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This project was aimed at determining the extent to which metabolism of trichloroethylene (TCE) to trichloroacetic acid (TCA) was responsible for its hepatotoxic and hepatocarcinogenic effects in rodents. Ethanol is to be used as a more or less selective means of decreasing the production of TCA. This information will be useful in determining whether these effects are relevant to human exposures to these chemicals.

During the first year of this project, it has been established that dichloroacetic acid (DCA), trichloroacetic acid and chloral hydrate (CH) all are capable of inducing single strand breaks in hepatic DNA of both mice and rats in vivo at much lower doses that could TCE. The mouse was found to be exceptionally sensitive to TCA in this regard, a finding that reflects the species differences in the hepatocarcinogenic effects of TCE.

Metabolism studies suggest that the production of TCA can be modified by

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(19. cont.) ethanol coadministration. At a 1:1 molar ratio, this effect is too inconsistent to depend upon for our repeated dose studies. Consequently, we are exploring the use of a 2:1 molar ratio at non-saturating doses of TCE (< 600 mg/kg doses).

Experiments conducted to evaluate the hepatoxic effects of repeated administrations of TCE metabolites have revealed that DCA produces a cytomegally that is seen uniformly throughout the liver of the mouse. This effect is characterized by massive accumulations of glycogen. It does not produce this effect in rats given even higher doses, nor is the effect duplicated completely by TCA. In addition, mice treated with DCA are displaying focal areas of change that appear preneoplastic after only 24 weeks of treatment with DCA. These results suggest that DCA may play a more important role in the hepatotoxic and hepatocarcinogenic effects of TCE than had been previously appreciated.

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ETHANOL-INDUCED CHANGES IN TRICHLOROETHENE TOXICITY

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INTRODUCTION

Trichloroethylene (TCE) is a industrial solvent that is used widely for degreasing of metallic surfaces. This use has made TCE a common chemical of occupational exposure within the Air Force and a frequent contaminant of ground waters in the vicinity of Air Force Bases. Regulation of TCE by civilian agencies (e.g. USEPA) has focused on its carcinogenic properties, although it produces a variety of other toxicological effects, most notably hepatotoxicity.

The designation of TCE as a carcinogen has been very controversial primarily because of it only appears to produce tumors in mice (NCI, 1976; NTP, 1983; NTP, 1985; Henschler et al, 1980; Fukuda et al., 1983) despite having been tested in seven species of rat. TCE is weakly mutagenic at best (Bull, 1985). As a result there has been considerable debate concerning potential mechanisms by which TCE produces tumors. A number of hypotheses have been developed around the observation that TCE is metabolized less extensively in the rat than the mouse <u>and</u> the fact that metabolic conversion of TCE is saturated at lower doses in the rat than the mouse (Prout et al., 1985).

It has been recently shown that trichloroethylene is capable of inducing proliferation of peroxisomes in the liver of mice, but it is less effective in inducing this effect in rats (Elcombe, 1985). This property of TCE is shared by trichloroacetic acid (TCA), a major metabolite of TCE (Green and Prout, 1985; Prout et al., 1985). TCA has been reported as capable of inducing peroxisomes in both mice and rats (Elcombe, 1985). Since peroxisome proliferation has been associated with a variety of other compounds that are hepatocarcinogenic in rodents, but which either lacked or possessed very minimal genotoxic activity (Reddy et al., 1983), attention has recently focussed on this as a potential explanation for the species specific nature of TCE's carcinogenic effects.

There are a number of steps that are common to the metabolism of ethanol and TCE. This led us to propose that interactions between the toxicological effects of ethanol might provide useful insights into the mechanisms involved in the hepatotoxic and hepatocarcinogenic effects of TCE. While the question of the interactions in the toxicology of ethanol and TCE are still of interest, some of our recent work has cast doubt on the current hypotheses that attempt to attribute the hepatotoxic and hepatocarcinogenic effects simply to trichloroacetic acid. This report includes data supplied in the following forms:

1. A paper, "Induction of DNA-strand breaks by trichloroethylene in rat and mouse liver <u>in vivo</u>."

recently submitted to Toxicology and Applied Toxicology.

2. A progress report on the work describing the effect of dose on the metabolism of TCE and the modifications induced in TCE metabolism by ethanol in the rat.

3. A report on work directed at identifying preneoplastic lesions in the mouse liver. This will include some preliminary data which suggests that dichloroacetic acid is a potent hepatoxin and hepatocarcinogen in mice.

RESULTS

Induction of single-strand breaks in hepatic DNA. Most of the data concerning the ability of TCE or its metabolites to produce single strand breaks (SSB) *in vivo* are included in the accompanying manuscript. Therefore, the data will only be summarized and discussed as they pertain to the further development of the project. It is important to note that measurement of SSB in these experiments was done 4 hours after the administration of TCE or its metabolites. This becomes important in interpretation of our results to date.

TCE was found to induce SSB in hepatic DNA of rats at high doses (e.g. in the range of 20-30 mmol or 3-4 g/kg body weight). In mice, the dose required was still high, but considerably lower than required in rats (e.g. 6 mmol or 0.75 g/kg).

As reported previously, pretreatment of rats with phenobarbital or low doses of TCE (below those required to induce SSB), decreased the subsequent (24 h after the last pretreatment) challenge doses of TCE required to induce SSB. Ethanol pretreatments did increase the rate of DNA unwinding, but not significantly. These data suggest that TCE requires metabolism through a phenobarbital and TCE-inducible pathway to produce SSB. Ethanol-sensitive pathways appear to be less involved, although a complex interaction between bioactivation and detoxification steps (e.g. phase I and II systems) cannot be ruled out at this time.

We have subsequently examined the ability of stable metabolites of TCE to induce SSB in hepatic DNA under similar circumstances. In the rat, dichloroacetic acid, trichloroacetic acid and trichloroacetaldehyde (chloral) were all capable of inducing SSB. Trichloroethanol was inactive. In mice, the same three compounds produced SSB. To compare the responses between the two species we provide an estimate (obtained graphically) of the dose of each metabolite that was required to increase the fraction of DNA

unwound in 2 hours to 0.4. The comparison of different metabolites on this basis is provided in Table 1. In general, most metabolites of TCE were somewhat more potent in B6C3F1 mice than in Sprague-Dawley rats. The most striking difference is the much higher potency activity of trichloroacetic acid in mice relative to rats. Approximately 1/20th of the dose is required to produce equivalent amounts of DNA damage in mice relative to rats.

Studies of TCE metabolism and its modification by coadministration of ethanol have progressed very rapidly in the past few months. We have examined the influence of using doses which apparently saturate the capability for metabolizing TCE vs. non-saturating doses. These data suggest that we need to evaluate an additional lower dose of TCE and to examine the influence of a larger dose of ethanol to TCE ratio in the dosing. Consequently, the data and the conclusions drawn from it this report should be considered preliminary.

Figures 1 through 5 display the differences in the time of appearance and disappearance of TCE and its three major metabolites when TCE is administered at saturating (22.8 mmol or 3.0 g/kg) or non-saturating (4.6 mmol or 0.6 g/kg) doses to the rat. In Figure 1 it is apparent that the peak levels of TCE in blood are not simply dependent upon dose since the concentrations in blood at the high dose were less than two-fold those observed at the low dose, despite the fact that the dose increased by five-fold. On the other hand, the concentration vs. time curve indicates that the area under the curve (AUC) is roughly proportional to the dose of TCE, increasing by a factor of 6.3 (Table 2).

The peak concentrations of the metabolites of TCE in blood were delayed and their persistance in the blood (Figures 2 to 5) appeared to depend largely upon the prolonged period of time that TCE was systemically available. In most cases the terminal elimination curves did not differ significantly at the two doses of TCE, suggesting that excretion of the metabolites was not rate limiting.

As might be surmized from the above data, the AUC for the metabolites of TCE were increased by the higher dose of TCE. However, this increase was not in direct proportion to the dose nor the AUC observed for TCE. In Table 2 it can be seen that increasing the dose of TCE from 0.6 to 3.0 g/kg increased the AUC of trichloroacetaldehyde and trichloroacetic acid by a factor of 2.3 and trichloroethanol by 3.0. The AUC of dichloroacetic acid was increased to the smallest extent, by a factor of 1.9 with the five-fold increase in dose.

In Table 3 the effect of the two doses of TCE on the amount of each metabolite that appears in the urine provides further perspective. Aside from the possibility of further metabolism, urinary excretion is probably the only significant route of excretion for the three metabolites, trichloroethanol (free alcohol and glucuronide values are combined in the table), dichloroacetic acid and trichloroacetic acid. The total elimination of trichloroethanol, including its glucuronide (determined by hydrolysis with beta-glucuronidase) was increased by a factor of only 1.4 with the five-fold increase in the dose of TCE. Conversely, the total urinary excretion of trichloroacetic acid was increased by a factor of 9.3, to the point that it is accounting for 3% of the total dose of TCE compared to 1.6% at the low dose. Dichloroacetic acid elimination was increased by a factor of only 3.1. Despite the varying effects on individual metabolites, it is clear that the proportion of trichloroethylene that is metabolized to products that are excreted in the urine is substantially reduced at the higher dose as well. Only 8.3% of the dose could be accounted for in these three compounds at the high dose, whereas 19.9% was found at the low dose.

The effects of ethanol coadministration on TCE metabolism has led to equivocal results. Consequently, we have not included in the report. In summary, we have found that concentrations of trichloroacetic acid in blood are decreased with ethanol coadministration, but these differences are not statistically significant. At the low dose of TCE, but not at the high dose trichloroethanol levels also appear to be increased. Again the data are too inconsistent to allow firm conclusions one way or the other. A similar trend is observed when these metabolites are measured in urine. The magnitude of the differences are too large to be satisfied that there is no effect. Therefore, we intend to pursue this issue until values converge or statistically significant results are obtained. One factor may be that we have only used a 1:1 molar ratio of ethanol. We intend to increase this ratio to 2 in our next experimental series to see if a more consistent effect can be produced.

Since the hypotheses we were pursuing is that trichloroacetic acid was involved in the hepatocarcinogenic and hepatotoxic effects of trichloroethylene, we began some long term studies to establish whether histological changes could be produced in the liver with trichloroacetic acid. Dichloroacetic acid has also been investigated because it is a minor metabolite of trichloroethylene and is closely related to trichloroacetic acid. It is important to realize that it is produced by a pathway which is relatively independent of the pathway that gives rise to trichloroacetic acid. This pathway does not involve an aldehyde intermediate and should thus be less susceptible to

modification by ethanol. Neonatal rats were initiated with diethylnitrosamine. When they were weaned (21 days of age) they were placed on drinking water containing 5 g/L dichloroacetic or trichloroacetic acids. Other animals received 500 mg/L phenobarbital as a positive control and a fourth group received distilled water as drinking water. Control groups that received no diethylnitrosamine were also included. Compounds that are peroxisome proliferators do not produce foci with the same phenotypes as is observed with phenobarbital promoted foci. Consequently, these experiments are meant primarily to provide tissue that contain enzyme altered foci with dichloroacetic and trichloroacetic acid so that their staining characteristics could be confirmed, so a minimal number of animals were involved. No sacrifices of these animals have yet been conducted.

A similar, but more complete study was begun in mice (40 animals per group), except the animals received no prior initiation with diethylnitrosamine. This latter approach was taken because of a preliminary report which indicated dichloroacetic and trichloroacetic acids were capable of producing hepatic tumors in B6C3F1 mice (Herren-Freund et al., 1987). Our intention is to sacrifice 5 animals with each treatment at approximately 15, 24, 36, 52 week intervals and to leave the remaining animals for overt tumor development. The data referred to here are taken from the first two sacrifice periods. Considering the results that were obtained at these time points we have begun an als on two lower doses so that dose-response relationships can be defined. The groups in the mouse study include the following:

Control - distilled water	
Dichloroacetic acid	- 1 g/L of drinking water
	2 "
Trichloroacetic acid	- 1 "
	2 "
Phenobarbital	- 0.5 "

Upon sacrifice, the most remarkable finding with dichloroacetic acid is the marked liver enlargement. At the single dose (2 g/L) for which data is currently available, liver weight as a percentage of the body weight is almost doubled (Table 4). A smaller increase is seen with 2 g/L trichloroacetic acid, but its effects are comparable to that observed with 0.5 g/L phenobarbital. The effects of both compounds is observed after both 15 and 24 months on study.

Histologically, livers from dichloroacetic acid show marked changes. The most prominent and consistent finding was the marked enlargement of liver parenchymal cells. This hypertrophy was associated with a marked accumulation of glycogen as observed with a periodic acid/

Schiff stain (Humason, 1967) which will be discussed later.

