with the accumulation of lipofuscin and the induction of single strand breaks in hepatic DNA in vivo. Evidence is presented that suggests that DCA acts primarily by increasing growth of hyperplastic nodules whereas TCA appears to accelerate progression of tumors. Together these data suggest that TCA was probably primarily responsible for the hepatocarcinogenic effects of TCE. However the cytotoxic effects of DCA-treatment may well contribute significantly to carcinogenic responses at very high doses of TCE.

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	UNCLASSIFIED
22a NAME OF RESPONSIBLE INDIVIDUAL: T. Jan Cerveny, Lt Col, USAF	22b TELEPHONE (Include Area Code): (202) 767-5021 / 22c OFFICE SYMBOL: NL

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE: UNCLASSIFIED

89 11 17 073

AFOSR-TR- 89-1325

IMPORTANCE OF DICHLOROACETATE AND TRICHLOROACETATE TO THE HEPATOCARCINOGENIC RESPONSE TO TRICHLOROETHYLENE IN B6C3F1 MICE

FINAL REPORT

U.S. Air Force Grant No.

AFOSR-86-0284

Principal Investigator:

Richard J. Bull, Ph.D.

College of Pharmacy
Washington State University
Pullman, WA 99164-6510

Project Officer:

Major T. Jan Cerveny, Ph.D.

Air Force Office of Scientific Research/NL
Building 410
Bolling AFB, DC 20332-6448

Project Period:

8/15/86 to 8/14/89

INTRODUCTION

Trichloroethylene (TCE) is one of the most ubiquitous environmental contaminants. Its widespread commercial use in degreasing operations and as a solvent has lead, through improper disposal, to its being the most common synthetic organic chemical contaminant of groundwater (CEQ, 1981; Fan, 1988). Consequently, it is a chemical that the U.S. Environmental Protection Agency has regulated under a number of authorities (Clean Water Act, Safe Drinking Water Act, CERCLA, etc).

TCE is a solvent of relatively low acute toxicity. Its acute oral LD_{50} is in excess of 3 g/kg and rats and mice survive lifetime doses in excess of 1 g/kg per day in carcinogenesis bioassays (NCI, 1976).

Of most concern to the regulatory community are reports that TCE is carcinogenic. The most sensitive bioassay system for its carcinogenic effects is the liver of B6C3F1 mice (NCI, 1976; NTP, 1983). It has also been shown to increase the incidence of pulmonary adenocarcinomas in female ICR mice (Fukuda et al., 1983) and lymphomas in female Han:NMRI mice (Henschler et al., 1980). The National Toxicology Program has subjected TCE to bioassay in 5 strains of rat and found very marginal evidence of increased renal and Leydig cell tumors (NTP, 1986). Other investigators have failed to demonstrate the carcinogenicity of TCE in rats (Henschler et al., 1980; Fukuda et al., 1983). TCE was also unable to induce tumors in Syrian hamsters (Henschler et al., 1980).

Since TCE appears to only increase tumors for which there is a substantial background incidence, the use of linear extrapolation models to estimate the risk it poses to the public has been controversial. Studies of TCE's ability to produce point mutation in bacterial or yeast systems has given inconsistent results (Crebelli and Carere, 1989). Where it has been found positive, it has required very high concentrations. TCE has been shown to induce micronuclei in mouse bone marrow, but no structural aberrations of chromosomes were observed (Duprat and Gradishi, 1980; Sbrana et al., 1985). It has been shown positive in the spot test (Fahrig, 1977) and at very high doses by inhalation it produced spermhead abnormalities in mice (Land et al., 1981). At very high oral doses TCE does induce single strand breaks (SSB) in hepatic DNA of mice and rats (Parchman and Magee, 1982; Walles, 1986; Nelson and Bull, 1987). On the other hand, TCE failed to induce unscheduled DNA synthesis in mouse hepatocytes (Mirsalis et al., 1985; Doolittle et al., 1987). Since some of these assays may respond to mechanisms other than direct interaction of TCE or one of its metabolites with DNA (e.g. SSB), it is difficult to clearly establish that TCE is a "genotoxic" carcinogen. An irreversible event, such as a mutation, is necessary to justify the use of the linear extrapolation model for estimating the carcinogenic risks involved (Crump et al., 1976).

Another means of approaching this problem is to identify and test alternative mechanisms by which TCE might induce cancer. TCE has long been recognized as a hepatotoxin, although it is a weak one. It requires doses that approach the LD_{50} to be effective (Klaassen and Plaa, 1967). Second, it has been observed that TCE is an inducer of peroxisome proliferation in mice and rats (Elcombe, 1985; Coldsworthy and Popp, 1987). This activity could be at least partially accounted for by metabolites of TCE, namely trichloroacetate (TCA)(Elcombe, 1985; Goldsworthy and Popp, 1987) and DCA (Nelson et al., 1989; DeAngelo et al., 1989). The finding that metabolism of TCE to TCA is much more efficient in mice at the high doses used in carcinogenesis bioassays (Prout et al., 1985; Dekant et al., 1986) made this an attractive hypothesis to account for the species differences in the sensitivity to the hepatocarcinogenic effects of TCE.

The primary objective of this project was to determine how important TCA was to the hepatotoxic and hepatocarcinogenic effects of TCE. Initial efforts were directed at developing a method for experimentally manipulating the portion of TCE that was metabolized to TCA by coadministration of ethanol in short term experiments. While the manipulation produced the predicted effects, the effects were too transient to make it a dependable experimental model. Consequently, it became necessary to spend more effort in documenting the hepatotoxic and hepatocarcinogenic effects of DCA and TCA in long term experiments to obtain definitive results.

METHODS

Materials:

Chemicals. TCE was purchased from Fisher Chemical Co., diethyl ether from J.T. Baker Co., polysorbate (Tween 80), beta-glucuronidase (Type VII), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), clofibrate, phenobarbital, DCA, TCA, TCOH and CH from Sigma Chemical Co. and [1-^{14}C]palmitoyl-CoA from New England Nuclear Products. Purity of administered chemicals was found to be 99+% using gas-liquid chromatographic methods described by Prout et al. (1985).

Animals. Male Sprague-Dawley rats weighing 300-400 g were obtained from the Washington State University Laboratory Animal Resource Center. Male and female B6C3F1 mice were obtained from Simonsen Laboratories (Gilroy, CA) and weighed between 25-30 g in acute experiments. In long-term studies, mice were begun on study at 36-38 days of age. A 12-hour light/dark cycle was utilized. Animals were maintained on Purina Laboratory Chow, Ralston-Purina Co. (St Louis, MO) provided ad libitum except when fasted overnight prior to drug administration. Access to water was unrestricted.

Experimental Design:

This project had three distinct phases of experimentation: 1. Metabolic studies directed at documenting influences of ethanol coadministration on the metabolism of TCE. 2. Induction of single strand breaks in hepatic DNA of rats and mice treated in vivo with TCE and its metabolites. 3. A chronic experiment to examine the hepatotoxicity and carcinogenicity of DCA and TCA.

Metabolism studies. In the metabolism studies all animals were dosed between 8:00 AM and 10:00 AM following overnight fasting. Only rats were used in these studies. Control rats received either 1.52, 4.56, or 22.8 mmol/kg body weight of TCE in a 1% aqueous suspension of Tween 80 by stomach tube. Rats with concurrent administrations of ethanol received equimolar doses of ethanol and TCE in the same suspension. Each treatment group consisted of 5 or 6 animals that were randomly assigned to these groups. The volume of solution administered was kept constant at 10 ml/kg body weight. After dosing the animals were housed singly in polycarbonate metabolism cages for collection of urine. Blood samples were collected via the tail vein at 1, 2, 4, 8, 12, 24, 48, and 72 hours after dosing. No anesthesia was utilized. Urine was collected at 24 hour intervals for 72 hours. Blood concentrations of TCE, TCA and free TCOH (i.e. unconjugated) were determined by gas chromatography utilizing the ether extraction method described by Prout et al. (1985). TCA was detected as the methyl ester following derivatization with diazomethane. Urinary metabolites were determined using the same methodology, but TCOH conjugated with glucuronic acid was determined in an aliquot of sample treated with beta-glucuronidase. A Varian Model 3700 gas chromatograph fitted with a 2 feet X 2 mm glass column packed with 0.1% AT 1000 on 80/100 mesh Graphpac-GC and an electron-capture detector. Column temperature was maintained at 130° C and nitrogen was used as the carrier gas at 25 ml/min. Under these conditions the retention times were: TCE 1.5 min., TCOH 4.3 min, and TCA-methyl ester 5.7 min. Standards were prepared by adding known amounts of TCE and its metabolites to blood and urine samples from untreated animals.

Induction of single strand breaks in hepatic DNA. Both mice and rats were utilized in these experiments. The basic experiment involved random assignment of animals to experimental groups (either 5 or 6 per group). Occasionally animals were inadvertently intubated in the lung, so animals that died or suffered respiratory distress were excluded from the results. In screening studies, the induction of SSB in hepatic DNA was determined 4 hours after dosing based on the time course of DCA accumulation and elimination from liver following oral intubation (Evans, 1982). Pretreatments involved daily administration of the indicated drugs (phenobarbital 50 mg/kg i.p. and TCE 0, 3.8 or 11.4 mmol/kg p.o.) for four days. On the fifth day, TCE was administered and the animals sacrificed 4 hours later for analysis of SSB-induction. Measurement of SSB was accomplished by the alkaline unwinding assay of Morris and Shertzer (1985).

This assay measures the rate of transition of native double-stranded DNA to single-stranded DNA during alkaline denaturation. Strand breaks provide sites at which DNA unwinding is initiated. Therefore, the rate of alkaline unwinding is directly related to the number of SSB (Rydberg, 1975). Sacrifice of animals, preparation of liver samples and the unwinding of DNA was conducted in subdued, indirect incandescent light to avoid introduction of SSB by uv irradiation. DNA was assayed using Setaro and Morley's (1976) modification of Kissane and Robins' (1958) diaminobenzoic acid fluorimetric assay. The fraction of DNA unwound during the two hour incubation at 0°C was calculated as:

$$\frac{(\text{Total DNA} - \text{DS DNA})_t}{(\text{Total DNA})} - \frac{(\text{Total DNA} - \text{DS DNA})_o}{(\text{Total DNA})}$$

where the subscript indicates the amount of double-stranded DNA at time "0" and time "t" of incubation in alkaline solution.

Serum enzyme determinations were measured in animals receiving parallel treatments in which SSB in hepatic DNA were measured to ascertain what role, if any, might be attributed to cytotoxic responses. These animals were all sacrificed 24 hours after the challenge dose of TCE or its metabolites and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) determined in serum. This time interval was chosen to match the peak of enzyme release in the usual time course following hepatic injury (Pappas, 1986). These enzymes were determined using the Sigma Chemical Co. kits. The results are reported in international units/liter of serum. One international unit of enzyme activity is defined as the amount of enzyme that produces 1 umol of NAD/min (Sigma procedure No. 59-UV)

Peroxisome proliferation was assessed enzymatically and morphologically. Enzymatic determinations were made by measurement of [^{14}C]-palmitoyl-CoA oxidation in 10% liver homogenates in 0.25 M sucrose by following its conversion to soluble [^{14}C]-acetyl CoA by the method of Lazarow (1981). The lack of carnitine, the addition of Triton X-100 and freeze/thawing make this assay peroxisomal-dependent. Peroxisomal counts were also made by electron microscopic examination of sections fixed in 2.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.2). Tissues were dehydrated using a series of acetone baths and embedded in Spurr's resin (Humason, 1967). 60 nm sections were cut with glass knives, the sections stained with uranyl acetate and lead citrate. Electron microscopic examinations were limited to two animals from each experimental group (vehicle, DCA and TCA-treated groups). Six micrographic fields of 184 um^2 each were randomly chosen and the number of peroxisome profiles counted.

Chronic study. Male mice were assigned to six different treatment groups of unequal sample size; a control group, two concentrations of DCA and TCA and a group receiving phenobarbital. Sufficient animals were included in the control

group and the groups receiving the highest concentrations (2 g/L) of DCA and TCA in their drinking water to allow interim sacrifices (5 per interval) and a group in which treatment was suspended after 37 weeks yet were sacrificed at 52 weeks with continuously treated animals (11 mice per group). This left 24 animals in each group receiving the high doses of phenobarbital, DCA and TCA for the full 52 weeks and 35 control animals. The two groups receiving the lower doses of DCA and TCA (1 g/L) contained 11 animals each. A smaller experiment with female mice involved random assignment of ten animals each to control, DCA 2 g/L, TCA 2 g/L and phenobarbital 0.5 g/L groups. All females were treated for 52 weeks.

Food and water consumption were measured biweekly and animals weighed weekly during the course of the study. The mice were sacrificed at the indicated intervals by cervical dislocation, liver and kidneys removed and weighed, examined for macroscopic lesions and the central lobe of the liver fixed and embedded in paraffin. The left lateral and right lobes of the liver were frozen (on dry ice) and stored at -70°C until frozen sections could be made for histological staining. At the terminal sacrifice (52 weeks), precedence was given to preserving suspected tumors for histological and histochemical examination. Thirty-one of the 65 animals whose livers were identified as containing lesions were randomly selected for histopathological examination. The liver tissue from remaining animals was set aside for subsequent molecular and biochemical studies (data not shown). Because of the small numbers and importance of correctly classifying lesions in the control group and groups in which treatment was suspended, 10 additional animals were examined in these groups to bring the total animals examined histologically to 41 mice. This resulted in close histological examination of 73 out of a total of 165 lesions identified at necropsy. Lesions were placed into four categories: lesions that displayed neither hyperplastic or neoplastic characteristics (normal), hyperplastic nodules, adenomas and hepatocellular carcinomas. The latter three categories are lumped as hepatoproliferative lesions in presenting macroscopic data.

At the time of interim sacrifices (15, 24 and 37 weeks of treatment), the central lobe was completely sectioned, stained with hematoxylin and eosin (H&E) and examined for the appearance of focal areas of cells with altered staining characteristics. When such foci were located, adjacent sections were stained with periodic acid/Schiff's (PAS) reagent (Humanson, 1967). Predigestion of such sections with a-amylase demonstrated that PAS-staining was attributable to glycogen when it was apparent.

Frozen sections taken at the interim sacrifices were stained to determine if enzyme-altered foci could be detected. Enzymes examined included ATPase using the method of Wachstein and Meisel (1957), glucose-6-phosphate dehydrogenase (G6PDH) under oxygen according to Altman as described by Chayen et al. (1973), glucose-6-phosphatase (G6P) by the method of Benner et al.

(1979), and gamma-glutamyltranspeptidase (GGT) as described by Rutenburg et al. (1969). Tissues taken from rats previously initiated by diethylnitrosamine and promoted by phenobarbital and previously identified as containing enzyme-altered foci were included with each GGTase staining run as a positive control. The other methods used were previously validated in our laboratory by demonstrating occurence in the same way, but were not run simultaneously with mouse tissues. At the terminal sacrifice, histochemical staining was limited to suspected tumors (i.e. enzyme-altered foci were not sought)

The intrinsic autofluorescence of lipofuscin was used to determine if there were significant accumulations of this pigment in the livers of treated vs. control animals. Formalin-fixed and paraffin-embedded liver tissues were sectioned at 6 um, deparaffinized and rehydrated. Sections were rinsed with 0.15 M phosphate buffered saline (pH 7.4) and mounted with nonfluorescent glycerol. For routine visualization, excitation wavelengths of 450-490 nm and emission wavelengths above 510 nm were used. The sections were examined in a Nikon fluorescence microscope for the yellow-orange fluorescence characteristically observed for lipofuscin (Reddy et al., 1982). The fact that the flourescence was truly attributable to lipofuscin, contiguous sections were stained with Kinyoun's Carbol Fuscin for 1 hour as described in the Armed Forces Institute of Pathology (Luna, 1988)

Statistical methods are noted with individual data sets.

RESULTS

Interactions between ethanol and TCE. The original intent of this project was to utilize interactions in the metabolism of ethanol and TCE to explore the importance of peroxisome proliferation to the hepatotoxic and hepatocarcinogenic effects of TCE. The first step was to determine whether ethanol would consistently produce the proposed modifications of TCE metabolism.

In Figure 1, the effect of ethanol on the time course of TCE, free TCOH and TCA in the blood of male Sprague-Dawley rats is shown. Three doses of TCE were utilized (mg/kg); 1.52 (200), 4.57 (600) and 22.8 (3000) mmol/kg body weight. In those groups in which ethanol was coadministered, it was given at equimolar doses with TCE.

At the lower doses, ethanol had little effect on blood concentrations of TCE. However, at the highest dose of ethanol and TCE the peak concentrations of TCE were significantly smaller, but the residence time of TCE in the blood was prolonged. These data indicate that at very high doses ethanol inhibits the absorption of TCE by some non-specific mechanism (i.e. because it was not observed at low doses).

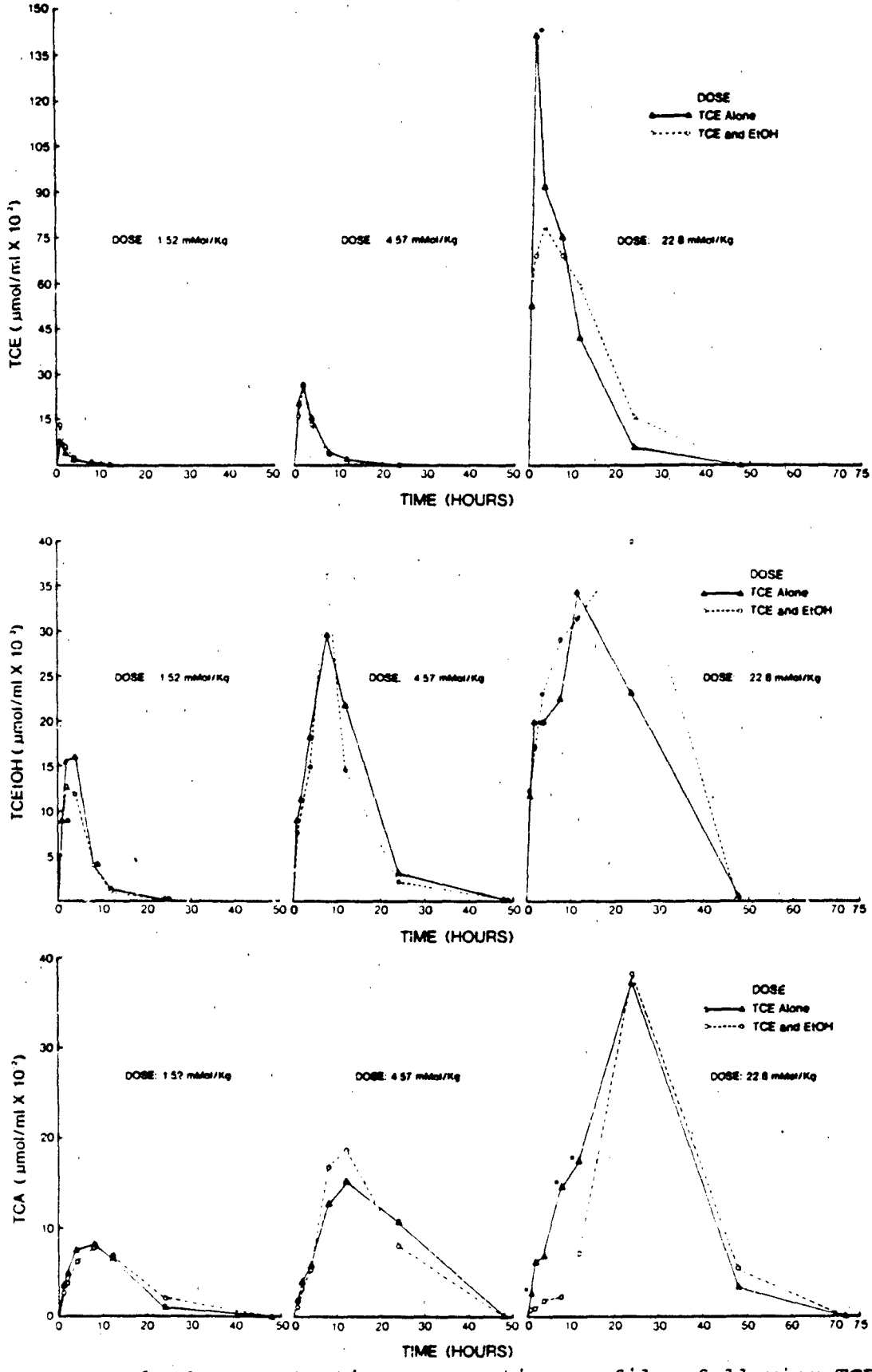


FIGURE 1. Blood concentration versus time profiles following TCE administration. Top panel, trichloroethylene; center panel, free-trichloroethanol; and lower panel, trichloroacetate. Astericks denote a statistically significant difference between the control and ethanol treatment groups ($P<0.05$).

Blood TCOH concentrations were not significantly affected by ethanol coadministration (Figure 1). This is not too surprising because TCOH is rapidly conjugated with glucuronic acid (Muller et al., 1972) and only the free form in blood was measured in these experiments.

The concentrations of TCA in blood were significantly modified by ethanol coadministration, but only at the highest doses of ethanol and TCE utilized. This effect was transitory, being evident for only the first 12 hours after dosing and the peak concentrations of TCA achieved were not affected. While interactions at the level of alcohol and aldehyde dehydrogenases may have contributed to this effect, ethanol's inhibition of TCE absorption at this dose obviously had a major impact on this result.

In urine, the total elimination of TCOH (TCOH + TCOH-glucuronide) was determined (Figure 2). Ethanol failed to significantly increase the net metabolism of TCE to TCOH. It is notable that the fraction of TCE that is converted to TCOH was substantially and significantly reduced at the highest dose of TCE administered irrespective of whether ethanol had been coadministered with TCE.

Despite the lack of significant effect on the conversion of TCE to TCOH, urinary levels of TCA were influenced by ethanol coadminstration (Figure 3). Some depression was observed at all doses. The ratio of TCOH to TCA in urine is increased by ethanol coadministration at all doses examined (Table 1). An apparently lesser effect at the intermediate dose appears to be an aberration in the control group (i.e. TCE only) rather than the group that received ethanol.

Induction of single strand breaks (SSB) in hepatic DNA. Presumably the hepatotoxic and hepatocarcinogenic effects of peroxisome proliferators is secondary to the production of hydrogen peroxide that accompanies peroxisomal beta-oxidation of fatty acids (Reddy and Lalwani, 1983). The proponents of this hypothesis suggest that hydrogen peroxide (or its metabolite, hydroxyl radical) is responsible for producing DNA damage that initiates carcinogenesis. Hydrogen peroxide is well known for its ability to induce SSB in DNA. Therefore, experiments were directed at:

1. Determining whether TCE was capable of inducing SSB in hepatic DNA of mice and rats in vivo.
2. Determining which metabolites of TCE might mediate SSB.
3. Determining whether the induction of SSB was dependent upon peroxisomal proliferation.

Data supplied in Table 2 show that high doses of TCE are indeed capable of inducing SSB in hepatic DNA of both B6C3F1 mice and Sprague-Dawley rats. Mice were more sensitive than rats. Significant increases in the rate of alkaline unwinding of DNA

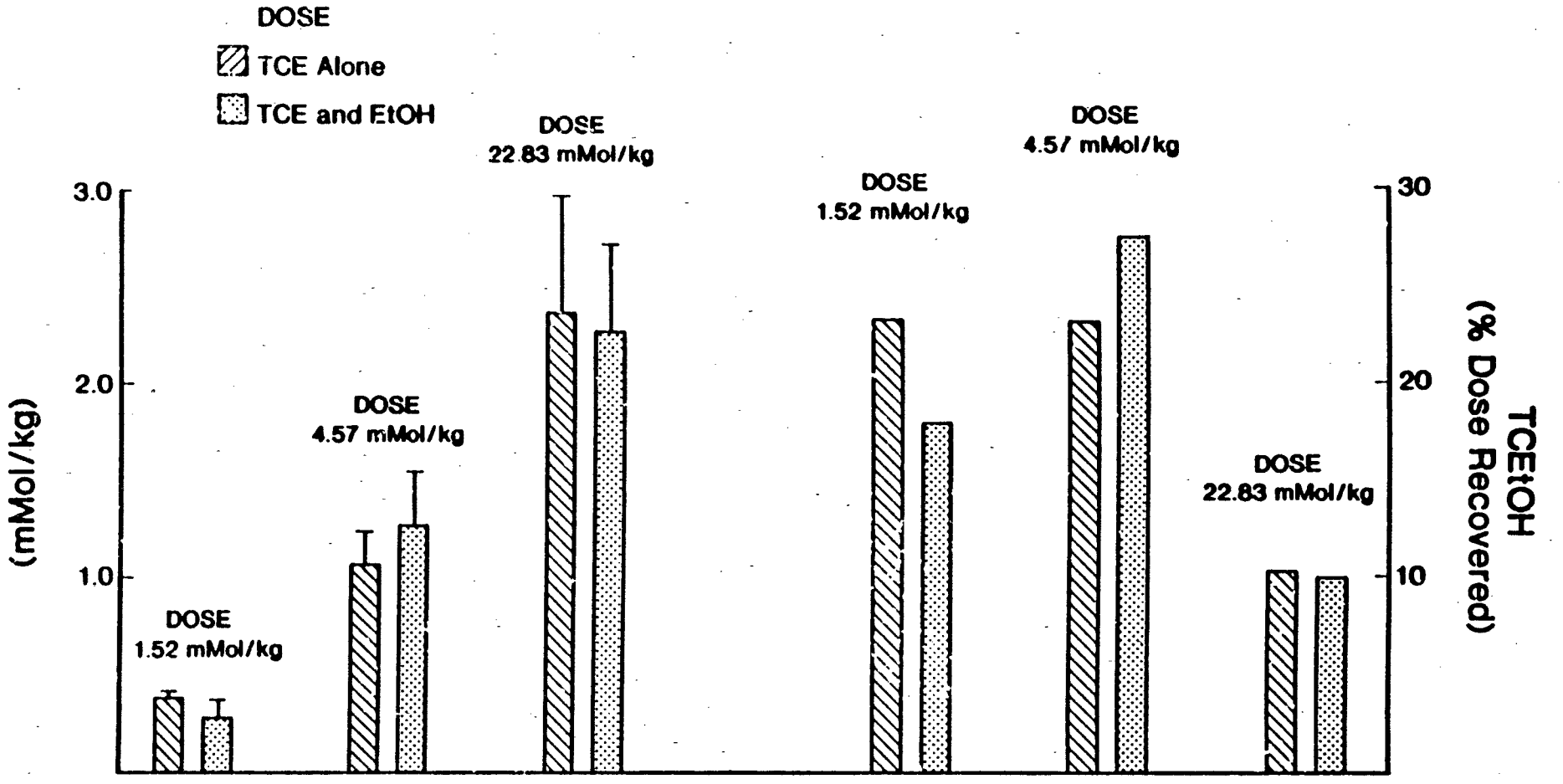


FIGURE 2. The effect of ethanol on the recovery of total trichhoroethanol in the urine. On the left is the total amount of trichloroethanol recovered in the urine over 72 hours. Brackets indicate mean ± S.E. for five to six rats. On the right, the amount of total trichloroethanol recovered is expressed as the percentage of the total dose of trichloroethylene administered.