At the 15 week sacrifice we noted a number of areas of focal staining for glucose-6-phosphate dehydrogenase (G6PDH) under oxygen. Alerted by the frequent association of this staining with what appeared to be locally infarcted areas on the frozen sections, we asked for assistance from Dr. Chuck Leathers, a veterinary pathologist. At his suggestion we investigated whether the local staining could be attributed to infiltrating lymphocytes. In figure 6 a frozen section of liver taken from an animal receiving dichloroacetic acid shows areas of positive staining for G6PDH. A contiguous section is shown in figure 7 that is stained for G6P (Benner et al., 1979) displays no corresponding area of staining. Examination of an adjoining section stained with H&E (figure 8, section contains some artifact because stain applied to frozen rather than fixed tissue), it is clear that the cells which are staining positive are not hepatocytes. Figures 9 and 10 simply show that cells with similar properties are easily identifiable in the spleen. These splenic cells did not stain for G6P (data not shown). The cells which stain for G6PDH are clearly leucocytes. In our view these data show that caution must be exercised in identifying areas of staining as being indicative of altered phenotype in liver parenchymal cells.

In addition to the G6PDH stain, frozen sections from the 15 and 24 month sacrifices have been stained for gamma glutamyl transferase (GGT; Rutenburg et al., 1968), canalicular ATPase (Wachstein and Meisel, 1957) and glucose-6-phosphatase (G6P; Benner et al., 1979) in frozen sections. To this point in time no foci have been identified using these stains. This is not necessarily contradictory with results with H&E staining, since these measurements have been made on random sections, whereas the H&E sections were done exhaustively from paraffin-embedded blocks. Thus, it is entirely possible that these lesions observed with H&E staining would stain for one or more of these histochemical markers.

Based on our experience at the 15 week sacrifice, we have sectioned completely through one lobe of the liver from each animal and stained every 20th section with hematoxylin and eosin (H & E). This was done in part to be certain that we were not missing some changes that would not be apparent in the frozen sections. In the dichloroacetic acid-treated animals areas of densely packed cells that appear to be mitotically active (Figure 11) were observed in 3 of 5 animals. Such lesions have not been observed in control, trichloroacetic acid or phenobarbital-treated animals. This section also makes clear the cellular hypertrophy that is induced by dichloroacetic acid (i.e. compare to normal areas in other H & E sections provided). Figure 12 staining for glycogen in a section contiguous to the section in figure 11

and illustrates the accumulation of glycogen in hypertrophied cells. These figures also illustrate that the focal area observed in figure 11 is glycogen poor compared to the surrounding cells.

A summary of the results of this sectioning is provided in Table 5. Essentially we observed that there are a number of preneoplastic lesions becoming apparent in dichloroacetic acid treated animals. While numbers of animals are too small to come to firm conclusions, there is a reasonably clear trend in the appearance of these lesions. Three of the five animals displayed these lesions in the dichloroacetic acid-treated groups whereas no evidence of these lesions has been observed in control animals or in those groups treated with trichloroacetic acid or phenobarbital.

DISCUSSION

The ramifications of our results in terms of identifying mechanisms by which TCE produces hepatotoxic and hepatocarcinogenic effects are not altogether clear. The ability of TCE and its metabolites to induce SSB in hepatic DNA may well be related to both hepatotoxic and hepatocarcinogenic effects, but this remains to be established. At present all that can be concluded is that the activity of these metabolites in mice and rats is consistent with the rleative sensitivity of these two species to the hepatocarcinogenic effects of TCE. In addition, the nature of the metabolites that produce this effect (particularly dichloroacetic and trichloroacetic acids) raises questions as to whether SSB are directly induced in hepatic DNA or whether a indirect mechanism is involved (e.g. inhibition of repair).

If the induction of SSB is involved in the hepatocarcinogenic effects of TCE, the present data indicate that the effects of TCE may not be mediated through reactive intermediates such as trichloroethylene oxide or dichloroacetyl chloride as has been suggested by other investigators (Henschler, 1977; Miller and Guengerich, 1983; Mathway, 1980). It is more consistent with the hypotheses put forward by Elcombe (1985) that trichloroacetic acid may be responsible for these effects. However, our data is inconsistent with the notion that SSB induced by TCE or its metabolites are secondary to the induction of peroxisome synthesis. It requires days to weeks for peroxisome proliferators to produce a maximal response, whereas our data show SSB to occur at four hours. In addition, the increased numbers of SSB appears to largely disappear within 24 hours. Work now underway indicates that the peak levels of SSB actually occurr within the first hour after administration of dichloroacetic acid, at least.

The results presented here with respect to the metabolism of doses of TCE that saturate metabolic affect TCE metabolism in a very complex way. It is clear from these results that measurements of the concentrations of TCE or its metabolites at one time point in blood or urine give a false impression. The modest increase in the concentration of TCE in blood (1.6 times) with a five-fold increase in dose was somewhat surprizing. The basis of this observation remains to be established. It is reasonable to assume that the damping of peak TCE concentrations is related to the partitioning of TCE into body fat. This would be consistent with the greatly extended period for which TCE remains at high levels in blood at the high dose. Essentially, fat could served as high capacity reservoir for TCE that would maintain blood levels over time. Metabolic or other excretory possibilities could not account for this behavior. The only other plausible explanation would be saturable mechanism for TCE absorption, a concept for which there is essentially no support.

Undoubtedly, the dampened peak concentrations of TCE and its persistance in blood at the high dose plays an important role in a similar dampening of peak concentrations of its metabolites in blood, their persistance in blood and prolonged excretion in urine. There also was an marked decrease in the overall metabolism of TCE at the high dose relative to the low dose. However, the decrease in the AUC for individual metabolites was not simply related to the overall decrease in metabolism. In the case of dichloroacetic acid, the AUC was increased by a factor of 1.9, but the amount excreted was increased by a factor of 3.0 when the dose of TCE was increased five-fold. The same circumstance resulted in a urinary excretion of trichloroethanol plus its glucuronide of only 1.4 fold, but the AUC for free trichloroethanol in blood increased by 2.3. The most interesting metabolite was trichloroacetic acid because its overall production as measured by urinary excretion actually increased by 9.3-fold, almost twice that which would have been expected from the five-fold increase in dose. On the other hand, its AUC was increased by only 2.3-fold.

Examination of the urinary metabolites of TCE provides a basis for speculating about processes that might be controlling changes in TCE metabolism at saturating doses. The conversion of TCE to trichloroethanol is inhibited at high doses to a much greater extent than the other metabolites. This cannot be simply attributed to saturation of cytochrome P450 dependent oxidations because trichloroacetic acid is derived from the same intermediate as trichloroethanol (i.e. trichloroacetaldehyde) and its production is actually increased relative to the dose administered. We suggest that inhibition of glucuronidation

of trichloroethanol occurs, probably as a result of depleted UDPGA stores. The smaller renal clearance of free trichloroethanol would favor oxidation of trichloroacetaldehyde to trichloroacetic acid which is more readily cleared by the kidney in an unconjugated form.

Prior studies of TCE metabolism at high doses have often focused on measurements of TCE or its metabolites in blood or urine at one point in time (Buben and O'Flaherty 1985; Green and Prout, 1985; Prout et al., 1985; D'Souza et al., 1985; Rouiase and Chakrabarti, 1986). Such comparisons would tend to exagerate the importance of differences in metabolism as being important in the toxicological effects of TCE, particularly if such measurements were confined to the first 24 hours after exposure. Consideration of the AUCs of these metabolites indicates that the exposure is not as different from what would have been predicted by the increase in dose. Finally, it is important to realize that the relationship between the AUC and the toxic and carcinogenic effects of TCE will depend substantially on the mechanism by which TCE induces these effects. Genotoxic effects directly exerted by TCE or one of its metabolites may be directly proportional to the AUC. A linear relationship between AUC and toxicity mediated through other mechanisms would not necessarily be expected if the effect was reversible.

The investigation of the effects of dichloroacetic and trichloroacetic acids on liver histology was undertaken to obtain a more realistic assessment of whether trichloroacetic acid could account for hepatotoxic and hepatocarcinogenic effects postulated by Elcombe (1985). Involvement of trichloroacetic acid in these responses is important to our hypothesized interactions with ethanol. Our original proposal was built around the notion that alterations in the production of trichloroacetic acid from trichloroethylene with ethanol would provide direct information as to its involvement in the hepatotoxic and hepatocarcinogenic effects of TCE. Our data on induction of strand breaks, particularly the large difference in potency between trichloroacetic acid in mice vs. rats, suggests that the hypothesis is reasonable. Our histological data (as preliminary as it is) and metabolic data cast some doubt as to whether the effects can be attributed specifically to trichloroacetic acid. They indicate that we must more specifically consider the role of dichloroacetic acid in these responses. Our metabolic data indicates that if there is an effect on trichloroacetic acid formation by ethanol (data is still inconclusive as described above), it will also effects dichloroacetic acid to roughly the same extent. If this trend is confirmed with futher experimentation, we will have to resort to more specific inhibitors of trichloroacetic acid formation (e.g. inhibitors of aldehyde dehydrogenase) to isolate its contribution to the

hepatotoxic and hepatocarcinogenic effects of TCE. The histological data strongly suggests that the potency of dichloroacetic acid as a hepatotoxin and possibly as a hepatocarcinogen may be considerably greater than for trichloroacetic acid in the mouse despite its greater ability to produce single strand breaks in this species. This reinforces the need to proceed systematically in this regard.

WORK PLAN FOR YEAR 2 AND BEYOND

It is our intent to continue pursuit of the general aims of our original proposal. Those were to determine what role trichloroacetic acid plays in the hepatotoxic and carcinogenic effects of trichloroethylene and to investigate the importance of peroxisome proliferation to these responses. These questions have practical as well as scientific implications. There is considerable amount of controversy over whether a chemical such as trichloroethylene should be regulated as a carcinogen. This is in part due to the fact that it has not been possible to show that it is carcinogenic in species other than mice. Because of its limited genotoxic activity there is also considerable question as to the mechanism by which trichloroethylene induces cancer even in mice. If its effects are mediated through a mechanism such as peroxisome proliferation, it is clear that no additional risk for cancer would exist at doses below those inducing peroxisome proliferation. If such was the case, then extrapolation of risks to low doses should not rely on linearized models of risk. Consequently, pinpointing the mechanism that is responsible can affect estimations of what are hazardous exposures by orders of magnitude.

What may have to change in our work is the method by which we pursue these questions. While it is premature to state this for certain, our data suggest 1) that the use of ethanol may not be the appropriate to isolate the role of trichloroacetic acid in these responses and 2) dichloroacetic acid may play a more important role in the responses trichloroethylene than has been previously appreciated. A logical modification in our experimental design to address point #1 would be to use more specific inhibitors of aldehyde dehydrogenase. This should decrease the formation of trichloroacetic acid, but should have little effect on the formation of dichloroacetic acid from its precursor dichloroacetyl chloride. The second question must be addressed by developing comparative dose-response information on the hepatotoxic and hepatocarcinogenic effects of dichloroacetic and trichloroacetic acids. As has been indicated above such studies are underway.

Therefore, over the next year our work will concentrate on the following research questions:

1. Confirm the non-linear relationships in TCE metabolism by including another, lower dose (e.g. 0.12 g/kg).

2. Extend the metabolism work to B6C3F1 mice as described in the original proposal.

3. Determine whether ethanol specifically modifies metabolism of trichloroethylene to trichloroacetic acid at less than saturating doses of both compounds. Work will include a higher ethanol to trichloroethylene ratio than the 1:1 molar ratio used to date.

4. Determine whether covalent binding of TCE and its metabolites change when trichloroethylene metabolism to trichloroacetic acid is modified with ethanol or inhibitors of aldehyde dehydrogenase (see #5). These experiments will be performed using ^{14}C-labelled trichloroethylene.

5. If ethanol proves not to be suitable as a relatively specific modifier of trichloroethylene metabolism, we will investigate the use of aldehyde dehydrogenase inhibitors such as Disulfiram to see whether the formation of trichloroacetic acid from trichloroethylene can be more specifically modified.

6. We will continue the ongoing experiments to produce tumors in B6C3F1 mice with trichloroacetic acid and trichloroacetic acid. This will allow their histochemical staining properties to be defined and validated for use as markers of preneoplastic change. This is needed to conduct interaction studies for periods of time less than that required for overt tumor development (e.g. months rather than years). At this point in time glycogen depletion and H&E stains are very promising. Based upon previous work of Herren-Freund et al. (1987) these look like they will be relatively low frequency lesions, and all may have a high liklihood of going all the way to hepatocellular carcinoma. This is attractive from a predictive point of view, but the low frequency precludes the use of random sections of the liver to demonstrate the lesion and may make it difficult to operate with an experimental period of less than 9 months. Such a long period will limit the amount of interaction work that can actually be done.

7. Assuming that our metabolic work provides data supporting a relatively straight-forward interaction study can be conducted with ethanol, we anticipate that experiment beginning about the first of April. If it is necessary to go to alternate means of modifying

trichloroacetic acid production, these studies would not be initiated until the first of June to allow working out of doses of aldehyde dehydrogenase inhibitors to be based on solid metabolic data.

8. We will attempt to clearly dissociate the effects of trichloroethylene and its metabolites for inducing strand breaks from peroxisome proliferation. Our data indicate that single-strand breaks occur before the liver is able to increase beta-oxidation of fatty acids. A time course for induction of SSB will be compared to the time course of increased capability for peroxisomal beta oxidation early in the second year. The assays are in hand and the positive control experiments with Clofibrate in B6C3F1 mice have demonstrated induction of peroxisome synthesis.

REFERENCES

Benner, U. et al., (1979) Histochem. 65:41-47.

Buben, J. A. and O'Flaherty, E. J. (1985) Toxicol. Appl. Pharmacol. 78:105-122.

Bull, R. J. (1985) Sci. Total Environment 47:385-413.

Chayen, J. et al. (1973) Practical Histochemistry. John Wiley and Sons, New York.

D'Souza, R. W., Bruckner, J. V. and Feldman, S. (1985) J. Toxicol. Environ. Health 15:587-601.

Elcombe, C. R. (1985) Arch. Toxicol., Suppl. 8:6-17.

Fukuda, K., Takemoto, K. and Tsuruta, H. (1983) Industr. Hyg. 21:243-254.

Green, T. and Prout, M. (1985) Toxicol. Appl. Pharmacol. 79:401-411.

Hathway, D. E. (1980) Cancer Letters 8:263-269.

Henschler, D., Romen, W., Elsasser, H. M., Reichert, D., Eder, E. and Radiran, Z. (1980) Arch. Toxicol. 43:237-248.

Herren-Fruend, S. L., Pereira, M. A. and Olson, G. (1987) The carcinogenicity of trichloroethylene and its metabolites. trichloroacetic acid and dichloroacetic acid, in mouse liver. Submitted to Toxicol. Appl. Pharmacol.

Humason, G. L. (1967) Animal Tissue Techniques. Walter Freeman & Co.

Miller, R. E. and Guengerich, F. P. (1983) Cancer Res. 43:1145-1152.

NCI (1976) Carcinogenesis bioassay of trichloroethylene National Cancer Institute Carcinogenesis Technical Report Series NCI-CG-TR-2. 76-02.

NTP (1983) National Toxicology Program technical report on the carcinogenesis bioassy of trichloroethylene in F344/N rats and B6C3F1/N mice. NIH publ. no. 83-1799, National Toxicology Program, Research Triangle Park, NC

NTP (1985) National Toxicology Draft Report on the carcinogenicity of trichloroethylene in several strains of rat.

Prout, M. S., Provan, W. M. and Green, T. (1985) Toxicol. Appl. Pharmacol. 79:389-400.

Reddy, J. K. and Lalwani, N. D. (1983) CRC Critical Rev. Toxicol. 12:1-58.

Rouisse, L. and Chakrabarti, S. K. (1986) Environ. Res. 40:450-458.

Rutenburg, A. M. et al. (1968) J. Histochem. Cytochem. 17:517-526.

Wachstein, M. and Meisel, E. (1957) Am. J. Clin. Path. 27:13-23.

Table 1. Activity of trichloroethylene and its metabolites in inducing single strand breaks in hepatic DNA of mice and rats.

Compound	Sprague-Dawley Rats	B6C3F1 Mice
Trichloroethylene	30*	**
Dichloroacetic acid	0.35	0.6
Trichloroacetaldehyde	2.9	2.7
Trichloroethanol	Inactive	--
Trichloroacetic acid	1.9	0.1

* Dose in mmoles/kg that was required to increase the fraction of DNA unwound in 2 hours at 0° C to 0.4.

** Although TCE induced SSB at lower doses in mice than rats, doses which would reach the criteria of 0.4 stated above were lethal in this species.

Table 2. Average area under curves (AUC) for trichlorothylene and its metabolites in rat blood following administration of saturating and non-saturating doses of trichloroethylene.

Compound	Dose of Trichloroethylene 4.6 mmoles/kg	22.8 mmols/kg	High Dose / Low Dose
Trichloroethylene	2102 ± 238*	13317 ± 1229	6.3
Dichloroacetic acid	536 ± 148	1036 ± 155	1.9
Trichloroacetaldehyde	7.8 ± 1.3	18.0 ± 2.3	2.3
Trichloroethanol	105 ± 17	316 ± 40	3.0
Trichloroacetic acid	5674 ± 1929	13030 ± 5620	2.3

* Area under the curve was calculated using the linear-trapezoidal rule. These values are expressed in terms of umole-h/L and represent the mean of 6 animals ± SEM.

Table 3. Urinary excretion of trichloroethanol, dichloroacetic acid and trichloroacetic acid following saturating and non-saturating doses of trichloroethylene.

Metabolite	Time	4.6 mmol/kg	22.8 mmol/kg
Trichloroethanol	0-24 h	790 ± 410*	796 ± 128
	24-48 h	26 ± 37	338 ± 228
	48-72 h	ND	1.1 ± 1.6
	Total	810 ± 410	1135 ± 106
(% of dose)		(17.8 ± 9.0)	(5.0 ± 0.5)
Dichloroacetic acid	0-24 h	18.4 ± 10.0	27.8 ± 10.7
	24-48 h	2.2 ± 1.8	33.9 ± 12.6
	48-72 h	ND	1.3 ± 1.1
	Total	20.4 ± 9.5	63.0 ± 19.0
(% of dose)		(0.45 ± 0.21)	(0.27 ± 0.08)
Trichloroacetic acid	0-24 h	61 ± 48	294 ± 139
	24-48 h	12.6 ± 11.9	341 ± 222
	48-72 h	ND	20 ± 26
	Total	73 ± 52	674 ± 221
(% of dose)		(1.6 ± 1.2)	(3.0 ± 1.0)

* Values expressed as mmol/kg body weight of the metabolite excreted.

Table 4. Effects of dichloroacetic and trichloroacetic acids on liver weight of male B6C3F1 mice.

Compound	Liver weight g	Liver weight % body weight
15 weeks		
Control (Dist. H_2O)	1.06 ± 0.05	3.2 ± 0.10
Phenobarbital 0.5 g/L	1.34 ± 0.16*	4.2 ± 0.35*
Dichloroacetic acid 2 g/L	1.80 ± 0.15*	5.6 ± 0.33*
Trichloroacetic acid 2 g/L	1.34 ± 0.09*	4.1 ± 0.14*
24 weeks		
Control	1.06 ± 0.04	2.8 ± 0.17
Phenobarbital 0.5 g/L	1.24 ± 0.04*	3.7 ± 0.09*
Dichloroacetic acid 2 g/L	1.78 ± 0.07*	5.4 ± 0.05*
Trichloroacetic acid 2 g/L	1.44 ± 0.05*	4.2 ± 0.14*

* Statistically different from corresponding negative control $P < 0.05$, n=5.

Table 5. Appearance of preneoplastic foci in the liver of B6C3F1 mice exposed to dichloroacetic and trichloroacetic acids for 24 months.

Treatment	N	Total # foci*	# Animals with foci
Distilled water	5	0	0
Dichloroacetic acid	5	4	3
Trichloroacetic acid	5	0	0

* Sections were taken every 150 μ through the central lobe of the liver. Focie were identified as areas of increased basophilia and glycogen depletion. Foci ranged from 300 - 700 μ in diameter.

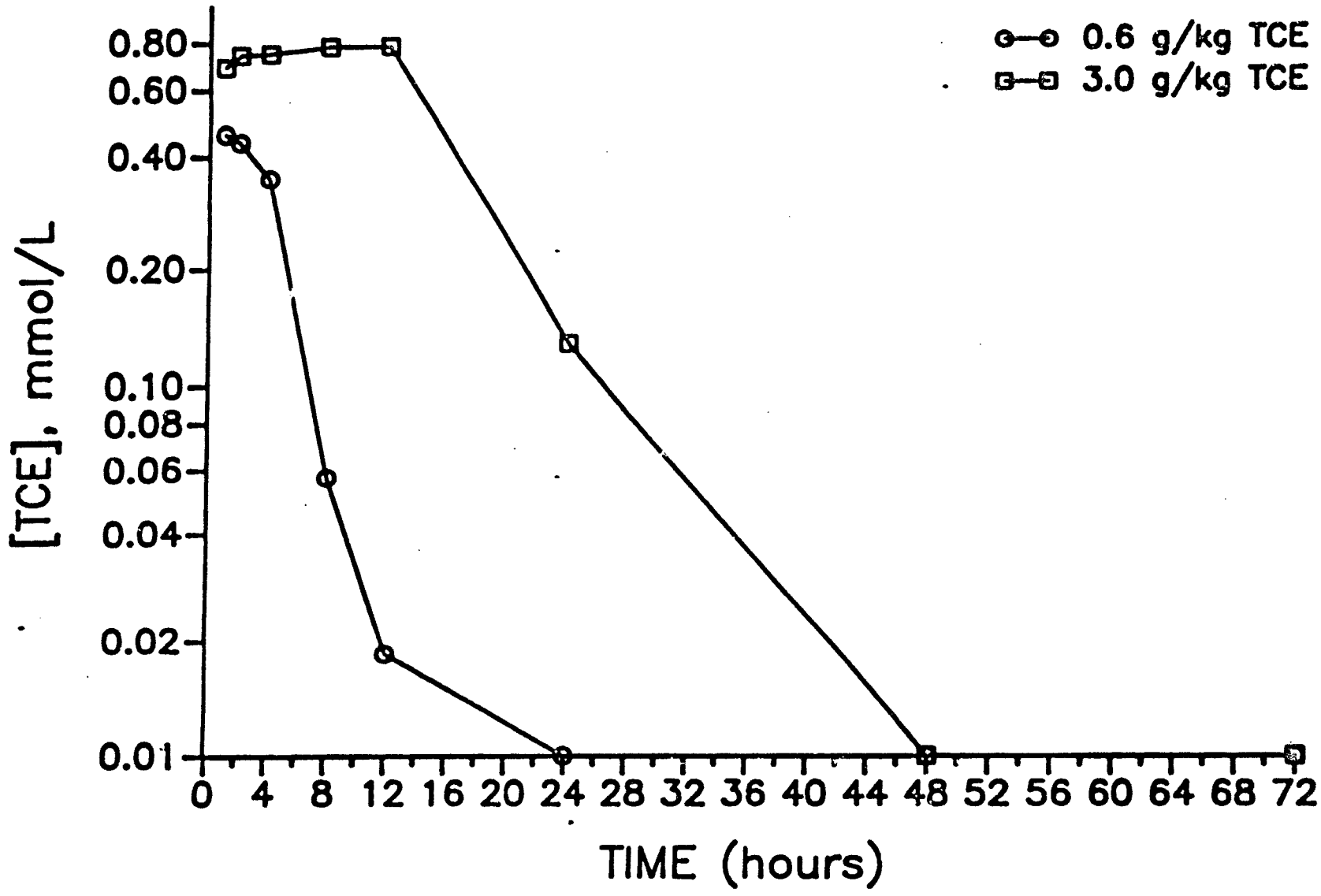

Figure 1. Blood concentrations of trichloroethylene in rats observed following administration of saturating (3 g/kg) and non-saturating doses of trichloroethylene. Each value represents the average of a minimum of 5 animals.