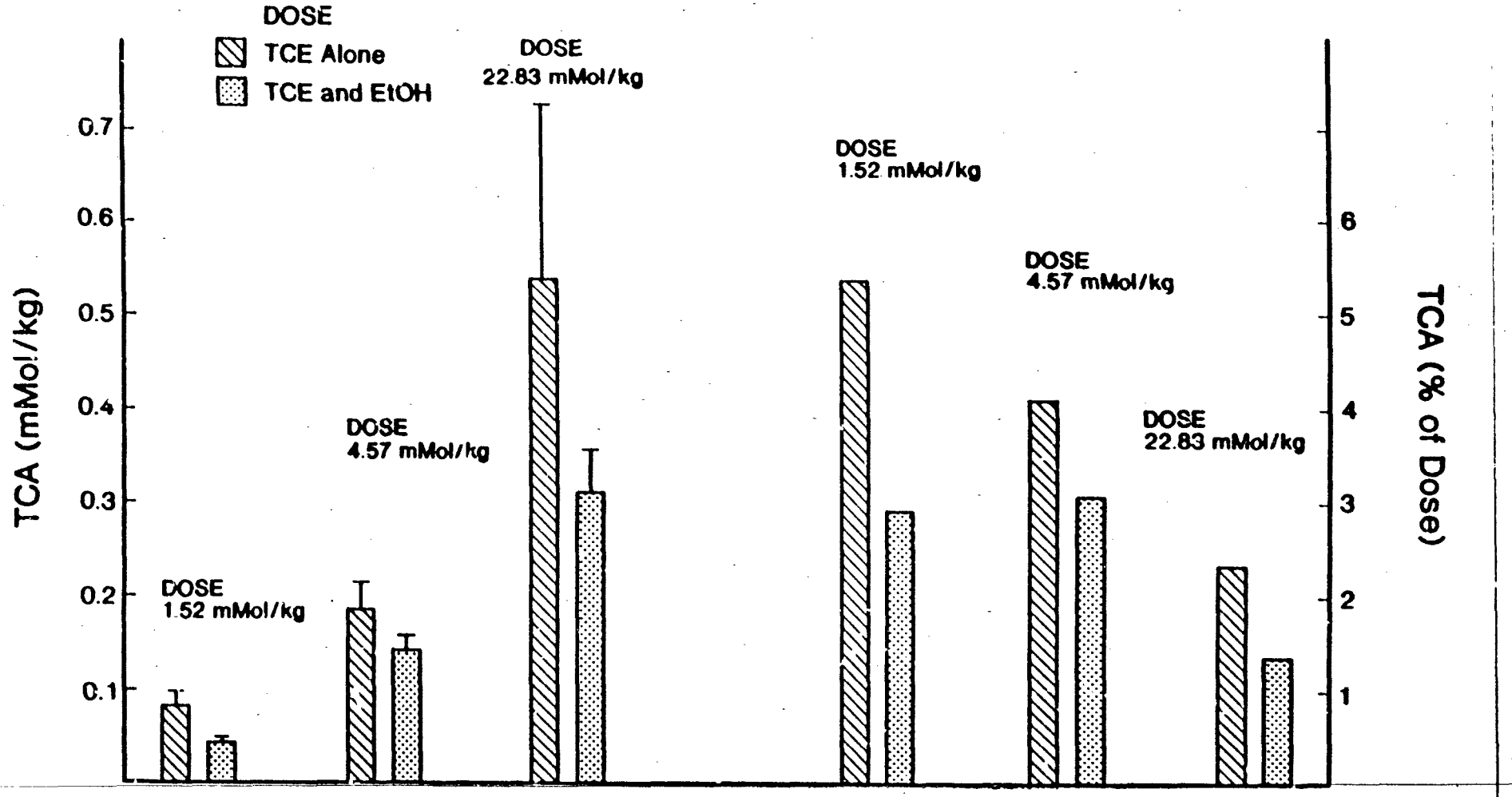


FIGURE 3. The effect of ethanol on the recovery of trichloroacetate in the urine. On the left is the tcal amount of trichloroacetate recovered in the urine over 72 hours. Brackets indicate mean ± S.E. for five to six rats. On the right, the amount of trichloroacetate recovered is expressed as the percentage of the total dose of trichloroethylene administered.

TABLE 1

THE EFFECT OF DOSE AND ETHANOL ON THE RATIO OF TCOH TO TCA REVOVERED IN URINE

Dose of TCE	TCOH/TCA without Ethanol	TCOH/TCA with Ethanol
1.52 mmol/kg	4.8 ± 0.5	6.5 ± 1.0
4.56 mmol/kg	6.2 ± 1.1	7.3 ± 1.1
22.8 mmol/kg	4.9 ± 0.4	7.2 ± 0.5

* Values are means ± standard errors for five to six rats.

TABLE 2

THE INDUCTION OF STRAND BREAKS IN RAT AND MOUSE HEPATIC DNA BY TCE IN VIVO[a]

Dose (mmol/kg)	N	Species	Fraction DNA Unwound/2 hr
0	7	SD-rats	0.22 ± 0.03[b]
3.9	5	SD-rats	0.25 ± 0.06
11.4	5	SD-rats	0.22 ± 0.03
22.9	7	SD-rats	0.34 ± 0.05[c]
30.4	7	SD-rats	0.47 ± 0.05[c]
0	5	B6C3F1-mice	0.13 ± 0.04
0.76	4	B6C3F1-mice	0.20 ± 0.03
5.8	6	B6C3F1-mice	0.22 ± 0.03
11.4	6	B6C3F1-mice	0.29 ± 0.03[c]
22.9	6	B6C3F1-mice	0.32 ± 0.04[c]

[a] Male Sprague-Dawley rats and male B6C3F1 mice were given single oral doses of TCE suspended in 1% Tween 80 in distilled water. Control animals received an equal volume of vehicle alone.

[b] Values are means ± SEM of N animals

[c] Significantly different from vehicle control, $P \leq 0.05$, by ANOVA and Duncan's multiple range test.

was observed in mice treated with 11.4 mmol/kg whereas 22.9 mmol/kg were required in rats.

The induction of SSB by TCE was enhanced by pretreatment with phenobarbital (Figure 4) and TCE (Figure 5). These data are consistent with the notion that the induction of SSB was dependent upon its metabolism. Subsequent experiments addressed whether four stable metabolites of TCE could be responsible for the effect; TCOH, CH, DCA and TCA.

The dose-response relationships involved in the induction of SSB in hepatic DNA of male Sprague-Dawley rats treated with TCOH, CH, DCA and TCA in vivo are depicted in Figure 6. SSB were induced by CH, DCA and TCA, but not with TCOH. Of those chemicals that were active, DCA was the more potent with doses as low as 0.23 mmol/kg producing increases in the rate of alkaline unwinding. TCA required a dose of 0.6 mmol/kg and CH a dose of 1.8 mmol/kg. The slopes of the dose-response curves observed in rats were very steep, but reasonably consistent between compound (± 95% C.L.): DCA, 0.29 ± 0.02; TCA, 0.31 ± 0.06; and CH, 0.37 ± 0.06.

The metabolites that induced SSB in the hepatic DNA of rats were also capable of inducing SSB in the hepatic DNA of mice (Figure 7). In mice, TCA was the more potent compound with 0.006 mmol/kg producing a significant increase in alkaline unwinding. A somewhat lesser response was observed with the same dose of DCA. CH was considerably less potent than the other two metabolites, with 1.0 mmol/kg as the lowest dose producing a significant effect.

It is notable that the slope of the dose-response curves for CH, DCA and TCA in B6C3F1 mice were less than half than that observed in Sprague-Dawley rats; 0.09 ± 0.04, 0.11 ± 0.02, and 0.13 ± 0.01, respectively. This suggests that the process involved in producing (or repairing) SSB is more complex in rats than it is in mice.

Induction of SSB in DNA can occur secondary to cell necrosis. To insure this was not occuring, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were monitored 24 and 48 hours after administration of TCE and its metabolites in the experiments described above. These data are supplied in Tables 3, 4 and 5. The only case in which significant increases in these serum enzymes was observed with a very high dose of TCE (30.5 mmol/kg) following phenobarbital pretreatment. The effect was not seen with the lower dose of 11.4 mmol/kg following phenobarbital pretreatment which was capable of inducing SSB. Induction of SSB was observed at much lower doses than those shown to be without effect on these serum enzyme activities with all other treatments. On this basis it can be concluded that SSB were not secondary to the production of necrosis.

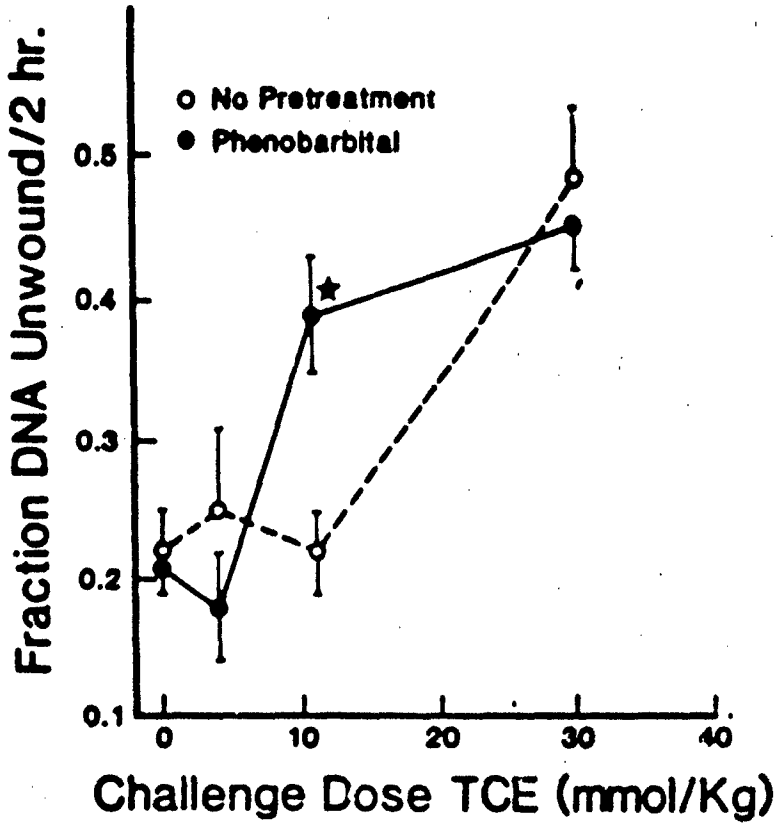


FIGURE 4. The induction of SSB in rat hepatic DNA by trichloroethylene and modification of this response by phenobarbital pretreatment. Phenobarbital was administered i.p. at a dose of 50 mg/kg for 4 consecutive days. On the fifth day animals received the indicated doses of trichloroethylene and sacrificed 4 hr later. For simplicity, data for vehicle control and phenobaribital control from individual experiments were combined and expressed as the means ± SEM for their respective control. However statisical analysis was done against the concurrent control. Values for vehicle/TCE and phenobaribital/TCE are the means from at least five animals ± SEM.

* Significantly different from vehicle pretreated animals administered the same dose of trichloroethylene (11.4 mmol/kg), $P \leq 0.05$, by ANOVA and Duncan's multiple range test.

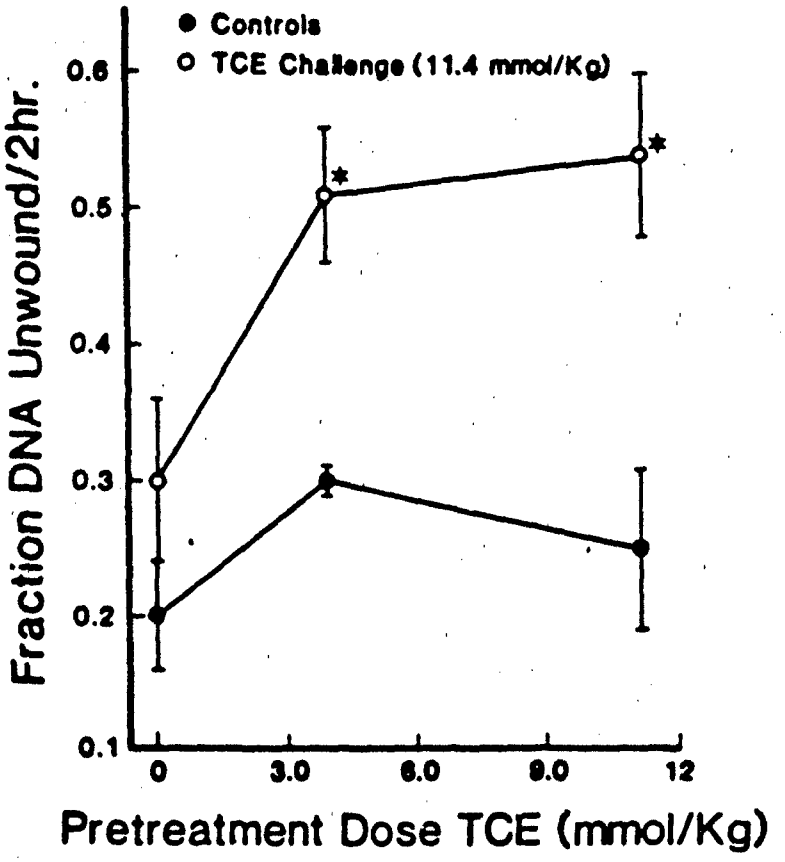


FIGURE 5. The induction of SSB in rat hepatic DNA by trichloroethylene and alteration of this response by trichloroethylene pretreatments. Animals were pretreated with the indicated doses of trichloroethylene by gavage for 4 consecutive days. On the fifth day the animals were given a dose of trichloroethylene (11.4 mmol/kg) by gavage and sacrificed 4 hr later. For simplicity, control data has been expressed and statisical analysis done as described for Fig 1. Values for TCE/Tween and TCE/TCE given are the means from at least five animals ± SEM.

* Significantly different from Tween/Tween concurrent control, Tween/TCE (11.4 mmol/kg), and TCE (3.8 mmol/kg)/Tween group, $P \leq 0.05$, by ANOVA and Duncan's multiple range test.