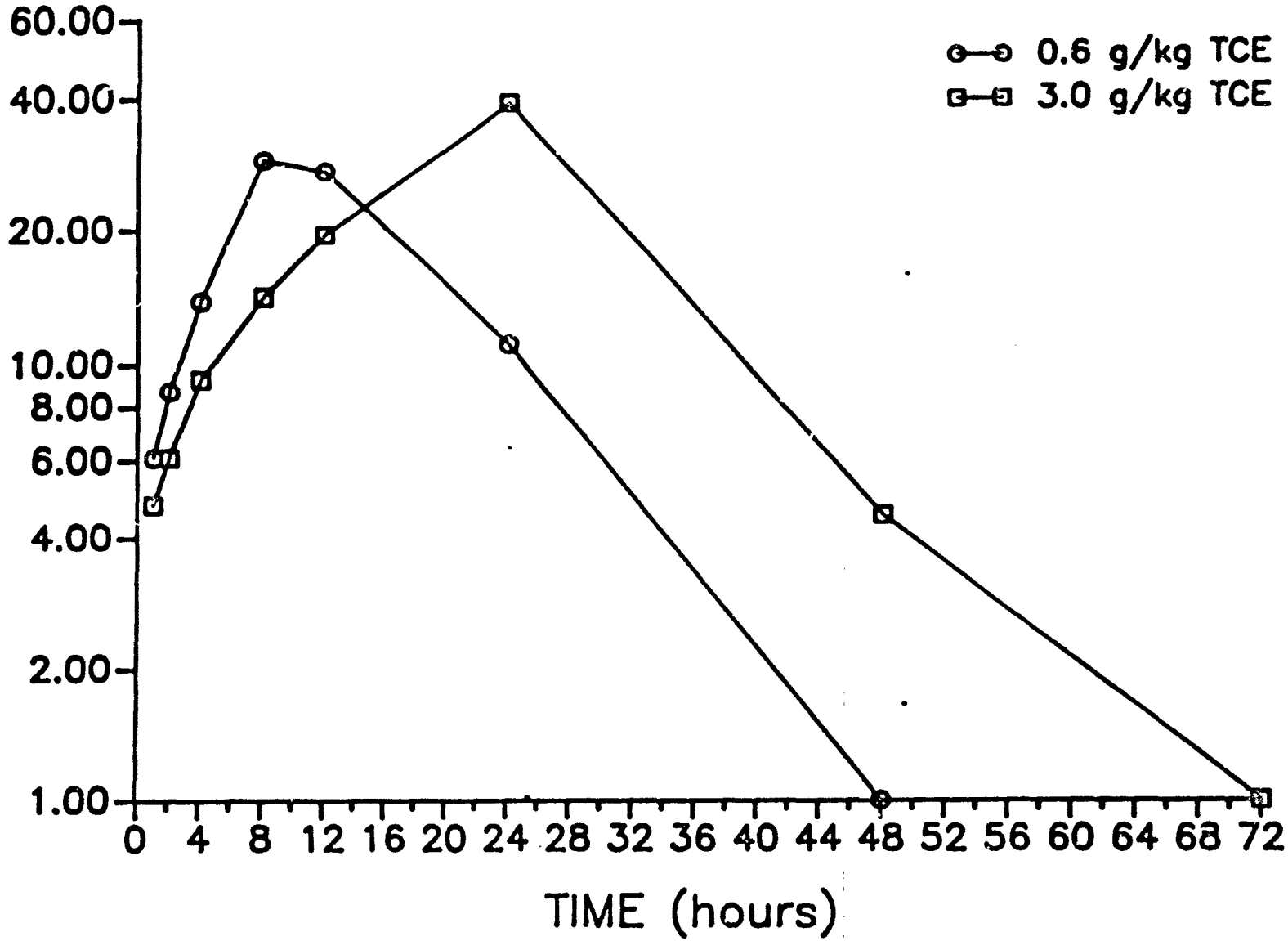

Figure 2. Blood concentrations of dichloroacetic acid in rats following administration of saturating (3 g/kg) and non-saturating (0.6 g/kg) doses of trichloroethylene. Each value represents the average of at least 5 animals.

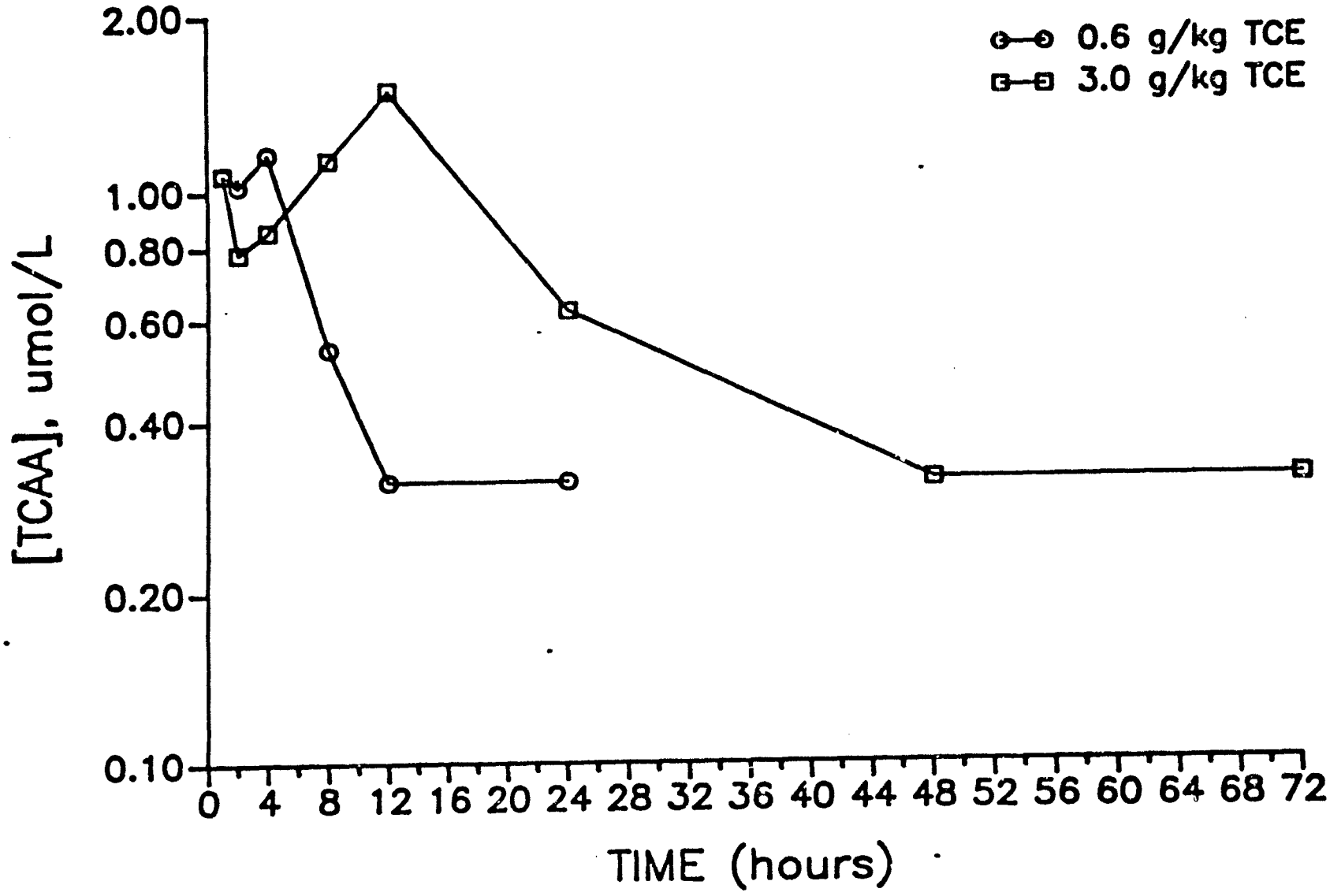

Figure 3. Blood concentrations of trichloroacetaldehyde in rats following administration of saturating (3 g/kg) and non-saturating (0.6 g/kg) doses of trichloroethylene. Each value represents the average of at least 5 animals.

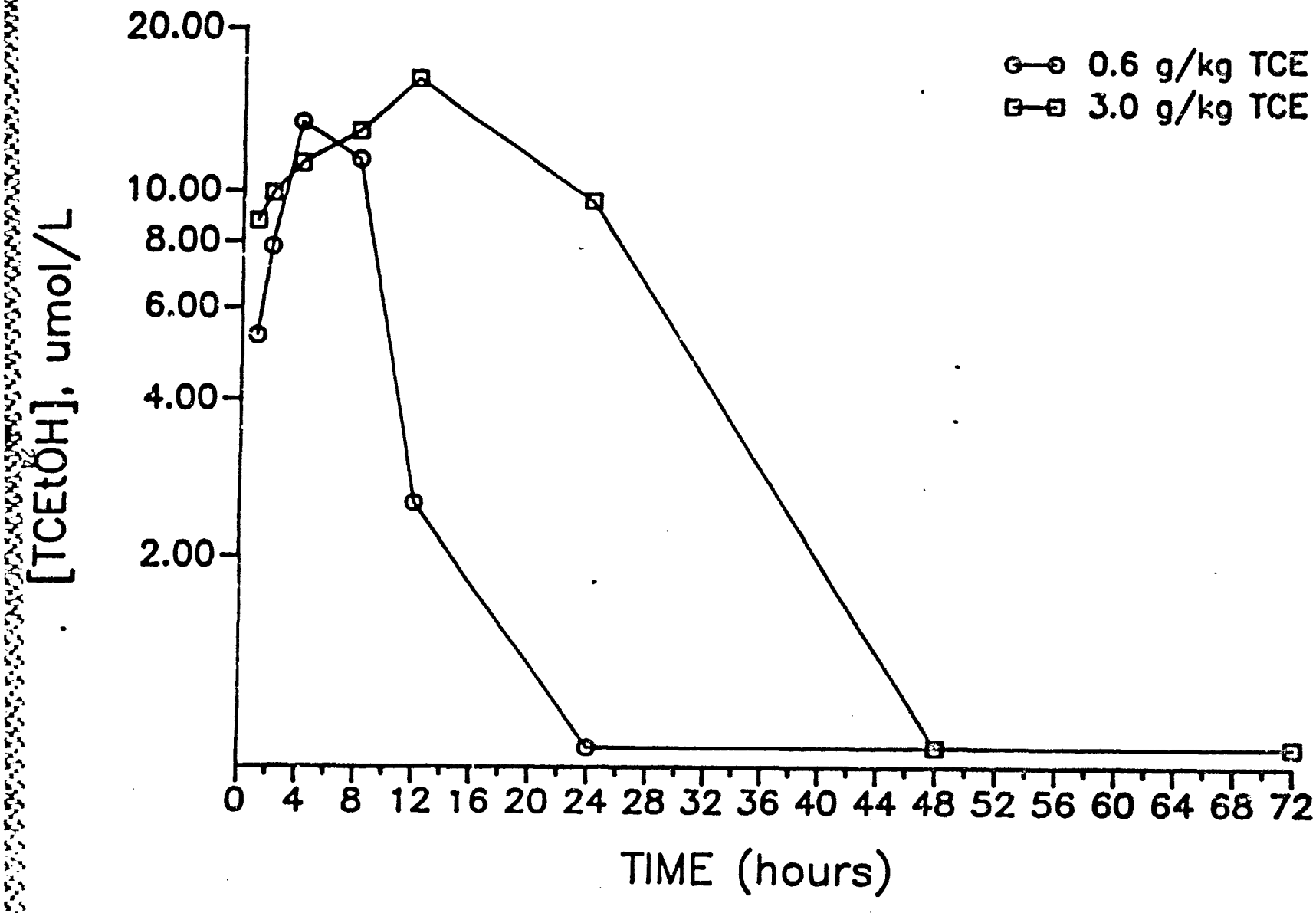

Figure 4. Blood concentrations of trichloroethanol in rats following administration of saturating (3 g/kg) and non-saturating (0.6 g/kg) doses of trichloroethylene. Each value represents the average of at least 5 animals.