**Significantly different from Tween/Tween concurrent control, Tween/TCE (11.4 mmol/kg) and TCE (11.4 mmol/kg)/Tween group, $P \leq 0.05$, by ANOVA and Duncan's multiple range test.

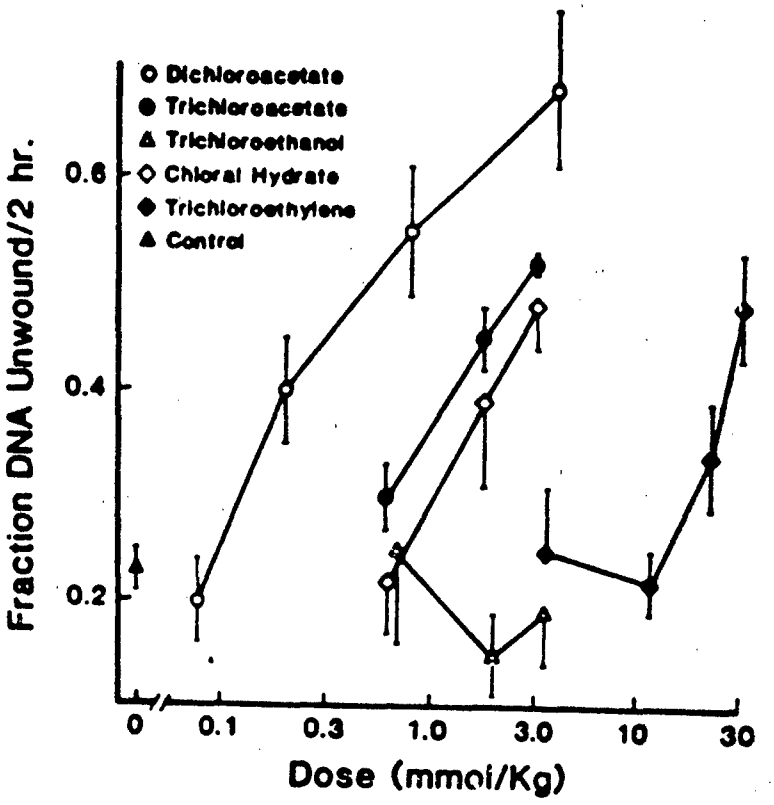


FIGURE 6. The induction of SSB in rat hepatic DNA after exposure to dichloroacetate (DCA), trichloroactate (TCA), chloral hydrate (CH) and trichloroethanol (TCOH) . The indicated doses of these metabolites were given by gavage and 4 hr later the animals were sacrificed. For simplicity, control data has been expressed and statisical analysis done as described for Fig 1. Each point represents ths mean from at least five animals ± SEM.

*Significantly different from concurrent vehicle control, $P \leq 0.05$, by ANOVA and Duncan's multiple range test.

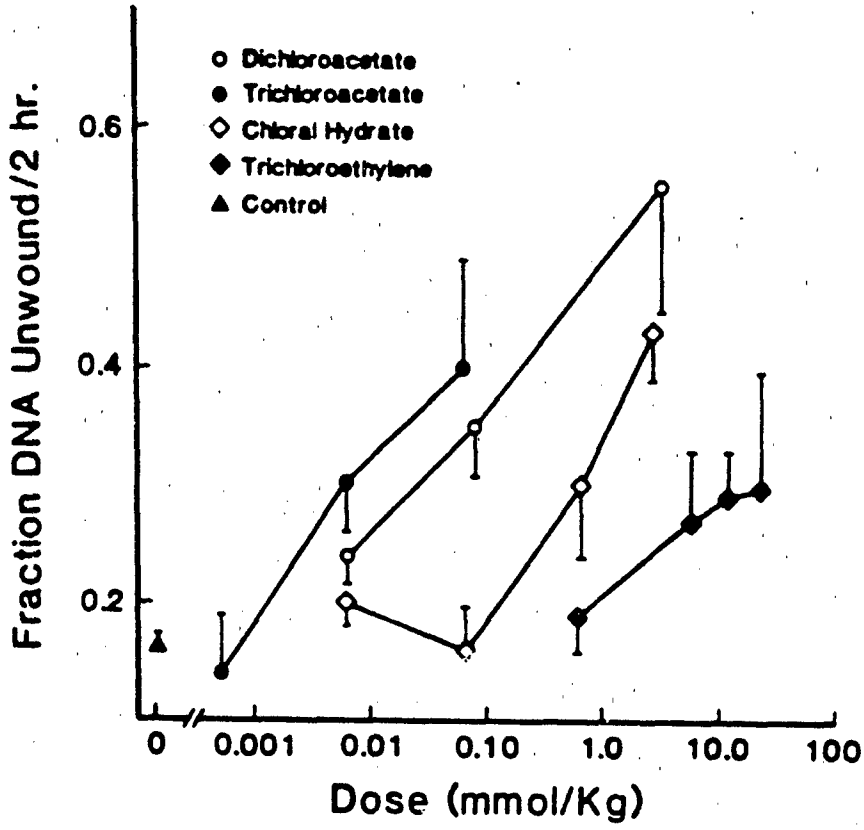


FIGURE 7. The induction of SSB in mouse hepatic DNA after exposure to dichloroacetate (DCA), trichloroacetate (TCA) and chloral hydrate (CH). The indicated doses of these metabolites were given by gavage and 4 hr later the animals were sacrificed. For simplicity, control data has been expressed and statisical analysis done as described for Fig 1. Each point represents the mean from at least five animals ± SEM.

*Significantly different from concurrent vehicle control, $P \leq 0.05$, by ANOVA and Duncan's multiple range test.

TABLE 3

EFFECTS OF TCE ON ASPARTATE AMINOTRANSFERASE (AST) AND ALANINE AMINOTRANSFERASE (ALT) ACTIVITIES IN SERUM OF RATS FOLLOWING PRETREATMENT WITH PHENOBARBITAL OR TCE[a]

Pretreatment/ Challenge Dose (mmol/kg)		AST (U/L)	ALT (U/L)
Saline/Tween	5	30 ± 6[b]	15 ± 6[b]
Phenobarbital/Tween	5	33 ± 2	14 ± 1
Tween/Tween	4	22 ± 2	3 ± 1
Saline/TCE			
(11.4)	5	36 ± 9	12 ± 2
(30.5)	5	24 ± 3	9 ± 1
Phenobarbital/TCE			
(11.4)	5	30 ± 4	13 ± 2
(30.5)	4	136 ± 3[c]	47 ± 16[c]
TCE/TCE (11.4)	5	27 ± 2	7 ± 1

[a] Male, Sprague-Dawley rats were given i.p. injections of 50 mg/kg (in saline) or 11.4 mmol/kg TCE (in 1% aqueous tween 80) per day for 4 days. Control animals received an equivalent volume of vehicle alone. Challenge doses of TCE were administred 24 hr after the last pretreatment dose. AST and ALT levels were determined 24 hr after the challenge dose of TCE.

[b] Values are expressed as means ± SEM of N animals.

[c] Significantly different from Saline/Tween and Phenobarbital/Tween groups, $P \leq 0.05$, by ANOVA and Duncan's multiple range test.

TABLE 4

EFFECTS OF DCA, TCA, TCEOH, AND CH ON ASPARTATE AMINOTRANSFERASE (AST) AND ALANINE AMINOTRANSFERASE (ALT) ACTIVITIES IN THE SERUM OF RATS[a]

Challenge Dose (mmol/kg)	N	AST (U/L)	ALT (U/L)
Tween	5	16 ± 2[bc]	5 ± 1[bc]
DCA (3.8)	4	16 ± 1	4 ± 1
TCA (3.1)	4	24 ± 7	6 ± 2
TCOH (3.3)	4	18 ± 2	3 ± 1
CH (3.4)	4	17 ± 0	2 ± 1

[a] Male Sprague-Dawley rats were administered agents by gavage in 1% tween 80 in distilled water solution. Control animals received an equivalent volume of vehicle alone. AST and ALT levels were determined 24 hr after the agents were administered.

[b] Values expressed as means ± SEM of N animals

[c] No significant differences found, $P \leq 0.05$, by ANOVA and Duncan's multiple range test.

TABLE 5

EFFECTS OF CH, DCA, TCA, AND TCE ON ASPARTATE AMINOTRANSFERASE (AST) AND ALANINE AMINOTRANSFERASE (ALT) ACTIVITIES IN THE SERUM OF MICE[a]

Challenge Dose (mmol/kg)	N	AST (U/L)	ALT (U/L)
Tween	5	23 ± 6[bc]	16 ± 4[bc]
CH (3.4)	5	18 ± 1	16 ± 4
DCA (3.9)	4	20 ± 3	15 ± 2
TCA (3.1)	5	24 ± 2	11 ± 1
TCE (11.4)	5	24 ± 3	24 ± 6

[a] Male B6C3F1 mice were administered agents by gavage in 1% Tween 80 in distilled water. Control animals equivalent volumes of vehicle. AST and ALT levels were determined 24 hr after the agents were administred.

[b] Values expressed as means ± SEM of N animals

[c] No significant differences found, $P \leq 0.05$, by ANOVA and Duncan's multiple range test.

The above results indicate that induction of SSB by TCE and its metabolites occured rather quickly, probably much more rapidly than could be accounted for by peroxisome proliferation. To confirm this the time course of induction and repair of SSB produced by DCA and TCA was examined. The data in Figure 8 show that the induction of SSB with both compounds appears to be maximal with both chemicals within one hour. The breaks were repaired rapidly such that the rates of alkaline unwinding were not significantly different from control 8 hours after treatment.

The data in Table 6 indicate that peroxisomal palmitoyl-CoA oxidation is not increased in the livers of B6C3F1 mice within the first 24 hours of administering DCA or TCA. Clearly peroxisomal proliferation does not account for the induction of SSB in DNA.

To be certain that DCA and TCA were capable of inducing peroxisome synthesis in the mice used in these studies, repeated doses were administered over a 10 day period. Clofibrate was included as a positive control. DCA increased peroxisomal palmitoyl-CoA oxidation in liver homogenates by more than 160%, and TCA increased it to 270% of control (Table 7). These increases were clearly less dramatic that was observed with clofibrate (517% of control), but consistent with reports of previous investigators (Elcombe et al., 1985; Goldsworthy and Popp, 1987).

Electron microscopic examination of frozen liver sections from the above experiment for peroxisomal profiles by revealed that DCA increased the numbers of peroxisomes by 2.4X whereas TCA increased the numbers by 2.6X (Table 7). These responses were essentially identical statistically and did not reflect the significantly smaller response of palmitoyl-CoA oxidation to DCA. However, peroxisomes from DCA-treated animals generally had poorly staining nucleoid cores, a structure associated with the oxidative activity of peroxisomes.

A final experiment was performed to determine whether the increased level of peroxisomes produced by repeated doses of TCA could itself contribute to strand breaks. The extent that DNA unwound under alkaline conditions 24 hours after the last dose of TCA was not significantly different from control (Table 8). This seems to exclude the possibility that peroxisome proliferation independently contributes to SSB in TCA-treated animals.

Chronic effects of DCA and TCA in B6C3F1 mice. When it became apparent that the induction of SSB by TCE and its metabolites were independent of peroxisome proliferation, a better characterization of the chronic effects of DCA and TCA on the liver of the B6C3F1 mice was obviously needed. A study was initiated that involved exposure of both male and female mice to 1 and 2 g of DCA or TCA/L of drinking water for 52 weeks. Five male mice were sacrificed at 15, 24, and 37 weeks to make histological examinations of the liver and to determine if foci

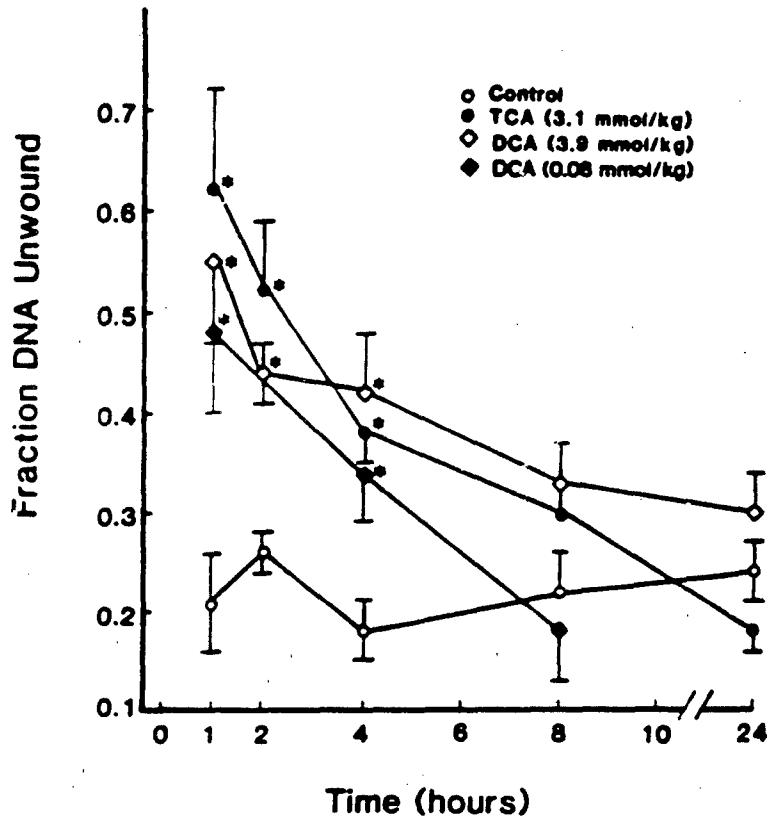


FIGURE 8. Time course for dichloroacetate (DCA) and trichloroacetate (TCA) induced single strand breaks in hepatic DNA *in vivo*. Mice were given a single oral dose of either DCA (0.08 or 3.9 mmol/kg) or TCA (3.1 mmol/kg). Controls received an equivalent volume of 1% aqueous Tween 80 vehicle. At the indicated time points, animals were killed and SSB determined. For simplicity, control animals were combined at a given time point (n=13). Each experimental point represents the mean of at least 6 animals ± SEM.