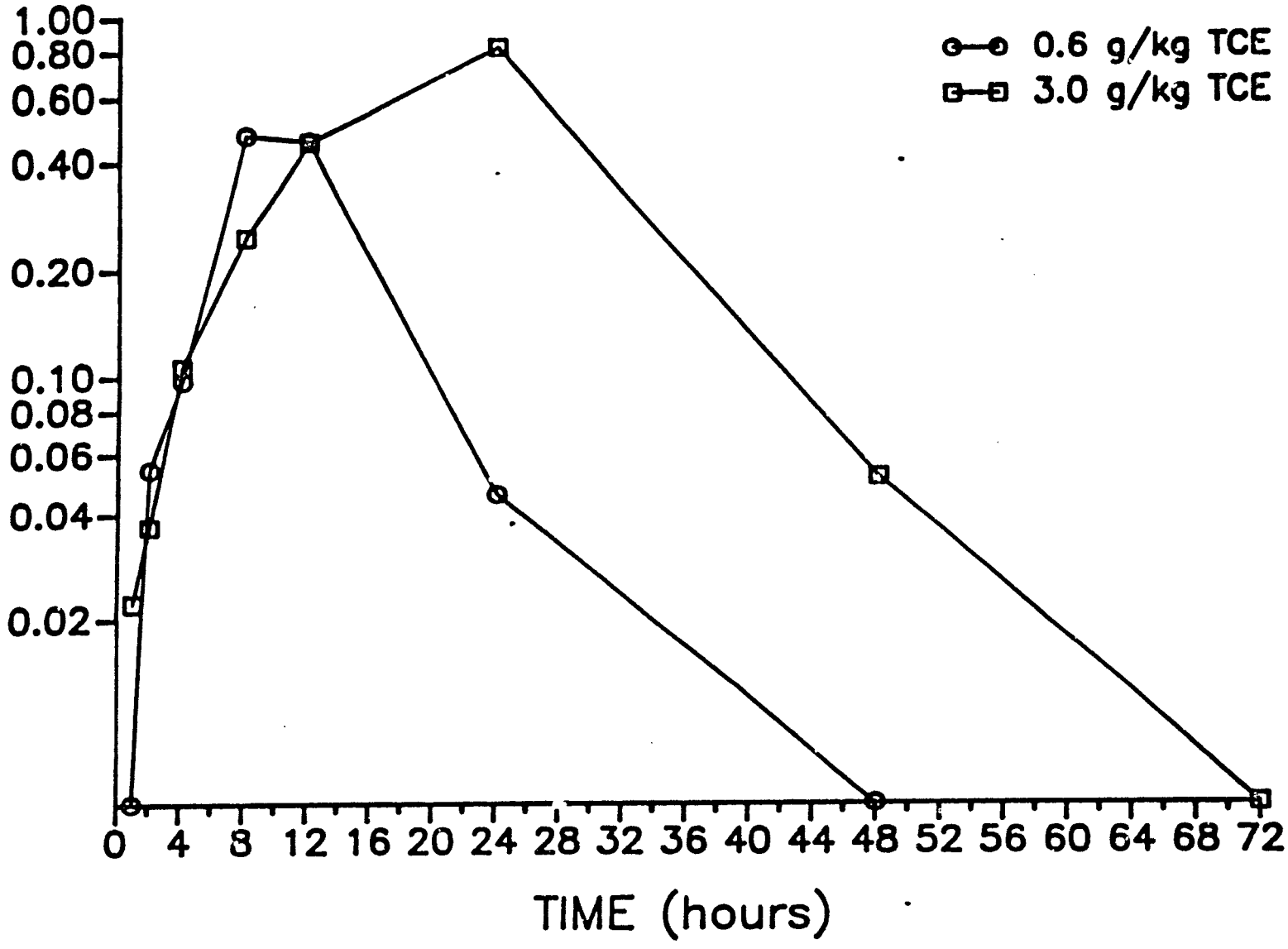

Figure 5. Blood concentrations of trichloroacetic acid in rats following administration of saturating (3 g/kg) and non-saturating (0.6 g/kg) doses of trichloroethylene. Each value represents the average of at least 5 animals.

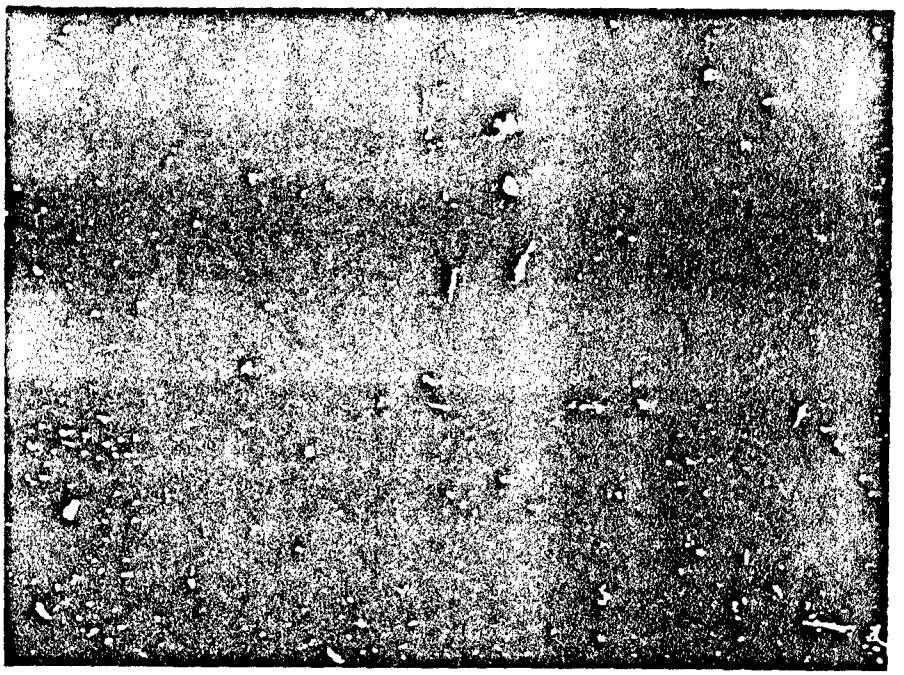

Figure 6. Section of liver from a B6C3F1 mouse that had received dichloroacetic acid its drinking water for 15 weeks showing focal areas of staining for G6PDH.

Figure 7. Contiguous section to that shown in figure 6 stained for G6P. No evidence of staining was observed in any region corresponding to those observed in figure 6.

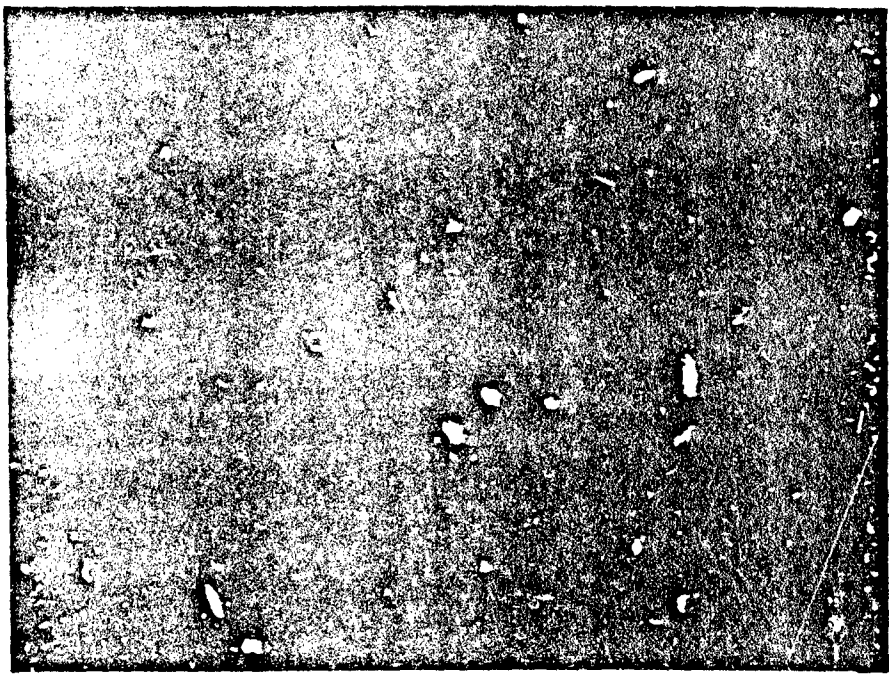

Figure 8. Hematoxylin and eosin staining of section adjacent to that shown in figure 6. Note that areas corresponding to G6PDH staining foci are not hepatocytes.

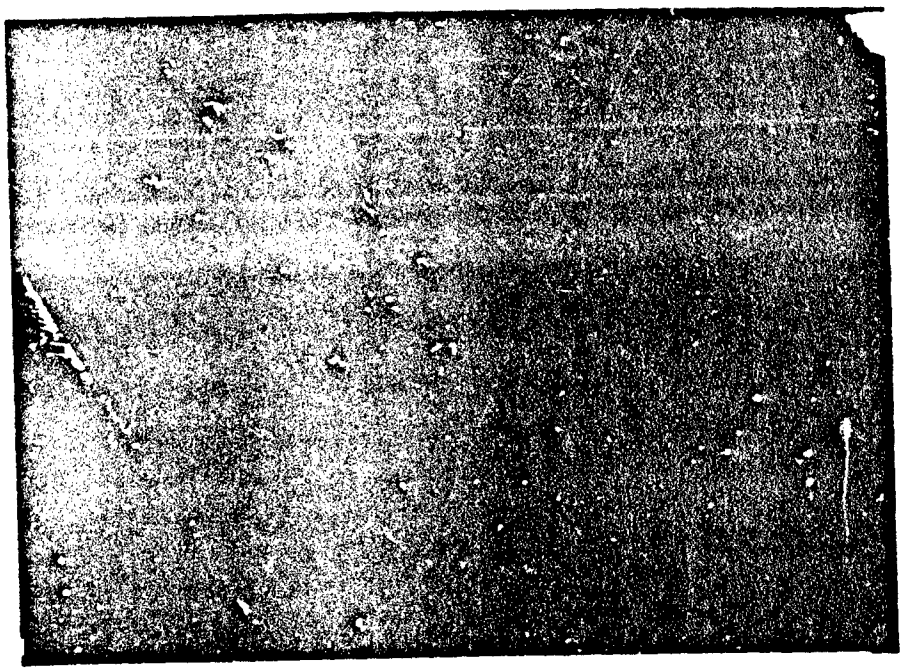

Figure 9. Section through the spleen of a B6C3F1 mouse stained for G6PDH showing focal areas of staining similar to that seen in the liver.

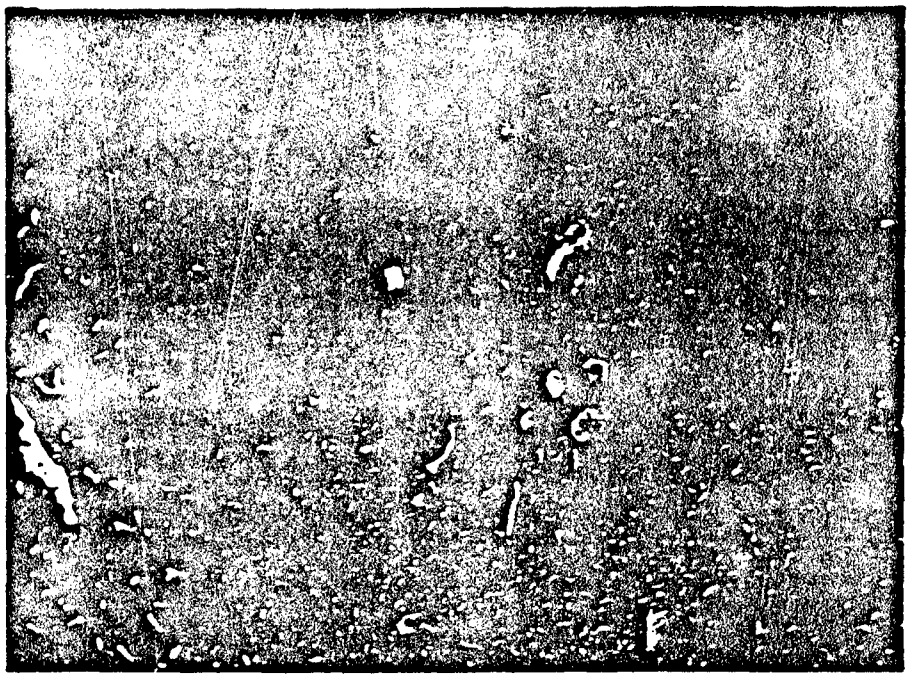

Figure 12. A contiguous section to the one in figure 11 which has been stained with periodic acid/Schiff stain for glycogen.

INDUCTION OF DNA-STRAND BREAKS BY TRICHLOROETHYLENE IN RAT AND MOUSE LIVER *IN VIVO*

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Induction of DNA-Strand Breaks by Trichloroethylene in Rat and Mouse Liver *In Vivo*. Nelson, M.A. and Bull, R.J. (1987). *Toxicol. Appl. Pharmacol.* 00, 0-000. The present study examines whether TCE is capable of producing single-strand breaks (SSB) in the DNA of rat and mouse liver *in vivo* and the extent to which metabolites might be responsible for these effects. After a single oral dose of TCE, SSB were measured in hepatic DNA of male Sprague-Dawley rats and B6C3F1 mice using the alkaline unwinding assay. High doses of TCE increased SSB in both species, but mice were more sensitive. To investigate the contribution of the metabolism of TCE on this response, rats were subjected to pretreatments of ethanol (3.0 g/kg, p.o.), phenobarbital (50 mg/kg, i.p.), TCE (3.6 mmol/kg or 11.4 mmol/kg, p.o.) or the appropriate vehicle for 4 days prior to challenge doses of TCE. Phenobarbital and TCE, but not ethanol pretreatments, reduced the dose of TCE required to produce significant increases in SSB. In a third series of experiments mice and rats were exposed to varying doses of trichloroacetic acid (TCA), dichloroacetic acid (DCA), chloral hydrate (CH), or trichloroethanol (TCEOH). TCA, DCA, and CH induced DNA-strand breaks in a dose-dependent manner in liver tissue from both species. TCEOH failed to produce DNA-strand breaks. In the rat DCA was much more active in producing SSB than TCA. TCA and CH appear to have about the same activity. In mice, TCA was as effective as DCA in inducing DNA-strand breaks. Both TCA and DCA were much more potent than CH. TCE and its metabolites increased SSB at doses that produced no observable hepatotoxic effects as measured by serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. These observations indicate that TCE is capable of inducing SSB in DNA *in vivo*, and that the effect can be duplicated at much lower doses by three stable metabolites of TCE. The ability of a major metabolite of TCE, TCA, to induce SSB in hepatic DNA *in vivo* appears to coincide with species sensitivity to the hepatocarcinogenic effects of TCE.

Trichloroethylene (TCE) is used extensively as an industrial degreasing agent and as a household cleaning fluid (Waters et al. 1977). It is commonly found in waste disposal sites and is a frequent contaminant of both surface and ground waters (Westrick et al., 1982).

TCE produces an increase in the incidence of liver neoplasms in mice but not rats (NCI, 1976 and NTP, 1983). A number of explanations have been offered for the difference in tumorigenic responses between the two species. First, the rate of TCE

metabolism is much greater in the mouse than in the rat. This is particularly noticeable at high doses (> 7.6 mmol/kg) where the proportion of TCE excreted unchanged markedly increases in the rat but less so in the mouse (Prout and Green, 1985). TCE is also more effective in inducing liver peroxisomes in mice compared to rats (Elcombe, 1985), a property which has been associated with hepatic carcinogenesis (Reddy et al., 1983). Trichloroacetic acid (TCA) is the metabolite of TCE that has been postulated to be responsible for peroxisome induction (Elcombe, 1985).

TCE is metabolized by the hepatic microsomal mixed-function oxidase system containing cytochrome P-450 to intermediates that can by rearrangement lead to the formation of chloral hydrate (CH) or dichloroacetyl chloride (DeKant et al., 1986; Miller and Guengerich, 1983). CH can be reduced to trichloroethanol (TCEOH) or oxidized to trichloroacetic acid (TCA). Hydrolysis of dichloroacetyl chloride leads to the formation of dichloroacetic acid (DCA_2) (Hathway, 1983).

TCE has been shown to covalently bind to DNA when incubated in the presence of hepatic microsomes or the S9 fraction <u>in vitro</u> (Van Duuren and Banerjee, 1976; Banerjee and Van Duuren, 1978; Cunningham et al., 1981; DiRenzo et al., 1981). However, the level of TCE-metabolite binding to hepatic DNA <u>in vivo</u> is very low (Parchman and Magee, 1981; Bergman, 1983). The lack of a demonstrable level of covalent interaction of TCE with DNA <u>in vivo</u> has led to suggestions that TCE is an epigenetic carcinogen

(Schumann et al., 1980; Stott et al., 1982). The present investigation examines whether TCE is capable of less direct interaction with DNA *in vivo* such that SSB are induced. Once this was established we determined whether this effect was modified by inducing cytochrome P-450 isozymes with phenobarbital, ethanol, or TCE-pretreatments and the extent it might be produced by metabolites of TCE.

METHODS

Chemicals

Phenol and TCE were obtained from Fisher Scientific. CH, DCA, TCEOH, and TCA were purchased from Sigma chemicals. Purity of administered chemicals was confirmed by gas-liquid chromatography. Diaminobenzoic acid (DABA) was obtained from Kodak chemical company.

Animals and Exposures

Male Sprague-Dawley rats weighing 300-400g (from Wash. St. Univ. Lab Animal Resource Center) and male B6C3F1 mice (from Simonsen Labs, Ca.) weighing 25-30g were used. The animals were housed in temperature controlled rooms with 12-hr. light/dark cycle. Food (Purina Laboratory Rodent Chow, Ralston-Purina Co., St. Louis, MO.) and water were provided ad libitum.

In the first set of experiments, single doses of TCE or individual metabolites were administered following overnight fasting p.o. in a total volume 10 ml of a 1% aqueous solution of Tween 80 kg body weight. Control animals received an equivalent volume of vehicle. Four hours after treatment, the animals were sacrificed, 10% liver suspensions prepared, and SSB determined as described below.

In a second series of experiments, rats were subjected to daily treatments of ethanol (0 or 3.0 g/kg, p.o.), phenobarbital (0 or 50 mg/kg, i.p.), TCE (0 or 3.8 or 11.4 mmol/kg, p.o.), or the appropriate vehicle pretreatments for 4 days prior to the

administration of challenge doses ranging from 3.8 to 30 mmol TCE/kg. Animals were sacrificed four hours after administration of the TCE challenge dose as described below.

Alkaline Unwinding Assay

To measure SSB in DNA, a modified version of the alkaline unwinding assay of Morris and Shertzer (1965) was used. This assay measures the rate of transition of double-stranded DNA to single-stranded DNA during alkaline denaturation. DNA-strand breaks serve as points at which unwinding is initiated (Rydberg, 1975). Therefore, the more DNA-strand breaks present, the more rapid the transition of double-stranded DNA to single-stranded DNA in alkaline solution. DNA-strand breaks may be present *in situ* or result from hydrolysis of alkaline-labile sites in the DNA molecule.

The following stock solutions were used in the alkaline unwinding assay: (I) 150 mM NaCl, 1.0 mM Na_2EDTA, pH 8.0; (II) 40 mM NaOH, 2.0 NaCl; (III) 100 mM NaH_2PO_4, 1.0 M NaCl.

Sacrifice of animals and preparation of liver samples was conducted in subdued lighting (indirect incandescent lighting) to avoid the introduction of strand breaks by UV irradiation. The livers were immediately removed from the animals, weighed, and rinsed in ice cold solution I. Connective tissue was removed by using an ice cold tissue press (Harvard Apparatus, Millis, MA) equipped with a stainless steel mesh sieve with 1.0 mm diameter holes. One portion of liver tissue was diluted with nine volumes (w/v) of ice cold solution I and tissue suspensions were prepared

with a single stroke of the pestle of an ice cold, hand held, 30 ml, Potter-Elvehjem tissue grinder. The samples were then placed on ice for one minute to allow debris to settle. After one minute, the upper most layer of the suspensions were withdrawn and vigorously pipetted into one ml of ice cold solution II. The samples were allowed to sit on ice for 2 hrs. without further agitation. The samples were neutralized by rapid addition of one ml portions of ice cold solution III. Samples were immediately sonicated for 5 seconds to shear the partially unwound DNA into small pieces of double-stranded (DS) and single-stranded (SS) DNA (Ahnstrom and Erixson, 1981; Rydberg, 1975). The neutralized samples were split and one portion was analyzed for total DNA content and the second analyzed for DS DNA following extraction of SS DNA with phenol. Protein and lipids were removed by extracting these samples with one ml portions of chloroform. DNA was assayed using Setaro and Morley's (1976) modification of Kissane and Robins' (1958) diaminobenzoic acid fluorometric assay. The fraction of DNA unwound was calculated as the fraction DNA unwound equal to:

$$\frac{[\text{Total DNA} - \text{DS DNA}]_t}{[\text{Total DNA}]} - \frac{[\text{Total DNA} - \text{DS DNA}]_0}{[\text{Total DNA}]}$$

where the subscript indicate the amount of double stranded DNA at time 0 and "t" refers to the incubation time in alkaline solution.

Serum Enzyme Determinations

Animals treated in a parallel fashion to those used to determine the SSB in hepatic DNA were sacrificed at 24 hours

after a challenge dose of TCE or its metabolites to determine whether increases in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzyme levels provide any indication of hepatic injury. Blood was drawn from the inferior vena cava and the serum separated by centrifugation and assayed for serum ALT and AST levels using diagnostic kits purchased from Sigma Chemical Co. These assays depend on spectrophotometric determination of the decrease of NADH that results from coupling the aminotransferase activity to the reduction of its products by malate dehydrogenase and lactate dehydrogenase respectively.

RESULTS

For simplicity, increases in the rate of DNA unwinding under alkaline conditions will be referred to as increases in single-strand breaks (SSB) in DNA. SSB have been demonstrated to be the major factor involved in increased rates of alkaline unwinding (Kydberg, 1979).

TCE induces SSB in rat hepatic DNA *in vivo* (Fig. 1). A single oral dose of 30 mmol/kg TCE significantly increased SSB relative to controls. Doses of 3.8 and 11.4 mmol/kg TCE were without effect. In a subsequent experiment (Fig. 2), the intermediate dose of 22.9 mmol/kg was also found to induce significant in SSB.

Phenobarbital pretreatment is known to alter the metabolism of TCE (Miller and Guengerich, 1983). To investigate the role metabolism of TCE might play in the induction of SSB rats were

pretreated with 50 mg/kg phenobarbital (i.p.) for 4 days. Phenobarbital pretreatment lowered the dose of TCE required to produce significant increases in SSB to 11.4 mmol/kg on the fifth day (Fig. 1).

Ethanol pretreatment is also known to modify the levels of P-450$_{IIE1}$ in the liver (Koop et al., 1982; Ryan et al., 1984), changing the metabolism of a different set of substrates. Therefore, we examined the ability of ethanol pretreatments (3.6 g/kg x 4 days) to modify the induction of SSB. An increase mean rate of alkaline unwinding was observed (Fig. 2), but it was smaller than that observed following phenobarbital pretreatment and was not statistically significant from the rate observed from vehicle-pretreated animals.

Given the observation that phenobarbital lowered the dose of TCE required to increase SSB, the influence of prior doses of TCE on this response was determined. As previously mentioned doses of 3.6 and 11.4 mmol/kg TCE were without effect when administered, these doses were used for the pretreatment. In animals pretreated with either 3.6 mmol/kg or 11.4 mmol/kg TCE, a challenge dose of 11.4 mmol/kg of TCE also increased the number of SSB in DNA (Fig 3). If one assumes that the fourth dose of 11.4 mmol/kg TCE was as effective as the fifth, it would appear from the control data that the SSB produced by the fourth pretreatment dose were repaired by the time of measurement (i.e. 16 hr. after the last pretreatment dose).

The above data suggested that a metabolite, produced at

higher rates in phenobarbital and/or TCE-pretreated animals, may be responsible for the SSB observed. Consequently, the ability of stable metabolites of TCE to cause SSB in hepatic DNA was evaluated. The dose required to increase the fraction of DNA unwound in 2 hr to 0.4 was used to compare the potency of the metabolites tested. In male Sprague-Dawley rats, DCA appeared to be the most potent of the four metabolites tested. A dose of 0.35 mmol/kg DCA, 1.9 mmol/kg TCA, or 2.9 mmol/kg CH produce as much DNA damage as 30 mmol/kg TCE. TCEOH did not increase the number of SSB at any dose tested (Fig. 4).

The time course of alkaline unwinding of hepatic DNA from rats treated with 3.6 mmol/kg DCA and control animals is shown in figure 5. DCA significantly increased the rate at which double-stranded DNA dissociated into single-stranded DNA. A similar pattern of response was observed by Morris and Sheitzer (1985) with N-nitrosodimethylamine.

To determine whether hepatic injury occurred with the doses used in the various SSB experiments serum ALT and AST levels were monitored. TCE increased the serum ALT and AST levels only after phenobarbital pretreatment and at the high dose of 30 mmol/kg (Table 1). Neither ethanol or TCE pretreatment resulted in any increases in these serum enzymes following the challenge doses of TCE administered (Table 2). CH, DCA, and TCA also failed to increase serum ALT and AST levels following acute administration (Table 3).

In B6C3F1 mice, TCE produced increased rates of DNA

unwinding following the administration of a dose of 5.8 mmol/kg TCE and above (Fig. 6). Therefore, mice are substantially more sensitive to the induction of SSB in hepatic DNA than rats. Doses above 22.9 mmol/kg TCE were lethal to mice.

The responses of mice to oral doses of the metabolites of TCE displayed a different order of potency than in rats. The doses required to increase the fraction of DNA unwound to 0.4 was 0.1 mmol/kg for TCA, 0.6 mmol/kg for DCA, and 2.7 mmol/kg for CH (Fig. 5). It is clear from these data that there is a substantial difference in species responsiveness to TCA-induced SSB in hepatic DNA.

DISCUSSION

The present study demonstrates that TCE is capable of inducing SSB in hepatic DNA of both mice and rats *in vivo*. In this investigation mice appear to be more sensitive to the effects of TCE than rats. Walles (1986) also demonstrated that there was a linear increase in the level of SSB in mouse liver DNA following i.p. administration of TCE. He also reported that the strand breaks are repaired within 24 hours, consistent with observations made in the rat in the present study.