*Different from concurrent control, $p \leq 0.05$, by Student's t test.

TABLE 6

EFFECTS OF A SINGLE ORAL DOSE OF DICHLOROACETIC ACID (DCA) OR TRICHLOROACETIC ACID (TCA) ON HEPATIC PALMITOYL-CoA OXIDATION ACTIVITY OF MALE B6C3F1 MICE[a]

Sacrifice Time (hr)	N	Control	N	Treated
		DCA EXPERIMENT		
1	6	0.78 ± 0.11[bc]	6	0.68 ± 0.06[bc]
2	6	0.79 ± 0.25	6	0.79 ± 0.18
4	6	0.82 ± 0.22	6	0.50 ± 0.04
8	6	1.04 ± 0.28	6	0.94 ± 0.14
24	6	0.77 ± 0.21	6	0.67 ± 0.19
		TCA EXPERIMENT		
1	6	0.74 ± 0.12[bc]	6	0.72 ± 0.12[bc]
2	6	0.51 ± 0.07	6	0.44 ± 0.07
4	6	0.61 ± 0.05	6	0.51 ± 0.05
8	6	0.38 ± 0.07	6	0.44 ± 0.02
24	6	0.73 ± 0.08	6	0.72 ± 0.08

[a] Mice were given a single oral dose of 3.9 mmol/kg DCA or 3.1 mmol/kg TCA. Control animals received 1% aqueous Tween 80 vehicle. At the indicated time points animals were killed and palmitoyl-CoA oxidation determined in liver homogenates.

[b] Mean values expressed as umol/min/g liver ± SE of N animals.

[c] No differences found, $p \leq 0.05$, by Student's t test.

TABLE 7

EFFECTS OF REPEATED ORAL DOSES OF DICHLOROACETIC ACID (DCA) AND TRICHLOROACETIC ACID (TCA) ON BODY AND LIVER WEIGHTS OF B6C3F1 MICE[a]

Treatment (g)	N	Liver wt wt Ratio X 100	Liver wt/Body oxidation[b]	Palmitoyl-CoA unit area[a]	Peroxisomes/
Control	6	1.3 ± 0.05[c]	4.9 ± 0.14[c]	0.63 ± 0.07[c]	9.8 ± 1.2[c]
DCA	6	2.1 ± 0.10[d]	7.5 ± 0.18[d]	1.03 ± 0.09[d]	25.4 ± 2.0[d]
TCA	6	1.7 ± 0.09[de]	5.7 ± 0.14[de]	1.70 ± 0.08[de]	23.6 ±1.8[de]
Clofibrate	6	--	--	3.26 ± 0.05[def]	

a Male mice were administered DCA (3.9 mmol/kg) or TCA (3.1 mmol/kg) by gavage for 10 consecutive days. Controls received an equivalent volume of 1% aqueous Tween 80 vehicle.

b umol/min per g liver

c Mean ± SE of N animals

d Different from control, $p < 0.05$, by ANOVA and Duncan's multiple range test.

e Different from DCA, $p < 0.05$, by ANOVA and Duncan's multiple range test.

f Different from TCA, $p < 0.05$, by ANOVA and Duncan's multiple range test.

TABLE 8

EFFECTS OF REPEATED ORAL DOSES OF TRICHLOROACETIC ACID (TCA) ON HEPATIC SINGLE STRAND BREAK INDUCTION IN B6C3F1 MICE[a]

Treatment	N	Fraction Unwound[b]
Control	5	0.23 ± 0.06
TCA	5	0.25 ± 0.03[c]

[a] Male mice were administered TCA (3.1 mmol/kg, 500 mg/kg) by gavage for 10 consecutive days. Controls received an equivalent volume of 1% aqueous Tween 80 vehicle. Twenty four hours after the last dose, animals were killed and hepatic DNA SSB measured.

[b] Mean values ± SE of N animals.

[c] No differences found, $p \leq 0.05$, by Student's t test.

of cells with altered enzyme activity could be detected. A group of 11 animals receiving each treatment was removed from treatment at 37 weeks and sacrificed along with the remaining animals at 52 weeks.

Figure 9 summarizes the effect of DCA and TCA treatments on the development of hepatoproliferative lesions (hyperplastic nodules + adenomas + hepatocellular carcinomas) in male mice. The data are expressed in total dose received so that the groups of animals whose treatments were suspended after 37 weeks could be included on the graph. Both DCA and TCA increased the numbers of hepatoproliferative lesions significantly. However, the characteristics of the dose-response and the effect of suspending treatments were quite different for the two compounds. Doubling the concentrations of DCA in the drinking water from 1 to 2 g liter (i.e. increasing the total dose from approximately 400 to about 850 mmol/kg) increased the response from one that was not significantly different from control to where an average of 3.8 lesions per animal was observed. In contrast, the response to TCA was essentially linear with dose. Animals that had received DCA for 37 weeks and were sacrificed after 52 weeks responded with a yield of lesions that would be predicted if the response was proportional to total dose (i.e. the point could not be differentiated from the line statistically). Conversely, the response to TCA was significantly below (i.e. it deviated from the linear model, $P = 0.022$) the response predicted by the total dose consumed.

No hepatoproliferative lesions were identified in female mice treated with either DCA or TCA after 52 weeks of treatment. This was despite the fact that the non-neoplastic changes observed in female mice were essentially identical to that observed in males (described below). Although the numbers females employed in this study were small, significant numbers of hyperplastic or neoplastic lesions should have been observed if their sensitivity was the same as the males.

Examination of a sampling of the lesions in the livers of male B6C3F1 mice at 52 weeks of treatment revealed that 64% were simple hyperplastic nodules, 9% were adenomas and 20% were hepatocellular carcinomas (Table 9). Four of the 73 lesions examined in this sampling displayed no signs of either neoplastic or hyperplastic change. Two of these were other types of abnormalities (see footnote b on Table 11) and the remaining two had apparently normal histology. Overall this provided a 95% correlation between hepatoproliferative lesions identified at autopsy and pathological classification. Therefore, we felt it unnecessary to examine the whole population of animals to establish the tumorigenic effects of DCA and TCA in B6C3F1 mice.

Because of the limited number of animals in the groups which had treatments suspended after 37 weeks, a larger fraction of the lesions observed were sampled. This lead to the observation that none of the hepatoproliferative lesions observed in animals

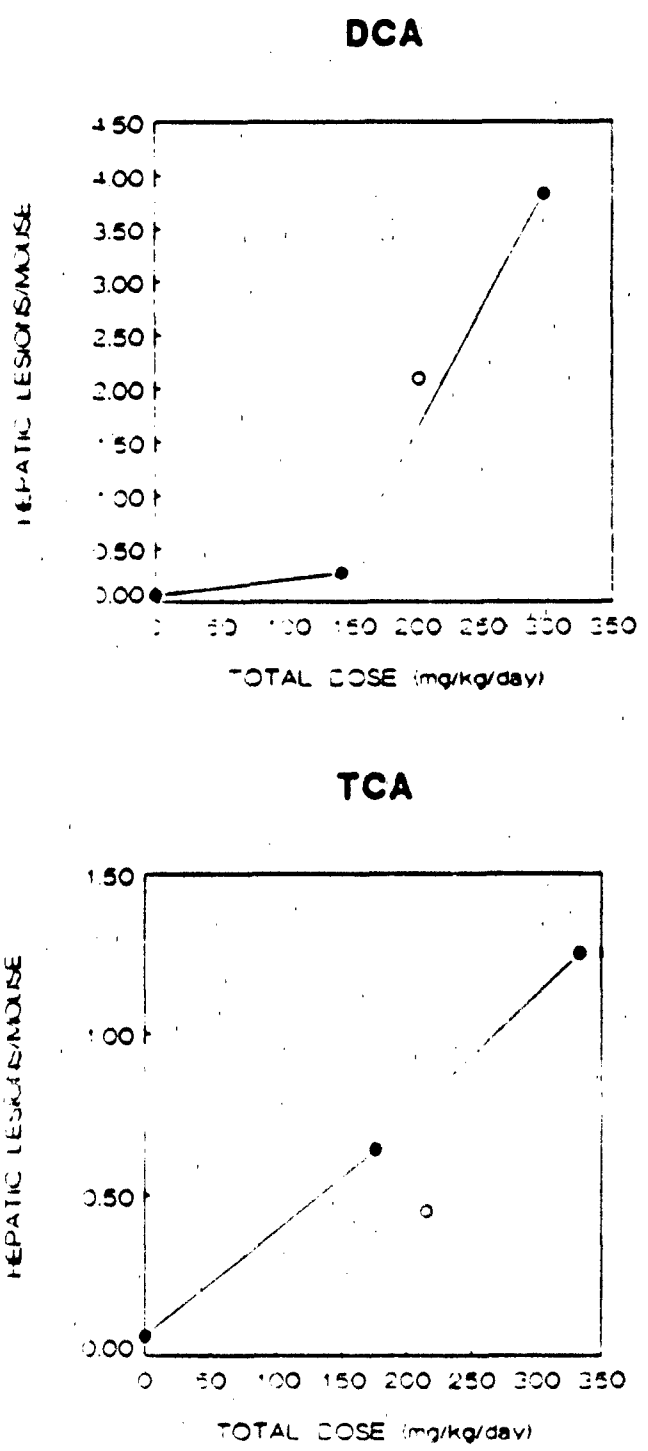


FIGURE 9. The induction of hepatoproliferative lesions in male B6C3F1 mice by dichloroacetate (DCA) and trichloroacetate (TCA). Points joined by the solid line include the animals treated for 52 weeks and the corresponding control group. The open symbol in each panel represent the tumor yield for animals whose treatment was suspended at 37 weeks and were sacrificed at 52 weeks. See Table 9 for categorization of lesions.

TABLE 9

HISTOLOGICAL CONFIRMATION OF HYPERPLASTIC AND NEOPLASTIC CHANGE IN THE LIVERS OF MALE B6C3F1 MICE EXPOSED TO DCA OR TCA IN DRINKING WATER.

Treatment[a]	N	# Mice Bearing Lesions	Total # Lesions	#Lesions Examined Histol. (# mice)	Normal[b]	Hyper-plastic nodules	Adenoma[c]	Hepato-cell. carc.[c]
Control	35	2	2	2 (2)	1 (1)	1 (1)	0	0
DCA,								
1 g/L X 52	11	2	3	1 (1)	0	1 (1)	0	0
2 g/L X 52	24	23	92	23 (10)	0	15 (9)	2 (2)	6[d] (5)
2 g/L X 37	11	7	23	19 (7)	2 (2)	15 (6)	2 (2)	0[d]
TCA,								
1 g/L X 52	11	5	7	7 (5)	0	3 (1)	2 (2)	2 (2)
2 g/L X 52	24	19	30	16 (11)	1 (1)	10 (9)	1 (1)	4 (4)
2 g/L X 37	11	4	5	5 (4)	0	2 (2)	0	3 (3)
Phenobarbital								
0.5 g/L X 52	24	0	0	0	0	0	0	0
Totals	151	63	162	73 (41)	4 (4)	47 (29)	7 (7)	15 (14

Doses were administered at the indicated concentration in the drinking water for the period of time indicated (X weeks).
The designation of normal indicates that the anomaly observed at necropsy lacked hyperplastic or neoplastic change histologically. Two of these tissues appeared histologically normal (one control and one DCA-37 weeks) one was a necrotic lesion (DCA-37 weeks) and one was an abcess (TCA-2 g/L
Adenomas and carcinomas were always found within or just adjacent to hyperplastic nodules.
Significantly different in carcinoma yield relative to total numbers of hepatoproliferative lesions when compared to animals receiving continuous treatement ($P = 0.05$).

treated with DCA for 37 weeks were hepatocellular carcinomas (0/19). This yield was significantly ($P < 0.05$) below the 24-29% yields obtained in animals that were continuously treated. Conversely, 60% of the hepatoproliferative lesions produced by TCA when treatment was suspended at 37 weeks were hepatocellular carcinomas. The total number of lesions (i.e. 5) were too small to demonstrate whether the proportion progressing to carcinoma were statistically different from the groups receiving continuous treatment. However, the relative yield of malignant tumors with TCA under these conditions was very different from the result obtained with DCA ($P << 0.01$).

DCA, TCA and phenobarbital significantly increased both absolute liver weights and liver to body weight ratios in male and female mice (Table 10) relative to control animals (data included for 37 week and terminal sacrifices only). The increases with DCA and TCA were both dose-related, but they were much more marked in DCA-treated animals throughout the treatment period. While the responses at one year appear to be somewhat greater in male than female mice, this is complicated by the appearance of multiple proliferative lesions in the males at this time point. No treatment produced significant changes in body weight or kidney weights of animals (data not shown). Suspension of treatments of DCA and TCA at 37 weeks led to substantial reversal of the effects on absolute and relative liver weights by 52 weeks. However, the liver weights in DCA-treated animals remained significantly higher than those observed in control animals. This irreversible component of DCA's effect was probably attributable to the high numbers of hyperplastic nodules in these animals.

Macroscopically, the livers of many mice receiving DCA in their drinking water displayed light colored streaks on the surface. On microscopic examination of H&E stained sections, these streaks were found to correspond to multi-focal areas of necrosis with frequent infiltration of lymphocytes (Figure 10). Such lesions are frequently observed in the interior of the liver as well and were found in varying numbers and sizes in all DCA-treated animals. These streaks were seen at every sacrifice period, suggesting that production and healing of these lesions is a continuous process in mice treated with high doses of DCA.

The necrotic lesions observed with DCA were rarely observed with TCA, the frequency being so low as to make it conjectural whether they were even treatment related. No such degenerative changes were observed in control animals or in animals receiving phenobarbital in the drinking water.