The increased number of SSB produced by TCE following phenobarbital pretreatment suggests that metabolic activation is necessary for DNA damage to occur. Several studies have reported that covalent binding to exogenous DNA takes place in the presence of liver microsomes (Cunningham et al., 1981;

DiRenzo et al., 1982; Bergman, 1983). The lack of demostrable binding *in vivo* suggests that the DNA-binding observed in these *in vitro* studies are not directly related to the induction of SSB reported in the present study. It is presumed that such binding would involve the epoxide or acid chloride intermediates in TCE metabolism, whereas the present data indicates that the more stable metabolites can produce the effect at low doses. The mechanism by which SSB are induced by these agents requires further study.

Unlike pretreatments with phenobarbital or TCE, ethanol only slightly increased the ability of TCE to cause SSB in DNA a change that was not statistically significant. The relative ineffectiveness of ethanol in this regard may be related to its capability for inducing different isozymes of cytochrome P-450 than phenobarbital. Ethanol administration is known to induce a unique P-450 isozyme with specific catalytic properties (Ko et al., 1987; Ryan et al., 1984; and Koop et al., 1982). Miller and Guengerich (1983) have shown that cytochrome P-450$_{PCN}$ is unusually efficient in converting TCE to chloral. The present results suggest that TCE induces a wider variety of P-450 isozymes than ethanol and that a pathway induced by phenobarbital pretreatment is important in metabolizing TCE to metabolites capable of producing DNA-strand breaks.

The most interesting aspect of this study was the substantial differences in sensitivity of mice and rats to TCA. While there was a tendency for mice to be more sensitive to all

the metabolites that tested positive, TCA was almost 20-fold more potent in mice than in rats. These results also parallel the sensitivity of these species to the induction of hepatic peroxisomes by TCA (Elcombe 1985; DeAngelo et al., 1986). Preliminary data indicate that the induction of SSB occurs long before there is any evidence of peroxisome proliferation indicating increased beta-oxidation of lipids by peroxisomes could not account for the SSB observed in the present study. Although these responses parallel one another, there may be a less obvious linkage between the two responses.

In summary, we have demonstrated that TCE is capable of inducing SSB in hepatic DNA of mice and rats <u>in vivo</u>. Pretreatment with phenobarbital or low doses of TCE, but not with ethanol, decreases the dose of TCE required to induce SSB. This suggests the involvement of a metabolite(s) which is produced by a pathway that is inducible by phenobarbital or TCE. The effect could be duplicated at much lower doses using the stable metabolites of TCE: DCA, TCA, and CH, suggesting that one or more of these compounds are responsible. The variation in the relative potency of TCA in mice and rats parallels the species specificity of the hepatocarcinogenic effects of TCE. The induction of SSB appears to be independent of the hepatotoxicity of these chemicals, since increased damage occurred in the absence of significant elevations in serum AST and ALT levels.

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REFERENCES

AHNSTROM, G., AND ERIXSON, K. (1981). Measurement of strand breaks by alkaline denaturation and hydroxyapatite chromatography. In Friedberg EC, Hanawalt PC (eds.), "DNA Repair", vol. 1, part B. New York: Marcel Dekker, ch 30, 403-416.

BANERJEE, S., AND VAN DUUREN, B.L. (1976). Covalent binding of the carcinogen trichloroethylene to hepatic microsomal proteins and to exogenous DNA in vitro. Cancer Res. 36, 776-780.

BERGMAN, K. (1983). Interaction of trichloroethylene with DNA in vitro and with RNA and DNA of various mouse tissue in vivo. Arch. Toxicol. 54, 181-193.

CUNNINGHAM, M.L., GANDOLFI, A.J., BRENDEL, K., AND SIPES, I.G. (1981). Covalent binding of halogenated volatile solvents to subcellular macromolecules in hepatocytes. Life Sci. 28, 1207-1212.

DEANGELO, A.B., HERREN-FREUND, S., PEREIRA, M.A., SCHULTZ, N.E., AND KLAUNIG, J.E. (1986). Species sensitivity to the induction of peroxisome proliferation by trichloroethylene and its metabolites. Toxicol. 6, 115.

DEKANT, W., SCHULTZ, A., METZLER, M., AND HENSCHLER, D. (1986). Absorption, elimination, and metabolism of trichloroethylene: a quantitative comparison between rats and mice. Xenobio. 16, 143-152.

DIRENZO, A.B., GANDOLFI, A.J., AND SIPES, I.G. (1981). Microsomal bioactivation and covalent binding of aliphatic halides to DNA. Toxicol. Lett. 11, 245-251.

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.

ELCOMBE, C.R., ROSE, M.S., AND PRATT, I.S. (1985). Biological, histological, and ultrastructural changes in rat and mouse liver following the administration of trichloroethylene: Possible relevance to species differences in hepatocarcinogenicity. Toxicol. Appl. Pharmacol. 79, 365-376.

GREEN, T., AND PROUT, M.S. (1985). Species differences in responses to trichlorethylene. II. Biotransformation in rats and mice. Toxicol. Appl. Pharmacol. 79, 401-411.

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

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.

KU, Y.Y., FARR, S.S., FATTEN, C., TAN, Y. HAH, Y.C., YANG, C.S., AND GELBOIN, H.V. (1987). Monoclonal antibodies to ethanol induced rat liver cytochrome P-450 that metabolizes aniline and nitrosamines. Cancer Res. 47, 3101-3109.

LOOF, D.R., MORGAN, E.T., TARR, G.E., AND COON, M.J. (1981). Purification and characterization of a unique isozyme of cytochrome P-450 from liver microsomes of ethanol-treated rabbits. J. Biol. Chem. 257, 6472-6480.

MILLER, R.E., AND GUENGERICH, F.P. (1983). Metabolism of trichloroethylene in isolated hepatocytes, microsomes, and reconstituted enzyme systems containing cytochrome P-450. Cancer Res. 43, 1145-1152.

MORRIS, S.R., AND SHERTZER, H.G. (1985). Rapid analysis of DNA strand breaks in soft tissues. Environ. Mut. 7, 671-680.

NCI (1976). Carcinogenesis bioassay of trichloroethylene. National Cancer Institute Carcinogenesis Technical Report Series, NCI-CG-TR-2, 76-802.

NTP (1983). National Toxicology Program draft report abstracts on nine chemical carcinogenesis animal bioassay. Chem. Hazol. Rep. 6, 767-768.

PARCHMAN, L.G., AND MAGEE, P.N. (1982). Metabolism of [^{14}C]-trichloroethylene to $^{14}CO_2$ and interaction of metabolites with liver DNA in rats and mice. J. Toxicol. Environ. Health 9, 787-813.

PROUT, M.S., PROVAN, W.M., AND GREEN, T. (1985). Species differences in responses to trichlorethylene I. Pharmacokinetics in rats and mice. Toxicol. Appl. Pharmacol. 78, 389-400.

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.

RYAN, D.E., IIDA, S., WOOD, A.W., THOMAS, P.E., LEIBER, C.S., AND LEVIN, W. (1984). Characterization of three highly purified cytochrome P-450 from hepatic microsomes of adult male rats. J. Biol. Chem. 259, 1239-1250.

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

SCHUMANN, A.M., QUAST, J.F., AND WATANABE, P.G. (1980). The pharmacokinetics and macromolecular interactions of perchloroethylene in mice and rats as related to oncogenicity. Toxicol. Appl. Pharmacol. 55, 207-219.

SETARO, F., AND MORLEY, 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.

STOTT, W.J., QUAST, J.F., AND WATANABE, P.G. (1981). The pharmacokinetics and macromolecular interactions of trichloroethylene in mice and rats. Toxicol. Appl. Pharmacol. 61, 139-151.

VAN DUUREN, B.L., AND BANERJEE, S. (1976). Covalent interactions of metabolites of the carcinogen trichloroethylene in rat hepatic microsomes. Cancer Res. 36, 2419-2422.

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

WATERS, E.M., GUSTNER, H.B., AND HUFF, J.E. (1977). Trichloroethylene: an overview. J. Toxicol. Environ. Health 2, 671-707.

WESTRICK, J.J., MELLO, J.W., AND THOMAS, R.F. (1981). The ground water supply survey: summary of volatile organic occurrence data. Technical Support Division, Office of Drinking Water, U.S. Environmental Protection Agency, Cincinnati, OH 45268.

TABLE 1

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

Pretreatment/Challenge		N	AST[a]	ALT[b]
Saline/Tween		5	66 ± 12	31 ± 13
Phenobarb/Tween		5	69 ± 5	30 ± 2
Tween/Tween		4	45 ± 5	7 ± 1
Phenobarb/TCE	11.4 mmol/kg	5	62 ± 8	27 ± 4
	30.5 mmol/kg	4	281 ± 74[c]	98 ± 33[c]
Saline/TCE	11.4 mmol/kg	5	76 ± 19	24 ± 4
	30.5 mmol/kg	5	50 ± 7	18 ± 2
TCE/TCE	11.4 mmol/kg	5	56 ± 5	14 ± 2

[a] Values are expressed as Karmen units/ml ± SEM

[b] Values are expressed as LaDue units/ml ± SEM

[c] $P \leq 0.05$ by ANOVA and Duncan's multiple range test

[d] Animals were pretreated with 11.4 mmol/kg TCE for 4 days prior to a challenge dose of TCE

TABLE 2

EFFECTS OF TCE ON ASPARTATE AMINOTRANSFERASE (AST) AND ALANINE AMINOTRANSFERASE (ALT) ACTIVITIES IN SERUM FOLLOWING PRETREATMENT WITH ETHANOL

Pretreatment/Challenge	N	AST[a]	ALT[b]
water Tween	5	62 ± 5[c]	17 ± 2[c]
EtOH Tween	5	54 ± 13	14 ± 1
EtOH TCE 11.4 mmol/kg	5	45 ± 5	16 ± 1
22.7 mmol/kg	5	46 ± 3	18 ± 3
water:TCE 11.4 mmol/kg	5	50 ± 5	21 ± 1
22.7 mmol/kg	5	46 ± 3	19 ± 1

[a] Values expressed as described in Table 1.

[b] Values expressed as described in Table 1.

[c] No significant differences found by ANOVA and Duncan's multiple range test.

TABLE 3

EFFECTS OF DCA, TCA, TCEOH, AND CH ON ASPARTATE AMINOTRANSFERASE (AST) AND ALANINE AMINOTRANSFERASE (ALT) ACTIVITIES IN SERUM

Challenge	N	AST[a]	ALT[b]
Tween	5	33 ± 5[c]	10 ± 3[c]
DCA (3.8 mmol/kg)	4	33 ± 3	8 ± 1
TCA (3.1 mmol/kg)	4	49 ± 15	13 ± 4
TCEOH (3.3 mmol/kg)	4	38 ± 4	7 ± 2
CH (3.4 mmol/kg)	4	35 ± 0	5 ± 1

[a] Values expressed as Karmen units/ml ± SEM

[b] Values expressed as LaDue units/ml ± SEM

[c] No significant differences found by ANOVA and Duncan's multiple range test.

Fig. 1. The induction of SSB in rat hepatic DNA by TCE and modification of this response by phenobarbital pretreatment. Values given are the means from at least five animals ± SEM.

* Value statisically different. $P \leq 0.05$ by ANOVA and Duncan's multiple range test.

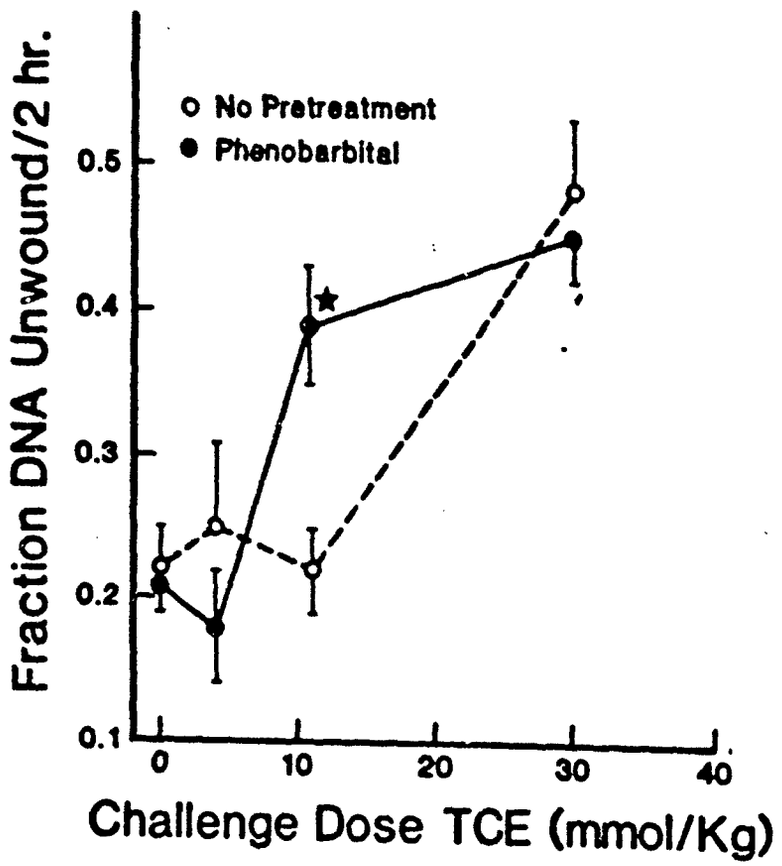

Fig. 2. The induction of SSB in rat hepatic DNA by TCE and the effect of ethanol pretreatments on this reponse. Values given are means from at least five animals ± SEM. No significant differences were found by ANOVA and Duncan's multiple range test.