Histologically, livers from mice treated with 1 or 2 g DCA/L displayed a marked cytomegaly (Figure 11). This response was seen uniformly throughout the liver. The intracellular swelling often rendered the cord-like structure and portal triads of the liver undiscernable. Although there was some cell enlargement in TCA-treated animals, the effect was much less dramatic than was

TABLE 10

EFFECTS OF DCA AND TCA ON LIVER WEIGHT OF B6C3FI MICE

Treatment	Liver weight	
	g	% of body weight
MALES		
37 weeks		
Control	1.6 ± 0.1	4.1 ± 0.3
Phenobarbital, 0.5 g/L	2.1 ± 0.2*	6.0 ± 0.4*
DCA, 2 g/L	2.5 ± 0.1*	7.3 ± 0.2*
TCA, 2 g/L	1.9 ± 0.1*	5.1 ± 0.1*
52 weeks		
Control	1.7 ± 0.1	4.6 ± 0.1
Phenobarbital, 0.5 g/L	2.0 ± 0.1*	5.6 ± 0.2*
DCA, 1 g/L X 52[a]	2.5 ± 0.1*	6.5 ± 0.2*
2 g/L X 52	5.1 ± 0.1*	10.5 ± 0.4*
2 g/L X 37	2.2 ± 0.1*	5.7 ± 0.3*
TCA, 1 g/L X 52	2.2 ± 0.1*	6.0 ± 0.3*
2 g/L X 52	2.7 ± 0.1*	7.5 ± 0.5*
2 g/L X 37	1.9 ± 0.1	5.4 ± 0.2
FEMALES		
52 weeks		
Control	1.3 ± 0.1	4.8 ± 0.3
Phenobarbital, 0.5 g/L	1.4 ± 0.0	5.4 ± 0.2*
DCA, 2 g/L X 52	2.6 ± 0.1*	9.0 ± 0.2*
TCA, 2 g/L X 52	1.7 ± 0.1*	6.0 ± 0.3*

* Statistically different from corresponding negative control, $P < 0.05$, by ANOVA. N = 5 at 37 weeks and is either 11 or 24 for males or 10 for females at 52 weeks.

[a] Weeks of treatment if different from overall group.

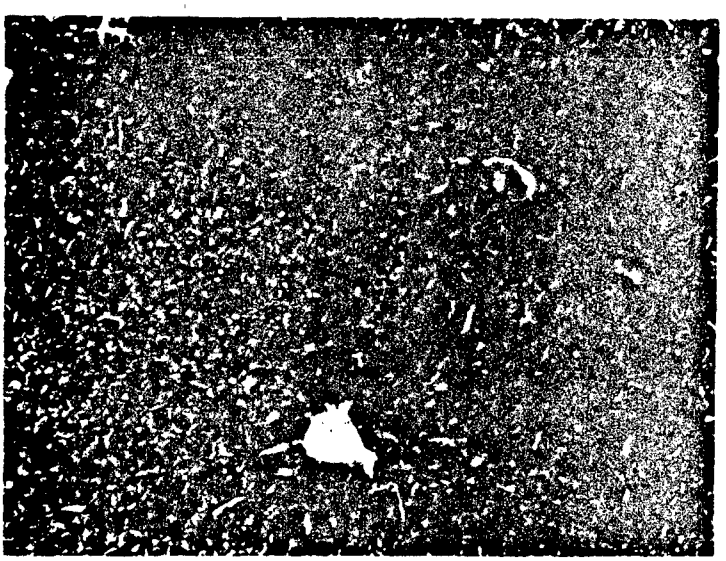


FIGURE 10. Area of coagulative necrosis produced by dichloroacetate in the B6C3F1 mouse liver (100X).

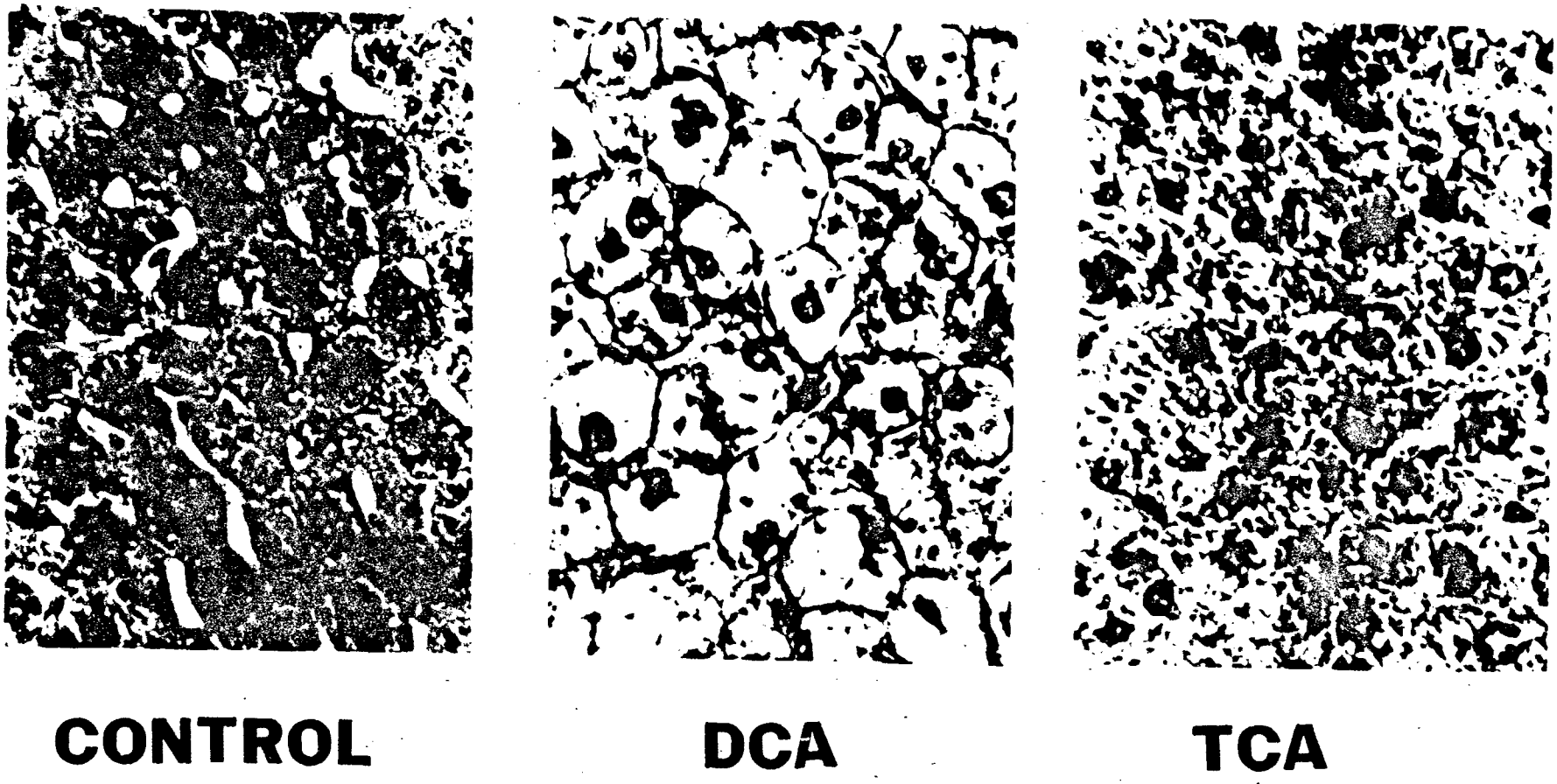


FIGURE 11. Hypertrophy produced by dichloroacetate (DCA) in B6C3F1 mice. Hematoxylin and eosin stained sections of hepatic tissue taken from mice treated for 26 weeks (400X). DCA and trichloracetate (TCA) administered at 2 g/L of drinking water.

observed with DCA. After 52 weeks of treatment the long axis of hepatocytes measured (mean + SEM) 24.9 ± 0.3, 26.1 ± 0.8, 38.5 ± 1.0 and 29.3 ± 1.4 microns in control, phenobarbital, DCA and TCA-treated animals, respectively. In parallel with effects on liver weight, the effect of DCA on cell size was largely reversible; mice that were treated for 37 weeks, then removed from treatment until sacrificed at 52 weeks had corresponding measurements of 23.5 ± 0.4, 26.8 ± 1.1 and 24.6 ± 0.4 microns in control, DCA and TCA-treated groups, respectively. PAS staining revealed that the enlarged cells observed with DCA-treatment contained very large amounts of glycogen (Figure 12). In this case, glycogen appeared distributed throughout the liver. The livers of TCA-treated animals displayed much less evidence of glycogen accumulation and it localized in periportal regions of the liver acinus.

Complete sectioning of the central lobe of livers of mice treated with DCA at a dose of 2 g/L revealed a low incidence of basophilic foci (Figure 13) of cellular alteration at the 24 and 37 week sacrifices (Table 11). These foci were not observed in TCA-treated, phenobarbital-treated or control mice. Contiguous sections stained with PAS (Figure 13) revealed that these foci were glycogen-poor relative to the neighboring tissue (which had been rendered glycogen-rich by DCA-treatment).

Attempts to identify enzyme-altered foci by staining frozen sections from other liver lobes for G6PDH, ATPase, G6Pase or GGTase were not successful. This was not surprizing because of the very low incidence of the basophilic foci in the central lobe and the fact that no more than 10 to 12 sections were examined per animal with these stains. Lesions clearly identified as tumors stained to a lesser degree than surrounding tissues for G6P and ATPase and to a greater extent for G6PDH. Staining for glycogen was markedly less in tumors taken from DCA-treated animals than in surrounding tissue. No alterations in GGTase was observed (data not shown).

Liver tissues taken from mice treated with TCA for 52 weeks had considerable, dose-related accumulations of lipofuscin (Figure 14). Mice exposed to DCA for the same period of time displayed some evidence of increased accumulation of lipofuscin relative to controls, but the amounts observed were minor in comparison with the changes seen in TCA-treated animals. It is interesting to note that lipofuscin accumulated in the highest concentrations in areas immediately surrounding hepatoproliferative lesions in TCA-treated animals, however, it was completely absent from the lesion itself. This difference was observed irrespective of whether the lesion was diagnosed as a hyperplastic nodule, adenoma or hepatocellular carcinoma. Finally, in mice whose exposure to TCA was suspended at 37 weeks, lipofuscin levels were not increased relative to controls.

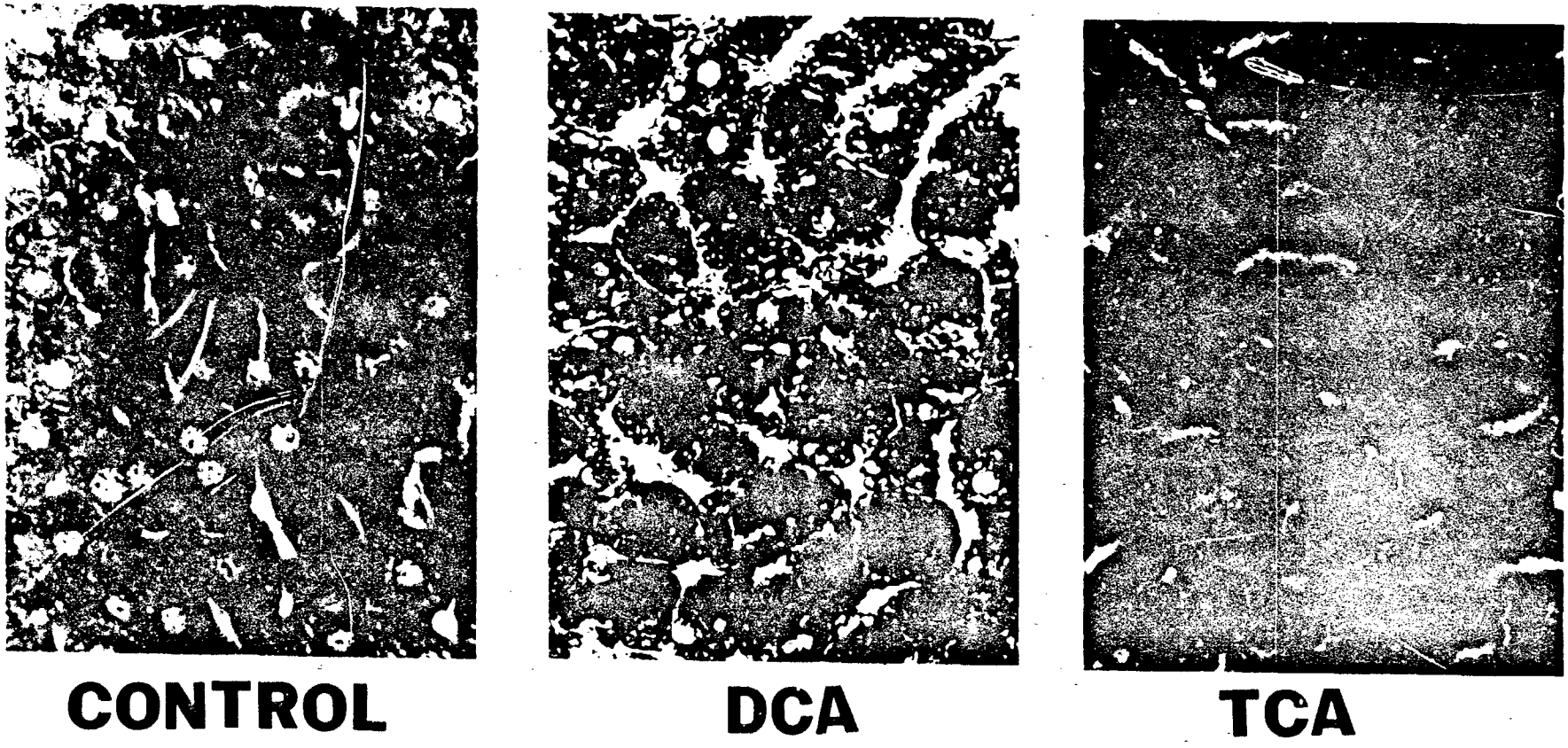
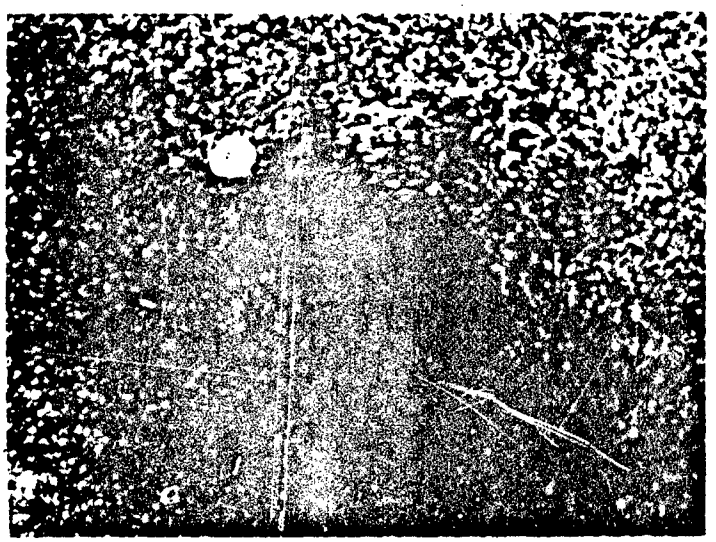
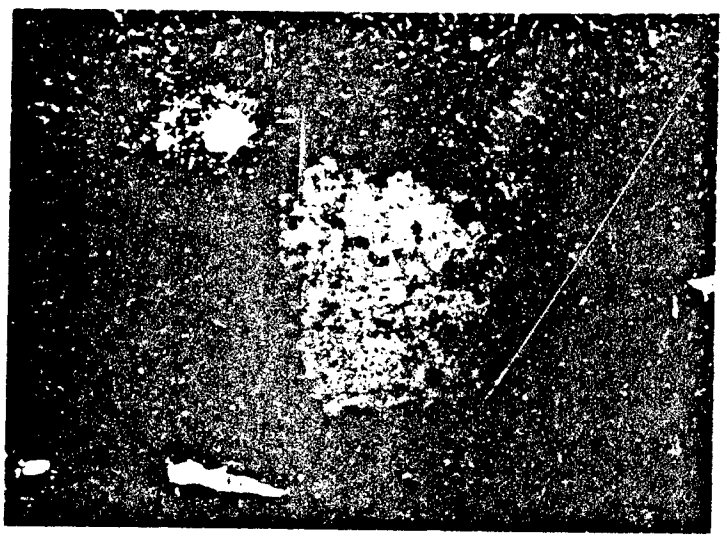


FIGURE 12. Glycogen accumulation in hepatocytes of B6C3F1 mice treated with dichloroacetate (DCA) and trichloroacetate (TCA). Periodic acid/Schiff staining of liver sections (400X) from mice receiving the following treatments for 26 weeks. DCA and TCA administered at 2 g/L of drinking water.



A



B

FIGURE 13. Basophilic and glycogen-poor (relative to surrounding tissue) focal areas observed in mice treated with dichloroacetate 2 g/L for a period of 26 weeks (40X). Hematoxylin and eosin (A) and periodic acid/Schiff stain (B).

TABLE 11

INDUCTION OF FOCI OF CELLULAR CHANGE IN THE LIVER OF MALE B6C3F1 MICE.

Treatment	24 weeks	37 weeks
	MALES	
Control		
No. of foci	0	0
No. mice with foci	0/5	0/5
Foci/central lobe	0	0
No. mice with tumors	0/5	0/5
DCA, 2 g/L		
No. of foci	4	8
No. mice with foci	3/5	5/5
Foci/central lobe	0.8	1.6
No. mice with tumors	0/5	0/5
TCA, 2 g/L		
No. of foci	0	0
No. mice with foci	0/5	0/5
Foci/lateral lobe	0	0
No. mice with tumors	0/5	1/5
Phenobarbital, 0.5 g/L		
No. of foci	0	0
No. mice with foci	0/5	0/5
Foci/lateral lobe	0	0
No. mice with tumors	0/5	0/5

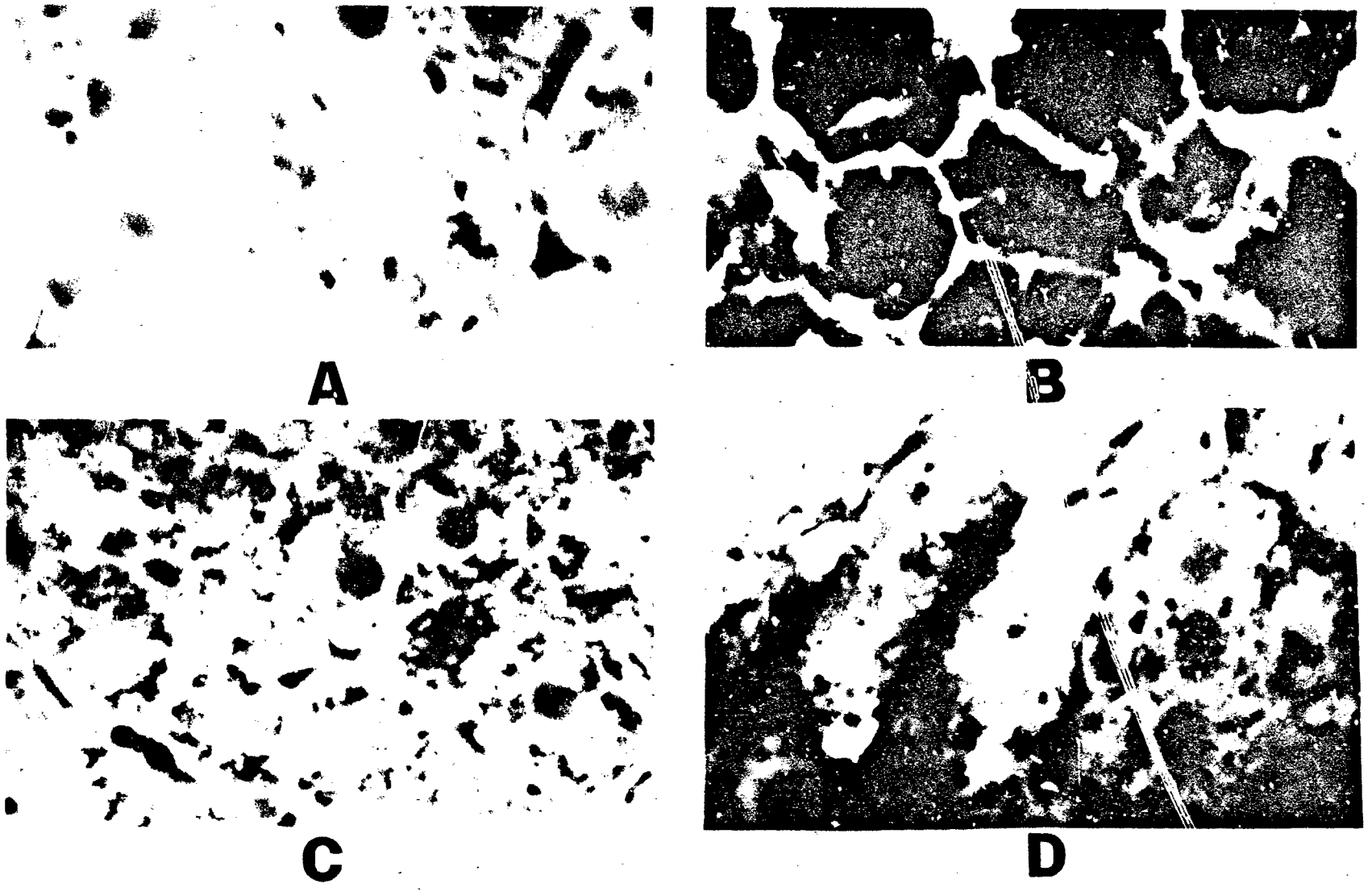


Figure 14. Accumulation of lipofuscin in non-tumorous and tumorous liver tissues of male mice treated with dichloroacetate (DCA) and trichloroacetate (TCA)) (1000X). Animals subjected to the following treatments for 52 weeks: Control, A; DCA 2 g/L, B; TCA 2 g/L, C. Panel D illustrates the sharp demarcation between normal (left) and tumorous tissue (right) taken from a mouse treated with TCA at 2 g/L for 52 weeks.

DISCUSSION

The original postulate of this study was that ethanol would shift the metabolism of TCE away from TCA and to TCOH. TCA has been shown by a number of investigators (Elcombe et al., 1985; Goldsworthy and Popp, 1987) to be a peroxisome proliferator and it was suggested that the higher conversion of TCE to TCA in mice accounted for the higher sensitivity of this species to TCE (Elcombe et al., 1985). Thus a shift in the metabolism of TCE to TCOH from TCA should reduce the hepatotoxic and hepatocarcinogenic effects of TCE. Although the increased ratio of TCOH/TCA in urine supports this hypothesis, it was not consistently reflected in the blood concentrations of TCA. Only at the highest dose was a consistent effect on TCA concentrations observed. Since the effect was observed for only the first 12 hours after dosing, it is very likely that a modification of TCE absorption by ethanol was largely responsible for this effect. As a consequence, we abandoned the notion that modification of TCE metabolism by ethanol can serve as a useful model for studying mechanisms of TCE-induced hepatotoxicity and hepatocarcinogenesis.

Subsequent experimentation did provide strong evidence that the hepatotoxic and hepatocarcinogenic activity of TCE is largely, if not entirely, attributable to DCA and TCA. This conclusion is based on the ability of smaller doses of DCA and TCA (ca. 150 mg/kg per day vs. the 1000 mg/kg per day required for TCE) to induce hepatic tumors at higher incidence and with a shorter latency (52 weeks vs. 78 weeks for TCE). Prior experience indicates that hepatocellular carcinomas increase in incidence exponentially with time ($t^{3.79}$) in male B6C3F1 mice (Portier et al., 1986).

Based simply on the fact that it is a major metabolite of TCE, TCA sould appear to be the most probable candidate to account for the hepatocarcinogenic effects of TCE in mice. DCA was immeasureable in the blood or urine of rats in the present study. However, previous investigators have demonstrated that it is a metabolite *in vivo* in mice (Hathway, 1980) and is clearly produced in *in vitro* systems (Miller and Guengerich, 1982; Dekant et al., 1986).

A major determinant of DCA's importance in the carcinogenic response to TCE is the extent to which it is further metabolized and how important this metabolism might be to the carcinogenic response. Although data is sketchy, it is relatively clear that DCA is much more rapidly metabolized than TCA (DeKant et al., 1986). Therefore, DCA could be more important to the hepatocarcinogenic effects of TCE than would be predicted from its concentrations in blood.

The data reported here indicates that DCA and TCA produce their carcinogenic effects by very different mechanisms. The curve describing the relationship between tumor induction and

dose of DCA has a markedly non-linear character, whereas the response to TCA appears to be linear.

The sharp increase in tumorigenic response to DCA is closely associated with the production of an unusual hepatomegaly not observed with TCA. It is characterized by liver weights almost twice normal, greatly enlarged cell size and large accumulations of glycogen. Most unusual was the finding that this pathology was observed throughout the liver. From the first interim sacrifice at 15 weeks and throughout the treatment period focal necrotic areas were observed. The obvious increased numbers of mitotic figures adjacent to these focal necrotic areas indicates there is a high rate of cell division surrounding these lesions. Their appearance at all interim sacrifices indicates that the production of these lesions is an ongoing process while the animals are on active treatment.

In sharp contrast to observations with DCA, TCA was seen to increase tissue levels of lipofuscin dramatically over the course of the 52 week treatment period. This is evidence that lipid peroxidation is occuring (Reddy et al., 1982). Lipid peroxidation is usually initiated by a radical produced directly by metabolism of the chemical (Recknagel, et al., 1977) or through the generation of oxygen radicals (Reddy et al., 1982). TCA has been shown to induce peroxisome synthesis (Elcombe, 1985; Goldsworthy and Popp, 1987) and it has been suggested that increased production of hydrogen peroxide by these organelles is responsible for the hepatocarcinogenicity of TCE in B6C3F1 mice (Elcombe, 1985). Present data confirm these observations with TCA and demonstrate that DCA is also capable of inducing peroxisome . However, it appears that peroxisomes synthesized in response to DCA are deficient in oxidative enzymes as indicated by the much weaker staining of the nucleoid core and ability to oxidize palmitoyl-CoA. This could account for the lesser accumulations of lipofuscin in DCA vs. TCA-treated mice. A second option is that homolytic cleavage of a chlorine-carbon bond or carbon-carbon bond in TCA could result in production of a free radical in a manner analogous to that reported for carbon tetrachloride (Recknagel et al., 1977). This would be a more probable route of metabolism for TCA than DCA and could also account for differences in lipofuscin accumulation.

Radicals, whether generated directly through metabolism of the chemical or indirectly by increasing hydrogen peroxide formation in peroxisomes, should be capable of inducing single strand (SSB) in hepatic DNA. TCE, DCA and TCA were found capable of inducing SSB in hepatic DNA of both mice and rats <u>in vivo</u>. The induction of SSB by these chemicals was independent of the induction of peroxisome proliferation. Peroxisome proliferation, itself, did not result in a higher level of SSB in hepatic DNA. If direct or indirect interactions with DNA are important to the hepatocarcinogenic responses to DCA and TCA, they appear to be independent of peroxisome proliferation.