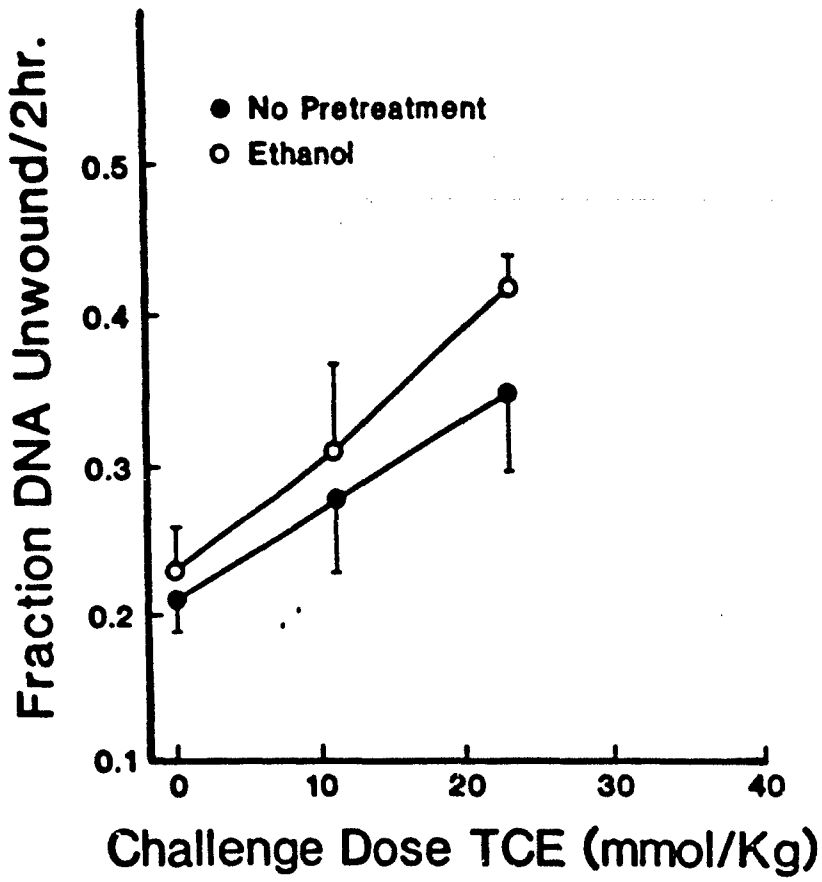

Fig. 3. The induction of SSB in rat hepatic DNA by TCE and alteration of this response by TCE pretreatments. Values given are the means from at least five animals ± SEM.

* Values are statisically different. $P \leq 0.05$ by ANOVA and Duncan's multiple range test.

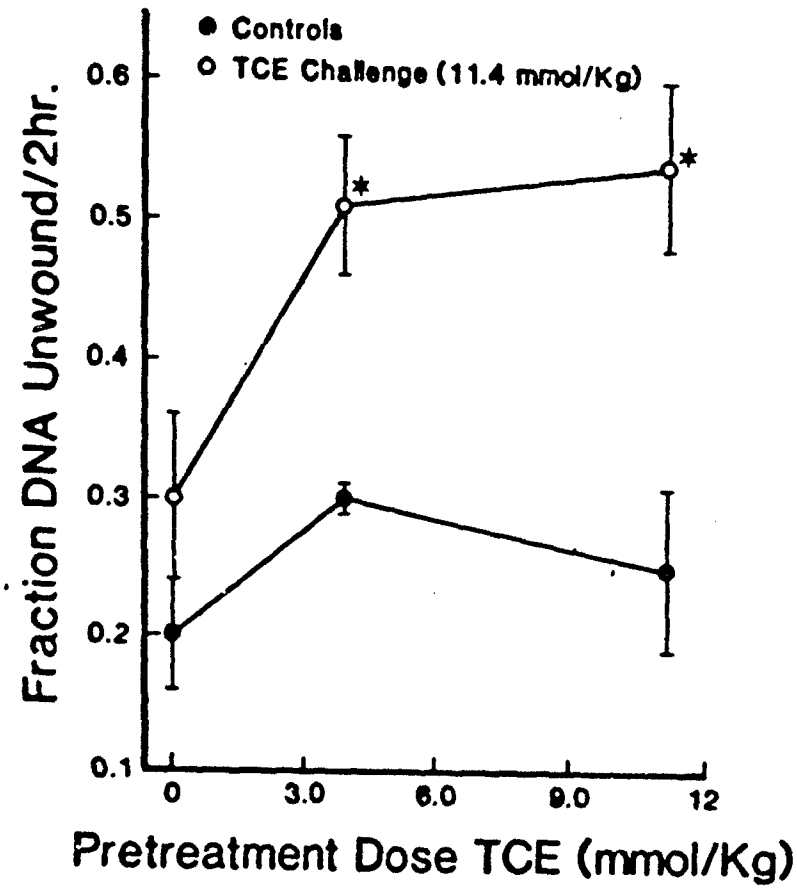

Fig. 4. The induction of DNA-strand breaks after exposure to DCA, TCA, CH, TCEOH, and TCE in rat hepatic DNA. Each point represents the mean from at least five animals ± SEM. The control value ( ) included 34 animals.

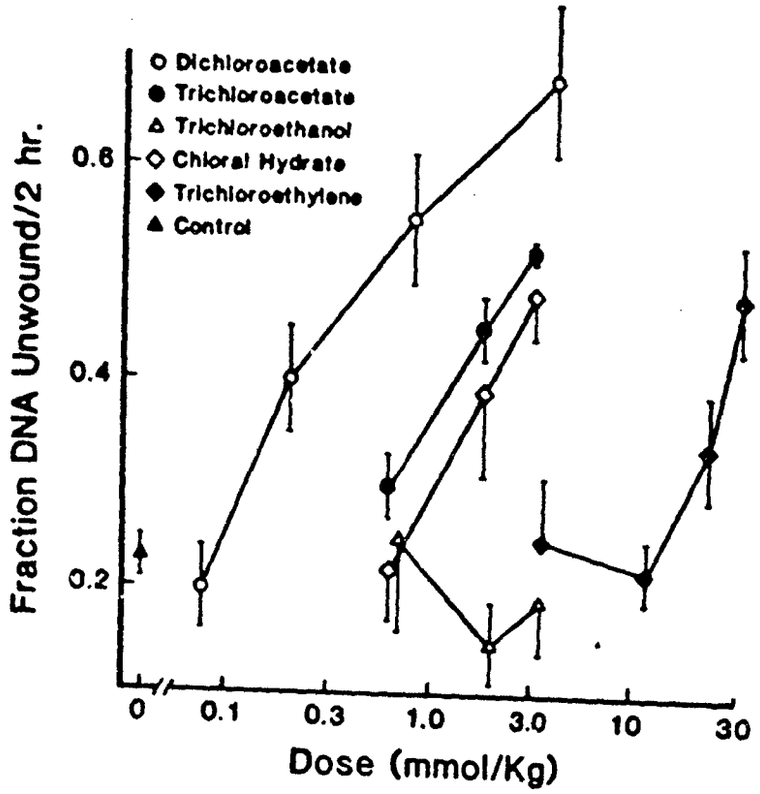

Fig. 5. The effect of DCA on the time course of alkaline unwinding of rat liver DNA. Values given are the means from at least four animals ± SEM.

* Value statisically different. $P \leq 0.05$ by ANOVA and Duncan's multiple range test.

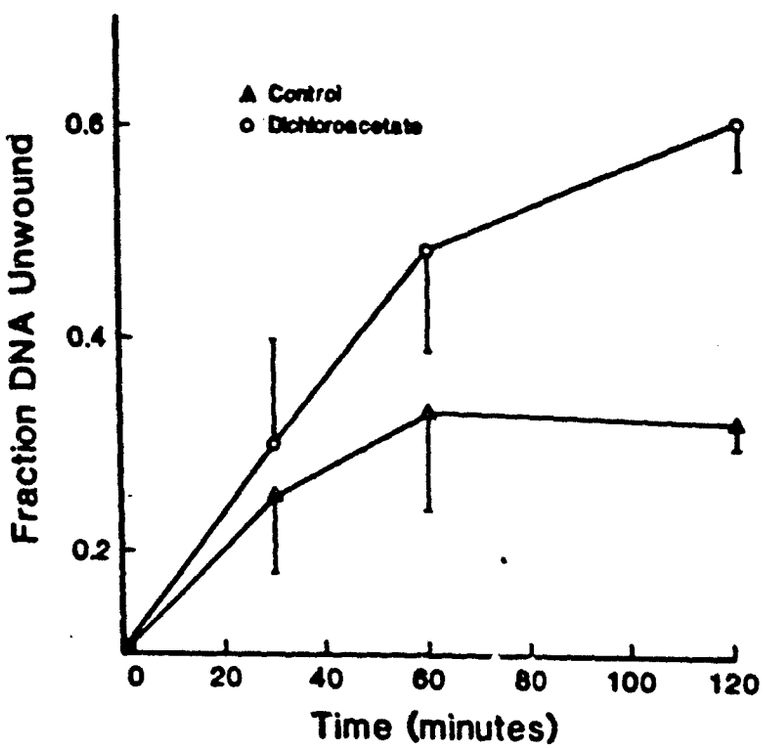
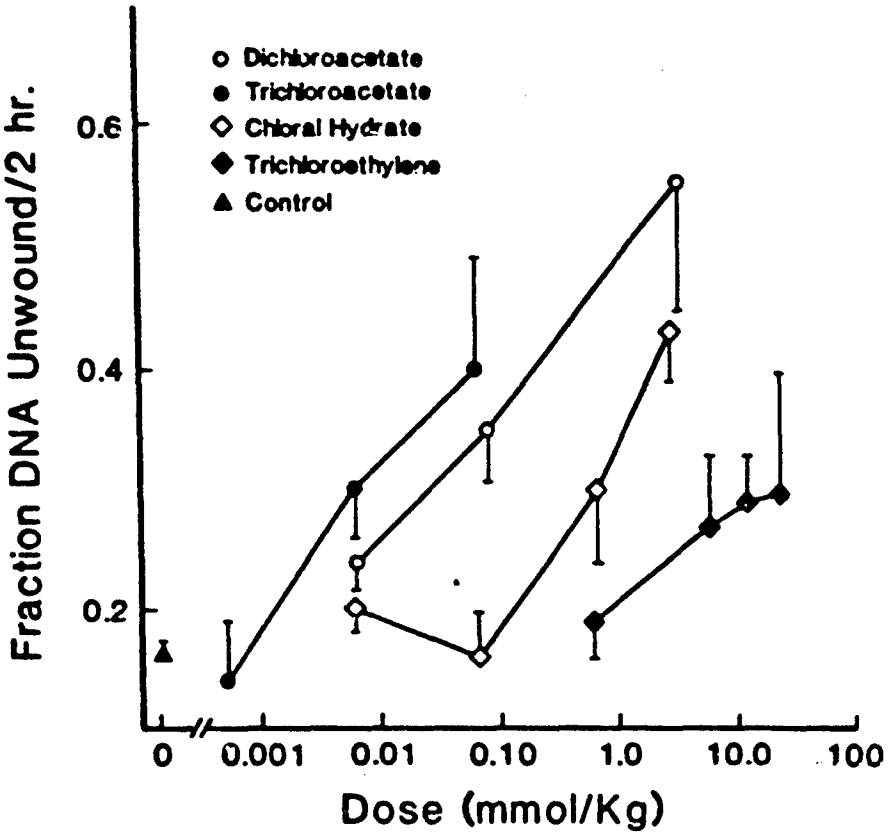
Fig. 6. The induction of SSB after exposure to DCA, TCA, CH, TCEOH, and TCE in mouse hepatic DNA. Each point represents the mean of at least five animals ± SEM. The control value ( ) included 35 animals.