While TCE, DCA and TCA were capable of inducing SSB in both mice and rats, the dose-response relationships in the two species were markedly different in slope. The more shallow dose-response relationship observed in mice resulted in detection of SSB at much lower doses in mice than in rats. DCA and TCA have not been extensively studied in rats. Nevertheless, they are clearly less potent in inducing hepatic tumors in rats than mice. Parnell et al. (1986) observed a small increase in the number of GGTase-positive foci in rats treated with DCA and TCA at doses of up to 5 g/L of drinking water for one year, but there was no indication of increased tumor incidence. A small number of Sprague-Dawley rats were treated in our laboratory (5 animals in each treated group and 5 in a control group) without any sign of developing hepatic tumors. Neither was there any sign of lipofuscin accumulation with TCA-treatment and only a minimal hepatomegaly was observed with DCA-treatment. Consequently, it is possible that the induction of SSB at very low doses in B6C3F1 mice is related to the hepatocarcinogenic effect in this animal.

The difference in potency of TCA to induce SSB in hepatic DNA of mice and rats is particularly notable. Whereas, TCA was considerably less potent than DCA in producing SSB in rats, TCA was actually slightly more potent in mice than in rats. Because of the linear response of tumorigenesis to TCA, it produces a greater response than DCA at lower doses. Within this narrow range, the tumorigenic response to the two chemicals parallels their ability ot induce SSB. We suggest that the responses to higher doses of DCA is complicated by the production of focal areas of necrosis and reparative hyperplasia, magnifying the tumorigenic response.

Finally, DCA and TCA were found to have very different effects on tumor progression. This was shown by the absence of hepatocellular carcinomas among the hepatoproliferative lesions (0/19) found after DCA-treatments were suspended and the very high percentage that resulted with suspension of TCA-treatment (3/5).

Interim sacrifices demonstrated that hyperplastic nodules were well established and approach the frequency observed at 52 weeks by the time DCA-treatment was suspended (37 weeks). The clonal nature of these early lesions and hyperplastic nodules seen at 52 weeks is suggested by the expression of apparently identical phenotypes. Since the number of lesions relative to total dose received indicated that remodelling had not occured, these lesions must have some of the properties of persistent nodules (Farber and Sarma, 1987). However, their ability to progress must depend on continued expansion of these clones. Thus, we believe that the activity of TCA depends primarily on stimulating the growth of hyperplastic nodules. This is supported by the close association of local necrosis and reparative hyperplasia with the development of tumors with DCA.

When TCA-treatment was suspended at 37 weeks either benign lesions that were present regressed or simply had not grown to a

size that would have been noticed on thorough histological examination. Nevertheless, it is clear that a few lesions were well established. The malignant phenotype was apparently responsible for the survival of most of the lesions when treatment was suspended. Based on these observations, we suggest that TCA affects progression of these tumors very early in their development. Its efficiency in this process is emphasized by the fact that hyperplastic nodules were not evident in TCA-treated animals at 37 weeks, thus their clonal size was much smaller than seen in animals treated for the same period with DCA.

The mechanism by which TCA might be affecting tumor progression is unclear. It is possible that it is related to its greater activity in inducing SSB in hepatic DNA of mice. However, DCA also produced SSB, but did not appear to affect progression of the lesions. Consequently, if strand breaks are involved they must differ in character significantly between the two chemicals. TCA did increase lipid peroxidation in mice, suggesting the involvement of a radical mechanism. The activity with DCA in this regard was quite marginal. TCA produced little evidence of such activity in rats, perhaps accounting for the striking difference in the induction of SSB by TCA between the two species. The higher concentrations of lipofuscin noted on the periphery of hyperplastic nodules and carcinomas suggests that cells immediately surrounding the tumors are very active in this regard. It is possible that this activity was sufficent to influence differentiation of the adjacent tumor tissue.

In summary, our results strongly implicate DCA and TCA in the hepatotoxic and hepatocarcinogenic of TCE. DCA appears to be much more hepatotoxic than TCA, producing a distinctive pathological picture. Our data suggest these hepatotoxic effects of DCA are primarily, if not entirely responsible for its carcinogenic activity in the liver of B6C3F1 mice. TCA clearly acts by a different mechanism, probably through metabolism to a radical species, or less likely, acting through its ability to induce peroxisome synthesis to produce an oxidative stress. Within the context of available data, TCA is probably the greatest contributer to the development of hepatic tumors in B6C3F1 mice.

REFERENCES

Benner, U., Hacker, H.J. and Bannasch, P. (1979) Electron microscopical demonstration of glucose-6-phosphatase in native cryostat sections fixed with glutaraldehyde through semi-permeable membranes. Histochem. 65:41-47.

CEQ (1981) Contamination of ground water with toxic chemicals. Executive Office of the President's Council on Environmental Quality, Washington, D.C..

Chayen, J., Bitensky, L.and Butcher, R.G. (1973) Practical Histochemistry. John Wiley & Sons, New York, pp. 204-209.

Crebelli, R. and Carere, A. (1989) Genetic toxicology of 1,1,2-trichloroethylene. Mutat. Res. 221:11-37.

Crump, K.S., Hoel, d.G., Langley, C.H. and Peto, R. (1976) Fundamental carcinogenic processes and their implications for low dose risk assessment. Cancer Res. 36:2973-2979.

DeAngelo, A.B., Daniel, F.B., McMillan, L., Wernsing, P. and Savage, R.E. Jr. (1989) Species and strain sensitivity to the induction of peroxisome proliferation by chloroacetic acids. Toxicol. Appl. Pharmacol. In press.

Dekant, W., Schulz, A., Metzler, M. and Henschler, D. (1986) Absorption, elimination and metabolism of trichloroethylene: A quantitative comparison between rats and mice. Xenobiotica 16:143-152.

Doolittle, D.J., Muller, G. and Scribner, H.E. (1987) The in vivo-in vitro hepatocyte assay for assessing DNA repair and DNA replication: studies in the CD-1 mouse. Food Chem. Toxicol. 25:399-405.

Duprat, P. and Gradiski, D. (1980) Cytogenetic effect of trichloroethylene in the mouse as evaluated by the micronucleus test. ICRS Med. Sci. 8:182.

Elcombe, C.R. (1985) Species differences in carcinogenicity and peroxisome proliferation due to trichloroethylene: a biochemical human hazard assessment. Arch. Toxicol. Suppl. 8:6-17.

Fahrig, R. (1977) The mammalian spot test (fellfleckentest) with mice. Arch. Toxicol. 38:87-98.

Fan, A.M. (1988) Trichloroethylene; water contamination and health risk assessment. Rev. Environ. Contamin. Toxicol. 101:55-93.

Farber, E. and Sarma, D.S.R. (1987) Hepatocarcinogenesis: A dynamic cellular perspective. Lab. Invest. 56:4-22.

Fukuda, K., Takemoto, K. and Tsuruta, H. (1983) Inhalation carcinogenicity of trichloroethylene in mice and rats. Industrial Health 21:243-254.

Goldsworthy, T.L. and Popp, J.A. (1987) Chlorinated hydrocarbon-induced peroxisomal enzyme activity in relation to species and organ carcinogenicity. Toxicol. Appl. Pharmacol. 88:225-233.

Hathway, D.E. (1980) Consideration of the evidence for mechanisms of 1,1,2-trichloroethylene metabolism, including new identification of its dichloracetic acid and trichloroacetic acid metabolites in mice. Cancer Lett. 8:263-269.

Henschler, D., Romen, W., Elsasser, H.M., Reichert, D., Eder, E. and Radwan, Z. (1980) Carcinogenicity study of trichloroethylene by longterm inhalation in three animal species. Arch. Toxicol. 43:237-248.

Herren-Freund, S.L., Pereira, M.A., Khoury, M.D. and Olson, G. (1987) The carcinogenicity of trichloroethylene and its metabolites, trichloroacetic acid and dichloroacetic acid, in mouse liver. Toxicol. Appl. Pharmacol. 90:183-189.

Humason, G. (1967) PAS and Feulgen techniques and related reactions. In: Humason (Ed.), Animal Tissue Techniques. W.H. Freeman and Co., London, pp. 303-309.

Kissane, J.M. and Robins, E. (1958). The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. J. Biol. Chem. 233:184-187.

Klaassen, C.D. and Plaa, G.L. (1967) Relative effects of various chlorinated hydrocarbons on liver and kidney function in dogs. Toxicol. Appl. Pharmacol. 10:119-131.

Land, P.C., Owen, E.L. and Linde, H.W. (1981) Morphological changes in mouse spermatozoa after exposure to inhalation anesthetics during early spermatogenesis. Anesthesiology 54:53-56.

Larson, J.L. and Bull, R.J. (1989) The effect of ethanol on the metabolism of trichloroethylene. J. Toxicol. Environ. Health In press.

Lazarow, P.B. (1981) Assay of peroxisomal B-oxidation of fatty acids. Methods Enzymol. 72:315.

Luna, L.G. (1968) Manual of Histological Staining Methods of the Armed Forces Institute of Pathology. Third edition, p. 186.

Miller, R.E. and Guengerich, F.P. (1982) Oxidation of trichloroethylene by liver microsomal cytochrome P-450: Evidence

for chlorine migration in a transition state not involving trichloroethylene oxide. Biochemistry 21:1090-1097.

Mirsalis, J.C., Tyson, C.K., Loh, E.N., Steinmetz, K.L., Bakke, J.P., Hamilton, C.N., Spak, D.K. and Spalding, J.W. (1985) Induction of hepatic cell prolideration and unscheduled DNA synthesis in mouse hepatocytes following in vivo treatment. Carcinogenesis 6:1521-1524.

Morris, S.R. and Shertzer, H.G. (1985) Rapid analysis of DNA strand breaks in soft tissues. Environ. Mut. 7:871-880.

Muller, G., Spassovski, M. and Henschler, D. (1972) Trichloroethylene exposure and trichloroethylene metabolites in urine and blood. Arch. Toxicol. 29:335-340.

Nelson, M.A. and Bull, R.J. (1988) Induction of strand breaks in DNA by trichloroethylene and metabolites in rat and mouse liver in vivo. Toxicol. Appl. Pharmacol. 94:45-54.

Nelson, M.A., Lansing, A.J., Sanchez, I.M., Bull, R.J. and Springer, D.L. (1989) Dichloroacetic acid and trichloroacetic acid-induced DNA strand breaks are independent of peroxisome proliferation. Toxicology In press.

NCI (1976) Carcinogenesis Bioassay of Trichloroethylene. NCI-CG-TR-2, DHEW Publ. No. (NIH)76-802.

NTP, (1983) NTP Technical Report on Carcinogenesis Studies of Trichloroethylene (without epichlorohydrin) in F344/N Rats and B6C3F1 Mice (Gavage Studies). NTP TR 243, NIH Publ. No. 83-1799.

NTP (1986) NTP Technical Report on the Toxicological and Carcinogenesis Studies of Trichloroethylene in Four Strains of Rat (ACI, August, Marshall, Osborne-Mendel)(Gavage Studies) NTP TR 273, NIH Publ. No. 86-2529.

Pappas, N.J. (1986) Source of increased serum aspartate and alanine aminotransferase: Cycloheximide effect on carbon tetrachloride hepatotoxicity. Clin. Chimica Acta 154:181-189.

Parchman, L.G. and Magee, P.N. (1982) Metabolism of ^{14}C-trichloroethylene to $^{14}CO_2$ and interaction of a metabolite with liver DNA in rats and mice. J. Toxicol. Env. Health 9:797-813.

Parnell, M.J., Koller, L.D., Exon, J.H. and Arnzen, J.M. (1986) Trichloroacetic acid effects on rat liver peroxisomes and enzyme-altered foci. Environ. Health Persp. 69:73-78.

Portier, C.J., Hedges, J.C. and Hoel, D.G. (1986) Age-specific models of mortality and tumor onset for historical control animals in the National Toxicology Program's carcinogenicity experiments. Cancer Res. 46:4372-4378.

Prout, M.S., Provan, W.M. and Green, T. (1985) Species differences in responses to trichloroethylene. I. Pharmacokinetics in rats and mice. Toxicol. Appl. Pharmacol. 79:389-400.

Reddy, J.K., Lalwani, N.D., Reddy, M.K. and Qureshi, S.A. (1982) Excessive accumulation of autofluorescent lipofuscin in the liver during hepatocarcinogenesis by methyl clofenapate and other hypolipidemic peroxisome proliferators. Cancer Res. 42:259-266.

Reddy, J.K. and Lalwai, N.D. (1983) Carcinogenesis by hepatic peroxisome proliferators: Evaluation of the risk of hypolipidemic drugs and industrial plasticizers to humans. CRC Crit. Rev. Toxicol. 12:1-58.

Rijhsinghani, K.S., Abrahams, C., Swerdlow, M.A., Rao, K.V.N. and Ghose, T. (1986) Induction of neoplastic lesions in the livers of $C_{57}BL \times C_3HF_1$ mice by chloral hydrate. Cancer Detect. and Prev. 9:279-288.

Rydberg, B. (1975) The rate of strand separation in alkali of DNA of irradiated mammalian cells. Radiat. Res. 61:274-287.

Rutenburg, A.M., Kim, H., Fischbein, J.M., Hanker, J.S., Wasserkrug, H.L. and Seligman, A.M. (1969) Histochemical and ultrastructural demonstration of gamma-glutamyl transpeptidase activity. J. HIstochem. Cytochem. 17:517-526.

Sbrana, I., Lascialfari, D. and Loprieno, N. (1985) TCE induces micronuclei but not chromosomal aberrations in mouse bone marrow cells. IV ICEM, Stockholm, 1985, Abstracts, p. 163.

Setaro, F. and Moreley, C.G.D. (1976) A modified fluorometric method for the determination of microgram quantities of DNA from cells or tissue cultures. Anal. Biochem. 71:313-317.

Wachstein, M. and Meisel, E. (1957) Histochemistry of hepatic phosphatase at a physiological pH with special reference to the demonstration of bile canaliculi. Am. J. Clin. Path. 27:13-23.

Walles, S.A.S. (1986) Induction of single-strand breaks in DNA of mice by tichloroethylene and tetrachloroethylene. Toxicol. Lett. 31:31-35.